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Uncovering the Neuroimmune Interactions in Neonatal Meningitis

Inês Lorga

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
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UNCOVERING THE NEUROIMMUNE INTERACTIONS IN NEONATAL MENINGITIS

Tese de Candidatura ao grau de Doutor em Biologia
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Categoria – Investigadora Principal

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Biologia Molecular e Celular

“Good, better, best. Never let it rest. ‘Til your good is better and your better is best.”

St. Jerome

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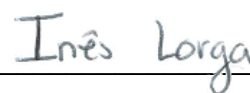
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DECLARAÇÃO DE HONRA

Eu, Inês Sampaio e Lorga, declaro que a presente tese é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico.

Porto, 20 de dezembro de 2024

A handwritten signature in blue ink that reads "Inês Lorga". The signature is written in a cursive style and is positioned above a horizontal line.

(Assinatura da Doutoranda)

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LISTA DE PUBLICAÇÕES

De acordo com o disposto no ponto nº 2 do Art.º 31º do Decreto-Lei n.º 74/2006, de 24 de março, aditado pelo Decreto-Lei n.º 230/2009, de 14 de setembro, o autor declara que na elaboração desta tese foram incluídos dados das publicações abaixo indicadas. O autor participou ativamente na conceção e execução dos trabalhos que estiveram na origem dos mesmos, assim como na sua interpretação, discussão e redação.

Lorga I, Geraldo R, Soares J, Oliveira L, Firon A, Bonifácio Andrade E. Development of bioluminescent Group B streptococcal strains for longitudinal infection studies. *Sci Rep*, 2024, 14.1: 24439.

Lorga I, Bonifácio Andrade E. Meningeal immunity: anatomy, function and neonatal unique features. Submitted for publication in the *European Journal of Immunology*. Unrevised version of the article. Invited Review.

Lorga I, Soares J, Bravo J, Mesquita P, Ribeiro N, Mesquita M, Oliveira L, Magalhães A, Summavielle T, Vilanova M, Ribot J, Bonifácio Andrade E. Meningeal $\gamma\delta$ T cells disrupt the blood-brain barrier during neonatal meningitis triggering long-term neurological sequelae. *In preparation*.

Lorga I, Teixeira A, Carvalho B, Soares J, Cunha J, Santos J, Silva R, Vilanova M, Bonifácio Andrade E. CGRP Suppresses SiglecF^{hi} Neutrophil Development in Group B *Streptococcus* Neonatal Pneumonia. *In preparation*.

APRESENTAÇÕES EM CONFERÊNCIAS

O presente trabalho foi apresentado pelo autor em diversas conferências, nacionais e internacionais. O autor participou ainda em trabalhos do grupo e de colaboradores, apresentados por outros em conferências.

Comunicações por Poster

Bravo J., Rodrigues J., Bonifácio Andrade E., Vieira R., Lorga I., Azevedo M., Summavielle T. IFN- γ as a modulator of Methamphetamine induced neuroinflammation. ImmunoHUB &NCBio ERA Chairs Annual Meeting. Porto, Portugal. December 2024.

Lorga I., Magalhães A., Teixeira, A., Vilanova M., Ribot J. C. and Bonifácio Andrade E. Altered meningeal $\gamma\delta$ T cells contribute to neurodevelopment impairment in neonatal bacterial meningitis. Congresso Internacional de Imunologia. Cape Town, South Africa. Nov-Dec 2023.

Lorga I., Magalhães A., Teixeira, A., Vilanova M., Ribot J. C. and Bonifácio Andrade E. Long-term behavioural sequelae associate with an altered meningeal immune compartment after bacterial neonatal meningitis. XLVIII Annual Meeting of the Portuguese Society for Immunology. Aveiro, Portugal. March 2023.

Soares J., Lorga I., Bravo J., Summavielle T., Vilanova M. and Bonifácio Andrade E. Microglia response to neonatal bacterial meningitis. XLVIII Annual Meeting of the Portuguese Society for Immunology. Aveiro, Portugal. March 2023.

Teixeira A., Lorga I., Vilanova M. and Bonifácio Andrade E. Study of the lung immune response to Group B Streptococcus neonatal infection. XLVII Annual Meeting of the Portuguese Society for Immunology. Lisboa, Portugal. April 2022.

Bravo J., Bonifácio Andrade E., Portugal C. C., Vieira R., Lorga I., Ribeiro I., Peralta G., Azevedo M., Sousa M., Magalhães A., Relvas J. and Summavielle T. Psychostimulants and neuroinflammation: finding critical players in the crosstalk between glial cells and neurons. V Symposium Portuguese Glial Network. Coimbra, Portugal. November 2021.

Comunicações Orais

Lorga I., Magalhães A., Teixeira, A., Vilanova M., Ribot J. C. and Bonifácio Andrade E. Meningeal $\gamma\delta 17$ T cells and blood-brain barrier disruption: implications for neonatal Group

B Streptococcus meningitis. European Congress of Immunology (ECI). Dublin, Ireland. September 2024. – Bright Sparks Award

Lorga I., Magalhães A., Teixeira, A., Vilanova M., Ribot J. C. and Bonifácio Andrade E. New players in neonatal bacterial meningitis: The role of meningeal $\gamma\delta$ T cells. 10th International $\gamma\delta$ T cell conference. Lisboa, Portugal. June 2023.

Lorga I., Magalhães A., Teixeira, A., Vilanova M., Ribot J. C. and Bonifácio Andrade E. Friend or foe: The role of $\gamma\delta 17$ T cells in neonatal bacterial meningitis. I ImmunoHub. Porto, Portugal. March 2023.

Lorga I., Magalhães A., Vilanova M., Ribot J. C. and Bonifácio Andrade E. Disclosing the role of meningeal immunity during Group B Streptococcus neonatal meningitis. 9th Molecular and Cell Biology Symposium. Porto, Portugal. February 2023. – Best Oral Presentation Award

Lorga I., Vilanova M., Ribot J. C. and Bonifácio Andrade E. Uncovering meningeal immunity upon Group B Streptococcus neonatal infection. XLVII Annual Meeting of the Portuguese Society for Immunology. Lisboa, Portugal. April 2022.

RESUMO

Os recém-nascidos são altamente suscetíveis a doenças infecciosas. Dado o ambiente quase estéril no útero e a sua limitada imunidade adaptativa, estes dependem principalmente do sistema imunitário inato para combater agentes patogénicos. A bactéria *Streptococcus agalactiae*, comumente referida como Estreptococos do Grupo B (EGB), continua como o principal agente de infeções bacterianas no período neonatal, frequentemente causando pneumonia, septicémia e/ou meningite. O EGB coloniza a mucosa vaginal de até 40% das mulheres grávidas em todo o mundo e é tipicamente transmitido verticalmente da mãe para o filho durante o parto. A mortalidade associada ao EGB pode alcançar cerca de 30% nos países em desenvolvimento, com uma média global de até 15%. Além disso, até 50% dos bebés que sobrevivem a esta infeção sofrem de Perturbações do Neurodesenvolvimento (PND). Contudo, os mecanismos subjacentes às PND permanecem pouco compreendidos. Embora estudos prévios com modelos de infeção localizados no cérebro e hematogénicos ou análises de amostras pós-morte tenham levado à hipótese de que o PND resulta de uma infiltração massiva de células imunitárias no cérebro, causando danos no tecido, o nosso modelo de murganho clinicamente relevante, que imita a transmissão vertical humana e a fisiopatologia da doença, não sustentou esta hipótese.

Esta tese teve como objetivo desvendar o mecanismo subjacente à PND na meningite neonatal causada por EGB. Para estudar a disseminação bacteriana e a progressão da doença, desenvolvemos estirpes bioluminescentes de EGB de modo a permitirem estudos longitudinais da doença causada por esta bactéria. Inicialmente, o EGB foi transformado com o plasmídeo pSL101P32, contendo o operão *luxABCDE* que codifica uma luciferase bacteriana e o seu substrato. No entanto, esta estirpe é inadequada para estudos *in vivo*, pois não foi detetado sinal bioluminescente. Em seguida, o EGB foi transformado com o plasmídeo pLZ12Km2-P23R:TA:ffluc, que codifica a luciferase do pirilampo com desvio para o comprimento de onda vermelho, necessitando por isso da administração de luciferina como substrato, mas proporcionando uma melhor penetração do tecido. No entanto, no nosso modelo de infeção vaginal, o EGB eliminou o plasmídeo tornando-o inadequado para estudos de longa duração *in vivo*. Por fim, o gene da luciferase foi integrado no genoma do EGB para ultrapassar a perda do plasmídeo. Contudo, a cópia única do gene da luciferase resultou num sinal bioluminescente mais fraco, insuficiente para detetar diferenças na colonização no cérebro dos murganhos infetados. Assim, os animais foram sacrificados nos primeiros 5 dias de vida para estudar a progressão da doença. Como esperado, os murganhos infetados apresentaram uma redução significativa

do peso corporal durante os primeiros cinco dias de vida. Contudo, a avaliação clínica da gravidade da doença e da previsão de mortalidade revelou-se inútil na meningite por EGB. Exceto em casos de extrema severidade, não foi possível prever a mortalidade, pois mesmo animais com pontuações baixas morriam. Os níveis bacterianos no cérebro mantiveram-se consistentemente altos desde o dia pós-natal (P) 1, atingindo o pico em P3. No entanto, as pontuações da avaliação clínica da doença não se correlacionaram com a carga bacteriana no parênquima cerebral, mostrando que a gravidade da infecção neste órgão não se relaciona com os níveis bacterianos. Estes resultados alinham-se com a imprevisibilidade da morte e a variabilidade nas cargas bacterianas observadas em infecções neonatais humanas por EGB, reforçando a relevância clínica do nosso modelo de murganho.

Utilizando este modelo de infecção para estudar os mecanismos da meningite neonatal que levam à PND, não encontramos evidências de infiltração de células imunitárias no parênquima cerebral, contrariamente ao descrito em estudos anteriores. Em vez disso, usando sequenciação de RNA de célula única e citometria de fluxo, os nossos resultados revelaram alterações significativas no perfil imune meníngeo durante a meningite bacteriana neonatal. Os murganhos infetados apresentaram uma acumulação de células T $\gamma\delta$ nas meninges. Embora o perfil transcricional de IL-17 destas células esteja alterado, com uma expressão reduzida da mesma, não existem diferenças no repertório do recetor das células T (TCR), que é maioritariamente pertencente ao subconjunto V γ 6, produtor de IL-17. De facto, o nível geral de IL-17 está aumentado nas meninges dos animais infetados. É importante notar que as células T $\gamma\delta$ produtoras de IL-17 (células T $\gamma\delta$ 17) mantiveram-se elevadas ao longo da vida após o episódio de meningite neonatal. Assim, para entender o papel das células T $\gamma\delta$ e da IL-17 nesta doença, realizámos estudos de infecção em murganhos deficientes em células T $\gamma\delta$ (Tcrd^{-/-}) ou IL-17 (IL-17^{-/-}). Ambos os genótipos não apresentaram diferenças na sobrevivência, mas exibiram níveis significativamente reduzidos de carga bacteriana no cérebro em P3. Adicionalmente, o bloqueio local da IL-17, através de injeção de anticorpos anti-IL-17 na cisterna magna em P1, resultou numa diminuição significativa dos números de EGB no cérebro, em comparação com murganhos que receberam o controlo isotípico IgG. Estes resultados revelam que as células T $\gamma\delta$ 17 afetam negativamente a proteção cerebral, facilitando a invasão do parênquima pelo EGB durante a meningite neonatal. Assim, hipotetizámos que as células T $\gamma\delta$ 17 poderiam comprometer a integridade da barreira hematoencefálica (BHE). A administração *in vivo* de FITC-dextrano por via subcutânea em P1 mostrou um aumento significativo da permeabilidade da BHE em murganhos da estirpe selvagem (do Inglês wild-type, WT) em comparação com os controlos não infetados. Por outro lado, os recém-nascidos Tcrd^{-/-}

apresentaram uma menor perturbação da BHE comparado com os animais WT com meningite, sugerindo que as células T $\gamma\delta$ facilitam a invasão cerebral por EGB através da ruptura da BHE. Ao analisar a microglia em murgos WT e Tcrd^{-/-} com meningite, os primeiros apresentaram uma diminuição significativa no número de microglia, mas estas estavam altamente ativadas. Contrariamente, os murgos Tcrd^{-/-} não apresentaram diferenças na microglia. Além disso, os murgos infectados com estirpes de GBS não invasoras do cérebro não apresentaram aumento de células T $\gamma\delta$, mostraram menor infecção cerebral e nenhuma ativação da microglia. Em suma, estes resultados sugerem um mecanismo entre as células T $\gamma\delta 17$, a produção de IL-17 e a ativação da microglia durante a meningite. Como a meningite por EGB está associada a PND e como as células T $\gamma\delta 17$ foram recentemente relacionadas à regulação da ansiedade, avaliamos os marcos de desenvolvimento e o comportamento dos animais WT e Tcrd^{-/-} sobreviventes à meningite neonatal. Durante o desenvolvimento, os sobreviventes WT adquiriram reflexos sensoriais significativamente mais cedo do que os animais controles e os Tcrd^{-/-}, podendo relacionar-se com défices de atenção e comportamentos semelhantes aos da perturbação de hiperatividade e défice de atenção (PHDA), visto que a hipersensibilidade é frequentemente encontrada em crianças com esta condição. Na idade adulta, tanto os sobreviventes WT como os Tcrd^{-/-} apresentaram défices cognitivos, mostrando que as células T $\gamma\delta$ não estão diretamente envolvidas nesta sequela neurológica. Contudo, apenas os sobreviventes WT exibiram hiperatividade, evidenciada por uma maior distância percorrida e uma maior velocidade média no teste de campo aberto (do inglês open field). Além disso, esta maior atividade foi restrita às bordas do campo, sendo que tempo passado em cada área não foi diferente, indicando um aumento da ansiedade. Estas características de hiperatividade e ansiedade estão frequentemente interrelacionadas e estão presentes em 25% das crianças com PHDA, reforçando os traços de PHDA observados durante o período de lactação. Concluindo, a meningite neonatal por EGB leva à acumulação de células T $\gamma\delta 17$ nas meninges, perturbando a BHE e facilitando a invasão cerebral pelo EGB. Além disso, a microglia é ativada em resposta à IL-17 e/ou ao EGB. Os sobreviventes da meningite neonatal por EGB exibem comportamentos semelhantes ao PHDA, incluindo hiperatividade e ansiedade. Estes resultados revelam um novo mecanismo de PND na meningite neonatal por EGB.

Dado que as infeções neonatais por EGB causam frequentemente pneumonia, estudamos as interações neuroimunes nos pulmões no nosso modelo de murganho. Demonstramos que as células T $\gamma\delta$ estão significativamente aumentadas em murgos infectados, assim como os neutrófilos, de acordo com o estado de infecção do tecido. Contudo, o fenótipo dos neutrófilos variou com a gravidade da doença. Em murgos com doença moderada,

observámos um aumento significativo de neutrófilos que expressam o marcador de superfície específico dos eosinófilos SiglecF, um fenótipo associado a uma maior capacidade fagocítica e eliminação bacteriana. Pelo contrário, os recém-nascidos gravemente doentes apresentaram uma maturação dos neutrófilos para o fenótipo SiglecF^{hi} reduzida, resultando numa menor eliminação bacteriana e maior progressão da doença. Além disso, demonstrámos que a gravidade da doença está associada ao aumento da expressão do péptido relacionado ao gene da calcitonina (CGRP) nos pulmões. O CGRP atua nos neutrófilos, suprimindo a sua ativação para o fenótipo SiglecF^{hi}. Assim, estes resultados sugerem que o EGB explora o eixo neuroimune pulmonar para escapar à imunidade do hospedeiro através da supressão ativação dos neutrófilos mediada pelo CGRP.

Concluindo, caracterizámos a meningite neonatal por EGB e as alterações associadas na imunidade meníngea, revelando um novo mecanismo subjacente ao PND. Além disso, revelámos interações neuroimunes nos pulmões de murganhos infectados por EGB, destacando a sua contribuição para a disseminação bacteriana e progressão da doença.

SUMMARY

Neonates are highly susceptible to infectious diseases. Given the almost sterile environment *in utero* and their limited adaptive immunity, neonates primarily rely on their innate immune system to fight pathogens. *Streptococcus agalactiae*, commonly referred to as Group B *Streptococcus* (GBS), is the leading infection agent during the neonatal period, frequently leading to pneumonia, sepsis, and/or meningitis. GBS colonises the vaginal mucosa of up to 40% of pregnant women worldwide and is typically transmitted vertically from mother to child during birth. GBS-associated mortality can reach approximately 30% in developing countries, with a global average of up to 15%. Moreover, up to 50% of survivors suffer from permanent neurodevelopment impairment (NDI). However, the mechanisms underlying NDI remain poorly understood. Although previous studies using diverse localised and haematogenous infection models, or post-mortem sample analysis led to the assumption that NDI results from massive infiltration of immune cells into the brain parenchyma causing tissue damage our clinically relevant mouse model, which mimics human vertical transmission and disease pathophysiology, did not support this hypothesis.

This thesis aimed to uncover the mechanism underlying NDI upon GBS neonatal meningitis. To study bacterial dissemination and disease progression, we engineered GBS bioluminescent strains to perform longitudinal disease studies. We initially transformed GBS with the plasmid pSL101P32, containing the *luxABCDE* operon encoding a bacterial luciferase and its substrate. However, this strain proved unsuitable for *in vivo* studies, as no bioluminescent signal was detected. We then transformed GBS with the plasmid pLZ12Km2-P23R:TA:ffluc, encoding for the red-shifted firefly luciferase, requiring luciferin administration as substrate, but providing improved tissue penetration. However, in our vaginal infection model, GBS was able to dispose of the plasmid, rendering it unsuitable for long-term *in vivo* studies. Lastly, we integrated the red-shifted firefly luciferase gene into the GBS genome to overcome plasmid loss. Despite this, the single copy of the luciferase gene resulted in a weak bioluminescent signal, insufficient to detect brain colonisation differences in infected pups. Thus, animals were sacrificed during the first 5 days of life to study the disease progression. Infected pups present an expected significantly reduced body weight during the first five days of life. However, clinical scoring, used to evaluate disease severity and predict mortality, revealed nonutility in GBS meningitis. Except in cases of extreme severity, predicting mortality was not possible, as animals often died even at minimal clinical scores. In addition, brain bacterial levels were consistently high from postnatal day (P) 1, peaking at P3. Importantly, clinical scores did not correlate with brain parenchyma bacterial load, indicating that the severity of the infection in this organ was unrelated to bacterial

levels. These results align with the unpredictability of death and variability in bacterial loads observed in neonatal human GBS infection, reinforcing the clinical relevance of our mouse model.

Using this infection model to uncover the mechanisms of neonatal meningitis leading to NDI, we find no evidence of parenchymal immune cell infiltration, contrary to what has been described in previous studies. Instead, using single-cell RNA sequencing and multiparameter flow cytometry, our findings revealed significant alterations in the meningeal immune landscape during meningitis. Infected pups exhibited an accumulation of $\gamma\delta$ T cells in the meninges. While the IL-17 transcription profile of these cells is altered, with decreased expression of IL-17, there are no differences in their TCR repertoire being biased towards the IL-17 producers V γ 6 subset. Indeed, the overall IL-17 level is increased in the meningeal environment of infected animals. Importantly, IL-17-producing $\gamma\delta$ T cells ($\gamma\delta$ 17 T cells) remained elevated throughout the lifespan following neonatal meningitis. To understand the role of $\gamma\delta$ T cells and IL-17, we performed infection studies in neonates deficient in $\gamma\delta$ T cells and IL-17. Both genotypes presented no differences in survival but exhibited significantly decreased levels of bacterial load in the brain parenchyma at P3. Additionally, local blockade of IL-17 via injection of anti-IL-17 antibodies in the cisterna magna at P1 resulted in significantly decreased GBS numbers in the brain compared to pups receiving the IgG isotype control. These results reveal $\gamma\delta$ 17 T cells detrimentally affect brain protection by facilitating GBS parenchymal invasion during neonatal meningitis. We thus hypothesised that $\gamma\delta$ 17 T cells could impair blood-brain barrier (BBB) integrity. *In vivo* administration of FITC-dextran subcutaneously at P3 showed significantly increased BBB permeability in wild-type (WT) pups compared to uninfected controls. Conversely, *Tcrd*^{-/-} newborns showed decreased BBB disruption compared to WT animals suffering from meningitis, suggesting $\gamma\delta$ T cells as facilitators of GBS parenchymal invasion through BBB breakdown. When analysing microglia in WT and *Tcrd*^{-/-} pups with meningitis, the firsts show significantly decreased microglia numbers, but these cells were highly activated. In contrast, *Tcrd*^{-/-} pups showed no microglial differences. Furthermore, pups infected with brain less invasive GBS strains presented no increase in $\gamma\delta$ T cells, reduced brain infection, and no microglia activation. Altogether, these results suggest a mechanism between $\gamma\delta$ 17 T cells, IL-17 production, and microglia activation during meningitis. As GBS meningitis is associated with NDI and $\gamma\delta$ 17 T cells have been recently linked to anxiety regulation, we evaluated the developmental milestones and adult behaviours of WT and *Tcrd*^{-/-} animals surviving meningitis. During development, WT pups suffering from meningitis acquire sensorial reflexes significantly earlier than both the controls and *Tcrd*^{-/-} pups, which may relate to attention deficit and hyperactivity disorder (ADHD)-like behaviour, as

hypersensitivity is commonly found in children with this condition. In adulthood, both WT and Tcrd^{-/-} survivors exhibited cognitive impairment, showing that $\gamma\delta$ T cells are not directly involved in cognitive deficits. However, only WT survivors displayed hyperactivity, evidenced by a significantly increased distance travelled and higher average speed in the open field arena. Additionally, this increased activity was confined to the borders of the arena, while no change in time spent in each area, being indicative of heightened anxiety. These hyperactivity and anxiety traits are commonly related and present in 25% of children with ADHD, reinforcing the ADHD traits observed during the lactation period. In summary, neonatal GBS meningitis leads to $\gamma\delta 17$ T cell accumulation in the meninges, disrupting the BBB and facilitating GBS parenchymal invasion. Moreover, microglia are activated in response to IL-17 and/or GBS. Survivors of neonatal GBS meningitis exhibited ADHD-like behaviour, including hyperactivity and anxiety. These results reveal a novel mechanism of NDI in GBS neonatal meningitis.

As GBS neonatal infections frequently lead to pneumonia, we aimed to uncover the neuroimmune interactions in the lungs using our mouse model. We show that $\gamma\delta$ T cells are significantly increased in infected pups, as well as neutrophils, in line with the tissue infection status. However, neutrophils' phenotype varies with disease severity. In pups with moderate disease, we observed a significant increase in neutrophils co-expressing the eosinophil-specific surface marker SiglecF (sialic acid-binding immunoglobulin-like lectin F), a phenotype associated with enhanced phagocytic capacity and bacterial clearance. Conversely, severely sick newborns present impaired neutrophil maturation into the SiglecF^{hi} phenotype, resulting in reduced bacterial clearance and increased disease progression. Moreover, we show that disease severity is associated with increased expression of calcitonin gene-related peptide (CGRP) in the lungs. CGRP acts on neutrophils, suppressing their activation into the SiglecF^{hi} phenotype. Thus, these findings suggest that GBS exploits the lung neuroimmune axis to evade host immunity through CGRP-mediated suppression of neutrophil activation.

In conclusion, we have characterised GBS neonatal meningitis and associated alterations in meningeal immunity, uncovering a novel mechanism underlying NDI. Moreover, we demonstrated new insights into the neuroimmune interactions in the lungs of GBS-infected pups, highlighting their contribution to bacterial dissemination and disease progression.

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ABBREVIATION LIST

Ach	Acetylcholine
ADHD	Attention deficit hyperactivity disorder
APC	Antigen-presenting cell
APP	Antimicrobial peptides and proteins
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BBB-ECs	BBB endothelial cells
BC	Before Christ
BCSFB	Blood-cerebrospinal fluid barrier
BLI	Bioluminescence imaging
BMP2	Bone morphogenic protein 2
B _{regs}	Regulatory B cells
CA	Cornu ammonis
CC	Clonal complex
CCL	C-C motif chemokine ligand
CCR	Chemokine receptor
CDC	Centers for Disease Control
CFU	Colony-forming unit
CGRP	Calcitonin gene-related peptide
CLN	Cervical lymph nodes
CNS	Central nervous system
CPS	Capsular polysaccharides
CSF	Cerebrospinal fluid
CXCR	CXC motif chemokine receptor
CXCR	CXC motif chemokine receptor
CysLTs	Cysteinyl leukotrienes
DC	Dendritic cells
DGAV	Direção Geral de Alimentação e Veterinária
EAE	Experimental autoimmune encephalomyelitis
EGFR	Epidermal growth factor receptor
ENS	Enteric nervous system
EOD	Early onset disease
FM	Foramen magnum

G	Gestational day
GABA	γ -aminobutyric acid
GAS	Group A <i>Streptococcus</i>
GBS	Group B <i>Streptococcus</i>
G-CSF	Granulocyte colony-stimulating factor
GDNF	Glial-derived neurotrophic factor
GF	Germ-free
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hpi	Hours post-infection
HvgA	Hypervirulent GBS adhesin
i.p.	Intraperitoneal
i.vag.	Intra-vaginal
IAP	<i>Intrapartum</i> antibiotic prophylaxis
IAV	Influenza A virus
ICAM	Intercellular adhesion molecule
ICAM-1	Intracellular adhesion molecule 1
Ig	Immunoglobulin
IL	Interleukin
IL-17R	IL-17 receptor
ILC	Innate lymphoid cell
INF	Interferon
ISF	Interstitial fluid
LB	Luria Bertani
LCMV	Lymphocytic choriomeningitis virus
LFA-1	Ligand leukocyte function-associated antigen 1
LOD	Late onset disease
LP	Lumbar puncture
LpR2	Lipophorin receptor 2
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LV	Lymphatic vessel
M-CSF	Macrophage colony-stimulating factor
MDSC	Myeloid-derived suppressor cells
MFI	Mean fluorescence intensity
MIA	Maternal immune activation
MMA	Middle meningeal artery

MOI	Multiplicity of infection
MS	Multiple sclerosis
NDI	Neurodevelopmental impairment
NET	Neutrophil extracellular trap
NICU	Neuroimmune cell unit
NK	Natural killer
NMU	Neuromedin U
OD	Optical density
ON	Overnight
OPC	Oligodendrocyte precursor cell
P	Postnatal day
PMA	Phorbol myristate acetate
PNEC	Pulmonary neuroendocrine cells
PPA	Pterygopalatine artery
PVL	Periventricular leukomalacia
PVN	Paraventricular nucleus of the hypothalamus
ROI	Region of interest
ROS	Reactive oxygen species
RT-qPCR	Real Time qPCR
S1P2	Sphingosine 1-phosphate receptor 2
SAS	Subarachnoid space
SCFA	Short-chain fatty acids
SEM	Standard errors of the mean
SFO	Subfornical organ
SL-1	Sulpholipid 1
SLYM	Subarachnoid lymphatic-like membrane
Srr2	Serine-rich repeat protein
TA	Toxin-antitoxin
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TH	Todd-Hewitt
THY	Todd-Hewitt yeast
TLR	Toll-like receptor
TNF	Tumour necrosis factor
T _{regs}	Regulatory T cells

VCAM-1	Vascular cell adhesion molecule 1
VIP	Vasoactive intestinal peptide
WHO	World Health Organisation
WT	Wild-type
ZO	Zonula occludens proteins
β2AR	β2-adrenergic receptor

PRESENTATION OF THE PROJECT

Improving newborn and child health is a World Health Organisation priority and a part of the Sustainable Development Goals for 2030, which aim to reduce both neurological burden and mortality associated with infection diseases.

This thesis focused on the characterisation and comprehension of the complex interactions between the immune and nervous systems during neonatal infection, involved in the neurodevelopmental impairment (NDI) observed in up to 50% of survivors of neonatal meningitis caused by Group B *Streptococcus* (GBS). The research seeks to elucidate the mechanisms underlying these impairments to contribute to better therapeutic and preventative strategies.

The results of this thesis focused on the main goals:

1. To study the kinetics of bacterial dissemination during neonatal GBS infection, alongside the onset of symptomatology and disease progression;
2. To characterise the dynamics of disease-specific immune cells and their transcriptional profiles in the meningeal spaces during neonatal meningitis;
3. To unravel the mechanisms responsible for GBS-induced NDI;
4. To characterise the neuroimmune interactions during neonatal GBS infection.

THESIS OUTLINE

The present thesis comprises three main chapters: Introduction, Results and Discussion.

Chapter I provides a general introduction that reviews the main topics addressed in this thesis, specifically the neonatal immune system, meningeal immunity, and neuroimmune interactions in tissue physiology.

Chapter II is the Results section, which includes a set of studies presented as three final manuscripts.

The general discussion of the work is presented in Chapter III, intending to illustrate the major contributions of this research to understanding the mechanisms responsible for the neonatal neurodevelopmental impairment caused by Group B *Streptococcus* meningitis.

CHAPTER I

INTRODUCTION

In the most medically and technologically advanced era, over 2.5 million infants under 5 years old die every year worldwide due to infections, with newborns accounting for more than 50% of deaths^{1,2}. Infant infections are common and often severe, ranking among the top three causes of neonatal death and representing approximately 20% of all neonatal mortality cases³⁻⁶. Moreover, despite advancements in reducing mortality, the incidence of neonatal infections is increasing, particularly due to increasing antibiotic resistance⁷.

The heightened susceptibility to infection during this particular period of life may be attributed to three key factors: 1. the unique characteristics of the neonatal immune system, which favour tolerance, 2. the limited time for infants to programme and mount a strong innate and adaptive immune response and 3. the fact that it is the first exposure to a gigantic number and diversity of microorganisms, as transiting from a close to sterile intra-uterine environment to a world led by microbes. Therefore, there is a demand for a better understanding of the neonatal immune system particularities and mechanisms contributing to infection susceptibility that will allow the development of new and efficient preventive therapeutics.

I. THE NEONATE

The uniqueness of the perinatal period

Humans are in constant contact and interaction with microorganisms. We are provided with physical barriers and an immune system that develops and specialises in distinguishing beneficial or prejudicial, ultimately leading to health or disease, respectively. However, developing specialised tissues and cells capable of fighting and eliminating pathogens is still in course during the first years of life. In addition to the genetically programmed maturation, the infant's immune system is profoundly influenced by the different antigens encountered throughout this stage of life, showcasing its tremendous plasticity power.

The first immune challenge begins even before the baby is born. During gestation, the foetus faces two types of potential harm: infection at the maternal-foetal interface and maternal pro-inflammatory responses. Therefore, the maternal immune system suffers major adaptations to confer immune tolerance to the foetus, while maintaining protection against pathogens, in order to avoid preterm birth or spontaneous abortion. The maternal-foetal communication starts immediately after embryo implantation. As the placenta forms, the embryo produces human chorionic gonadotropin, leading to an anti-inflammatory environment. This process includes increased activity and number of regulatory cells (T_{regs}), reduced levels of T helper (Th) 17 and cytotoxic $CD8^+$ T cells, decreased reactive state of dendritic cells (DCs), suppression of inflammatory cytokines, and increased production of anti-inflammatory interleukin (IL)-10⁸⁻¹⁵ (Figure 1). Trophoblasts, which form the majority of the placenta, produce interferon (IFN)-stimulated genes to polarise macrophages from the M1 to M2 phenotype, favouring Th2 responses and decreasing inflammation^{16,17}. Moreover, the complete formation of the placenta constitutes a strong barrier against infections per se¹⁸⁻²⁰, conferring to the foetus a close to sterile environment. Foetal barriers, such as the skin, intestine, nasal cavity and blood produce antimicrobial proteins and peptides (APPs) and proteins with microbicidal and microbial-neutralising properties, important for host defence²¹ (Figure 1). Under intrauterine infectious conditions, a pro-inflammatory environment accelerates labour. Up to 40% of preterm labours are due to infections, a likely underestimated value given the difficulty in detecting intrauterine infection²² and the stigma regarding reporting stillbirths even in high income countries²³. Additionally, infections are responsible for 50% of stillbirths²⁴.

The second major immune challenge occurs at birth. After a successful pregnancy in an almost sterile environment, the baby is born into a drastically different environment, being immediately exposed to a wide variety of antigens. Some of these antigens are essential for the immune system development, while others may be detrimental to their health as they

can cause severe infections. Within moments after birth, the skin²⁵ and the intestine^{26–28} become colonised by microbiota. As the skin and gut colonisation are extremely important for protection against pathogens and for the development of the immune system, the neonatal immune system works in two apparently opposite directions, fighting several potential microbes while tolerating others (Figure 1).

During the first days of life, the neonate is covered by a waxy coat, called vernix caseosa²⁹. This coat is produced by the foetal sebaceous glands *in utero* to confer protection during and upon birth³⁰. The gut also increases the production of APPs, and significant structural changes occur, including the development of crypts and Paneth cells³¹. Moreover, the maternal antibodies circulating in the newborn's blood confer additional protection during this early period³² (Figure 1). Breastfeeding extends this protection while the neonatal immune system matures as exposure to environmental microbes drives its development^{33,34}.

Preterm babies face a heightened risk of severe infections, as the skin and gut barriers are not fully developed to prevent infection gateways into the newborn's body. Additionally, their immune system is unprepared to encounter and properly combat pathogens, leaving them particularly vulnerable during this crucial stage.

The neonatal immune system

The human immune system starts to develop *in utero* by the third-fourth week of gestation and is still not fully developed at birth³⁵. During gestation, the foetus has minimal environmental exposure to antigens, limiting the development of immune memory. As a result, neonates are born without adaptive immune memory and must initially rely on their innate immune system for protection³⁶. However, at birth, the immune system faces sudden and overwhelming exposure to a wide range of antigens while still maturing. Furthermore, neonatal innate immunity itself is underdeveloped, with innate immune cells being less polyfunctional and producing fewer cytokines compared to their adult counterparts³⁷. Consequently, the neonate's immune response is slower and less robust during this critical period, increasing the vulnerability to severe infections. Newborns produce low levels of immunoglobulin (Ig) G³⁸ and present limited capacity for antibody responses to bacterial capsular polysaccharides, making them particularly susceptible to encapsulated bacteria^{39–41}. In accordance, this type of bacteria is predominantly responsible for bacteraemia and meningitis^{42,43}. Therefore, neonates largely depend on the maternal transfer of IgG across the placenta, mainly during the third trimester, and IgA through breast milk^{44,45} (Figure 1).

These maternal antibodies provide passive immunity, helping in protecting neonates against enteric and other infections during early life.

The neonatal immune system is predominantly regulated by immunosuppressive mechanisms to prevent massive inflammatory responses that could harm the neonate, given the high exposure to bacterial colonisation³². Antigen-presenting cells (APCs) produce lower levels of pro-inflammatory cytokines, such as Tumour Necrosis Factor (TNF- α) and IL-1 β , reducing Th1-derived responses⁴⁶. This immune modulation may partly explain the particular vulnerability to intracellular pathogens. Additionally, neonatal monocytes and DCs produce higher levels of anti-inflammatory IL-10 compared to adults, reinforcing the tolerogenic environment that supports microbiota colonisation in early life³² (Figure 1).

Neutrophils are the dominant leukocytes at birth and exceed the number of adult neutrophils in circulation during the first week of life (Figure 1). However, neonatal neutrophils have a reduced ability to adhere and extravasate from the bloodstream and lower chemotaxis activity^{37,47}, limiting their recruitment to sites of infection and inflammation. Their capacity to release bactericidal substances such as reactive oxygen species, to form neutrophils extracellular traps (NETs), and perform phagocytosis is also diminished^{37,47}, increasing the risk of bacteraemia. Pathogen killing is further weakened in the neonatal period due to the significantly lowered cytotoxicity of natural killer (NK) cells, which the poor levels of activating cytokines may explain³⁷.

Myeloid-derived suppressor cells (MDSC) accumulate in the maternal circulation and placenta^{48,49}. These cells play a key role in maternal-foetal tolerance, as women with miscarriage presented decreased MDSC levels^{49,50}. As their name says, they contribute to the suppression of T cell and NK T cell functions, polarising towards a Th2 tolerance response (as reviewed elsewhere⁵¹). These cells also accumulate in the cord blood and persist in the neonate until 6 weeks after birth, displaying similar functions to those of maternal MDSC, thereby maintaining immune tolerance in the neonate^{49,51,52} (Figure 1).

Complement-mediated immunity is also impaired during the neonatal period, with lower concentration of complement proteins and reduced complement-mediated opsonic capabilities compared to adults^{53–55} (Figure 1). This deficiency hinders cell recruitment to infection sites, opsonisation and pathogen lysis^{53–55}. Similarly, neonatal $\gamma\delta$ T cells are also less functional than those from adults, displaying a decreased proliferation rate and reduced ability to produce cytokines, perforin and granzyme B⁵⁶.

The neonatal adaptive immune system starts to develop before birth but with poor functionality, further contributing to neonatal susceptibility to infections. Neonatal T cells, unlike adult ones, are partly derived from the foetal liver⁵⁷. Neonatal T cells are fewer in

number compared to adult T cells, although they exhibit high proliferative potential upon antigen stimulation^{57,58}. However, these cells present a limited T cell receptor (TCR) repertoire and lower capacity to differentiate into long-lasting memory T cells, which is a challenge in protecting the host against the multitude of pathogens encountered in early life^{57,58}. Additionally, CD4⁺ T cells in the neonatal period are biased towards Th2 and T_{regs} differentiation, favouring immune tolerance and microbiota colonisation^{55,57}, while simultaneously increasing vulnerability to certain pathogens.

Neonatal B cells are predominantly naïve and immature, primarily consisting of transitional 1 and 2 B cells⁵⁹. Their antibody responses are delayed, with reduced ability to undergo

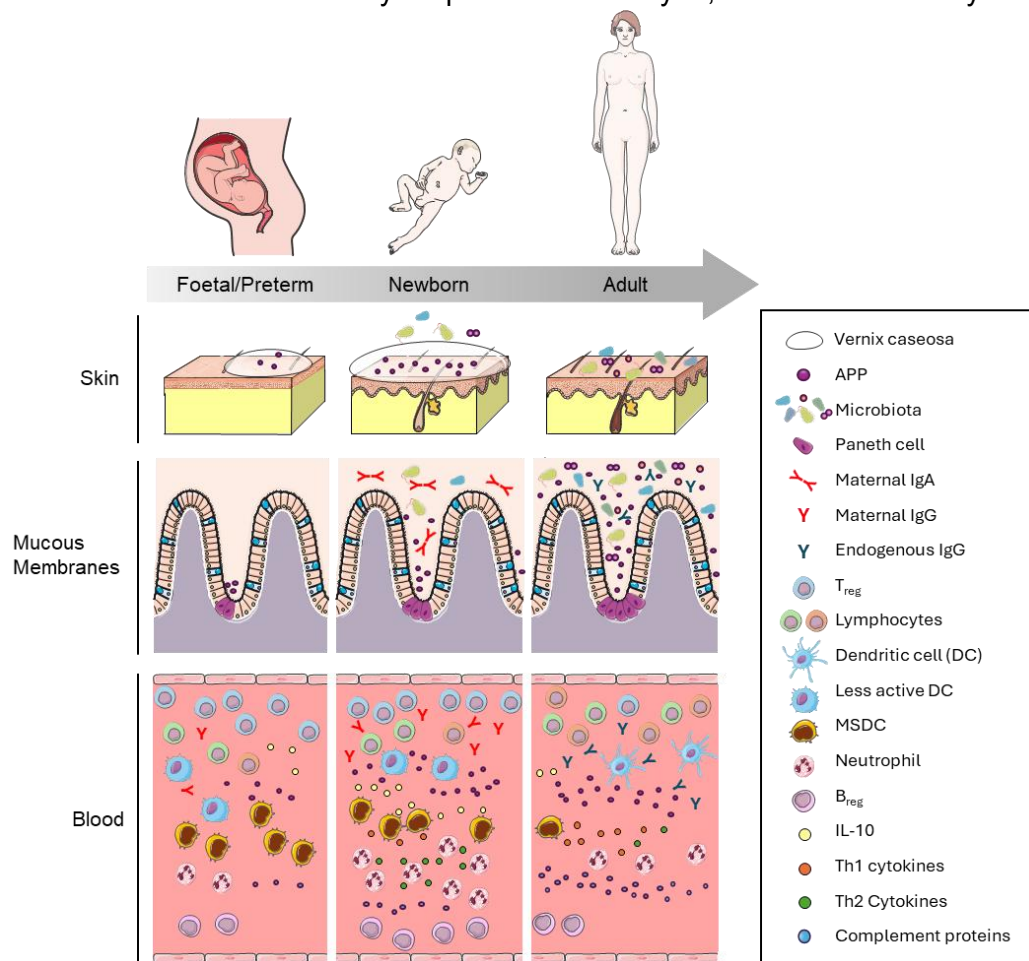


Figure 1. The perinatal period is equipped with structural and immune particularities to favour a tolerant environment. During gestation, the newborn starts to produce antimicrobial proteins and peptides (APPs) and a skin coat called vernix caseosa to protect against pathogens during and right after birth. In parallel, the mucous membranes, specifically in the gut, change dramatically with increased crypts and Paneth cells, heightening APPs production. Moreover, during the neonatal phase, the gut becomes colonised and maternal IgA is present. In blood circulation, increased T_{regs}, myeloid suppressor-derived cells (MSDCs), neutrophils and B_{regs} are present compared to adults. The dendritic cells (DCs) and lymphocytes are less active, and neutrophils present decreased phagocytic and migration capacities. Th1 cytokines are suppressed in opposition to Th2, and IL-10 is significantly expressed during the neonatal period. Additionally, complement proteins are also produced. This illustration is for explanatory purposes and is neither drawn to scale nor depicting immunity with precise proportions.

class switching⁶⁰. Furthermore, naïve B cells express low levels of cell-surface receptors, such as CD40, CD80, and CD86, required for activation, proliferation and germinal centre formation⁶¹. This immaturity contributes to weaker and slower adaptive immune responses during the neonatal period. Additionally, CD24^{hi}CD38^{hi} regulatory B cells (B_{regs}) are increased in neonates^{62,63} (Figure 1). These cells produce high levels of IgM and IL-10, limiting Th1 responses and thus favouring a tolerogenic neonatal environment while risking higher neonatal susceptibility infection^{62–64}.

Overall, the neonatal period is characterised by unique immune features regarding both innate and adaptive immunity, often perceived as immature or impaired compared to adults. However, these differences are key for tolerating intestinal colonisation and supporting the development of a fully functional immune system *per se*.

Neonatal bacterial meningitis

Meningitis is defined as an inflammation of the membranes covering the brain and spinal cord, called meninges⁶⁵. The first symptoms of what meningitis could be came from no other than the father of medicine, Hippocrates (c. 460-370 BC), who noticed an inflammation in the lining of the brain⁶⁶. Later, in 1661, Thomas Willis (1621-1675) wrote one of the earliest definitions of meningitis, by observing patients with “continual raving or the depravation of the chief faculties of the brain arising from inflammation of the meninges with a continual fever”⁶⁷. However, the term “meningitis” was only introduced at the beginning of the 19th century, by the Scottish and French physicians John Abercrombie and Félix Herpin, respectively, combining the word “meninges” with the suffix “-itis”, which implies inflammation^{68,69}.

Neonatal meningitis is a leading cause of neonatal mortality and morbidity worldwide. The global incidence varies significantly by region and is influenced by healthcare infrastructures, access to vaccination, hygiene practices, and neonatal quality care. Mortality rates are high, especially in low- and middle-income countries, reaching 25-58%. Of note, in low-income countries, numbers may be underestimated given the lack of diagnostic facilities and resources. In contrast, high-income countries report lower mortality rates, generally ranging from 10-15%⁷⁰. Besides mortality, neonatal meningitis is associated with significant morbidity. Among survivors, up to 50% of the individuals suffer long-term neurological injury, including seizures, hearing and vision loss, cognitive impairment, neuromotor disability and memory or behavioural changes⁷¹.

Neonatal meningitis is predominantly caused by bacteria, including Group B *Streptococcus* (GBS), *Escherichia coli* and, to a considerably lower extent, *Listeria monocytogenes*^{70,72,73}. Common symptoms such as headache (84%), fever (74%), stiff neck (74%), and nausea (62%)⁷⁴ are non-specific and can be observed in many other illnesses like gastroenteritis, flu and other infections, making early diagnosis challenging. Additionally, performing lumbar puncture, the gold standard procedure for confirming meningitis, is invasive, technically difficult, and often postponed in neonates with unstable clinical conditions⁷⁵. Currently available treatment for bacterial meningitis rely on antibiotic therapy⁷⁶. However, antibiotic resistance by pathogens is rising, especially in developing countries⁷⁶, where neonatal meningitis presents a higher incidence and mortality. Therefore, further and deeper comprehension of disease progression, symptoms appearance, and the mechanisms underlining pathogen entrance and tissue damage is mandatory to develop novel therapeutic strategies in order to mitigate the burden of neonatal meningitis.

To cause meningitis, bacteria must reach the central nervous system (CNS), a process involving several key steps: host colonisation/invasion, survival in the bloodstream, and crossing the blood-brain barrier (BBB) and/or the blood-cerebrospinal fluid (CSF) barrier (BCSFB). Bacterial pathogens causing meningitis commonly enter the host through the respiratory, gastrointestinal, or genital tracts.^{77,78} To survive in the bactericidal environment of the bloodstream, they benefit from the presence of polysaccharide capsules and bacterial surface molecules capable of neutralising complement factors⁷⁷. Lastly, CNS invasion occurs when bacteria cross the BBB, a structural barrier located at the CNS microvessels composed of highly specialised microvascular endothelial cells and a basal membrane of pericytes, microglia and astrocytes endfeet^{79,80}. Endothelial cells are connected by tight junctions that control the flux of ions, oxygen, nutrients and cells between the blood and brain parenchyma and minimise pathogen entrance^{81,82}. The tight junctions are a unique feature of these endothelial cells and are composed of four integral membrane proteins (occludin, claudin, junctional adhesion molecules and cell-selective adhesion molecule) connected to the actin cytoskeleton through cytoplasmic proteins (zonula occludens proteins ZO-1, -2 and -3 and cingulin)^{82,83}. Similarly, the BCSFB, located at the blood-choroid-plexus interface, comprises a single layer of cuboidal epithelial cells connected by apical tight junctions. Additionally, this endothelium is responsible for CSF production⁷⁹.

Bacteria can cross the BBB and reach the meninges via three approaches: transcellularly, paracellularly or intracellularly within infected phagocytes (Trojan horse mechanism). In the transcellular route, bacteria penetrate the endothelial cells without evidence of intercellular tight junction disruption. During the paracellular traversal, the endothelial tight junctions loosen or disrupt, enabling bacterial passage into the CNS. Lastly, in the Trojan horse

mechanism, bacteria migrate through the barrier inside infected phagocytes⁸¹. Once inside the CNS, bacteria can multiply, induce inflammation and further disrupt the BBB integrity⁸⁴, resulting in brain injury and leading to the neurological impairment commonly observed in meningitis survivors.

The mechanisms underlying brain injury and long-term neurological impairment in neonatal meningitis are complex and not fully understood. It remains unclear whether a single mechanism leads to a specific neurological disability or a combination of multiple processes resulting in different disabilities. Moreover, because an infant's immune system and brain are still developing and differ from those of adults, it is expected that the mechanisms of brain injury are age dependent. Various pathogens enter the BBB through different routes, secrete distinct molecules, and consequently induce varied types of brain damage. It is crucial to identify the specific relationship between the pathogen, the mechanisms of brain injury and the neurological or behavioural outcomes in order to develop new targeted therapies.

Once bacteria enter the CNS and replicate, secretion and spontaneous autolysis liberate bacterial compounds such as lipopolysaccharide (LPS), lipoteichoic acids (LTAs), lipoproteins, peptidoglycans and bacterial DNA that further fuels the inflammatory response from the host^{85,86}. These microbial molecules are perceived by pattern recognition receptors expressed on the local host cells, such as the Toll-like receptors (TLRs) on microglia, astrocytes and paravascular macrophages, triggering the host immune response. While this response is crucial for pathogen control, by the production of anti- and pro-inflammatory cytokines, reactive oxygen and nitrogen species, excessive inflammation can also contribute to brain injury⁸⁵⁻⁸⁷. Additionally, during the neonatal period, microglia play critical roles in brain development and function, participating in the neurogenesis, synaptogenesis, neuron myelination and proper vascularisation of the parenchyma. Therefore, disruptions to microglial activation may not only directly damage the brain but also impair critical functions for adequate brain development, leading to long-term neurological deficits⁸⁸. Besides resident cells, recruited leukocytes from bloodstream exacerbate CNS inflammation through cytokine secretion and reactive oxygen and nitrogen species that may be cytotoxic for the parenchyma, further damaging neurons^{85,89}.

The mechanisms of neuronal injury imply neuron apoptosis or necrosis and most likely result from a combination of events: dysfunctional cerebral blood flow, detrimental effects of inflammatory mediators and infiltrating cells, cytotoxicity, increased CSF outflow resistance, and excitatory amino acids, which finally lead to neuronal death^{86,89}. The hippocampus is particularly sensitive to neuronal damage, although it is not observed in

100% of meningitis patients and mice, this feature not being fully understood⁷⁷. Neuronal damage in the dentate gyrus mainly occurs through apoptosis, whereas necrosis is the primary mechanism in the cornu ammonis (CA) regions and the cortex^{77,85,86}.

In summary, neonatal bacterial meningitis is characterised by high incidence, mortality and long-term burden, with limited treatment options, especially to minimise neurologic disability. The most plausible reason is the lack of deep knowledge of the mechanisms underlying brain injury and its link towards neurologic and behavioural deficits. Therefore, scientists must persist, and science must advance towards new clinically efficient therapies, either in neonatal infection prevention or minimising parenchymal injury.

Group B *Streptococcus*

Streptococcus agalactiae, commonly referred to as Group B *Streptococcus* (GBS), is the leading cause of neonatal meningitis⁷¹. In newborns under two months old, GBS accounts for over 80% of meningitis cases⁹⁰.

A brief history of GBS

GBS was first described in 1887 by two French veterinarians, Nocard and Mollereau, as a pathogen causing contagious bovine mastitis⁹¹. Subsequently, in 1896, German scientists Lahmann and Neumann named this pathogen *Streptococcus agalactiae* (agalactiae meaning “no milk”) as they observed that infected cattle had diminished milk production⁹². During the early 20th century, many scientists had identified different *Streptococcus* strains, but there was confusion in classifying them due to their similar morphologies and overlapping pathogenic traits. A clear system to distinguish between the strains was lacking^{93,94}. Rebecca Lancefield, an American microbiologist, extensively studied haemolytic streptococci and proposed a classification system based on the surface capsular polysaccharides (CPS) in the different *Streptococcus* strains^{93,95,96}. In Lancefield’s classification, *S. agalactiae* was the only strain belonging to the serogroup B⁹⁵, leading to its worldwide designation as Group B *Streptococcus* (GBS). Additionally, Lancefield subdivided GBS into three different serotypes (I-III) based on rabbit sera immune reaction to different CPS⁹⁵, with serotype III predominantly associated with neonatal infections^{97,98}. However, as molecular technologies advanced, such as multilocus sequence typing (MLST), serotype classification decayed as it did not account for genetic diversity or clonal ancestry^{99,100}. In the late 1990s and early 2000s, Nicola Jones established a clonal complex (CC) classification system using the MLST technique¹⁰¹. This approach identifies genetically related strains descending from a common ancestor, helping to identify not just serotypes but also the strains’ genetic lineage and potential pathogenicity.

The first reports on human GBS infections date back to the 1930s^{102–104}. However, it took 30 additional years for the first reports of GBS isolation from neonatal humans, but only 10 years for GBS to emerge as the leading neonatal pathogen, in the 1970s^{105–107}, which remains until today.

GBS transmission and disease

GBS is a commensal microorganism colonising the genitourinary and gastrointestinal tracts of about 50% of healthy adults¹⁰⁸. Up to 40% of pregnant women carry GBS in their vaginal mucosa⁷¹, translating to nearly 20 million women worldwide¹⁰⁹. The source for vaginal colonisation is usually the gastrointestinal tract, the primary GBS reservoir^{110,111}. Vaginal colonisation represents a major risk for vertical transmission to the newborn. This transmission, occurring in 50% of cases¹¹², can occur *in utero*, as GBS can ascend to the uterus and cross the placenta, or during birth when the newborn contacts the mother's vaginal canal and acquires bacteria through inhalation or ingestion of infected vaginal fluids^{108,113}.

Two distinct syndromes may arise from neonatal GBS infection, according to the age at which symptomatic manifestations occur. Early-onset disease (EOD) occurs during the first week of life, and clinical signs usually present in the first 24-48 hours. Late-onset disease (LOD) manifests in infants over one week old and until three months of age¹¹⁴. These two diseases differ in their clinical manifestations. EOD is characterised by pneumonia and respiratory failure complicated by bloodstream infection and septicaemia. In contrast, LOD is associated with bacteraemia without focus and a 50% incidence of meningitis⁷¹. Mortality rates of EOD and LOD are 5-27% and 4-12% respectively, being the wide ranges attributed to the difference between developed and developing countries¹¹⁵. Among the survivors, 50% suffer from permanent neurological impairment, including deafness, seizures, hydrocephalus, hearing loss, cortical blindness, and speech and language delay¹¹⁶.

EOD pathogenesis is attributed to GBS ascendance from the genital tract into the amniotic cavity to initiate infection *in utero* or during passage through the vaginal canal at delivery, through contact with contaminated fluids^{71,117}. GBS replication and adherence to the respiratory epithelium, followed by host neutrophil influx, cause pulmonary injury¹¹⁸, which then may drive GBS leakage to the bloodstream, leading to septicaemia. In addition to vaginal colonisation, several other risk factors for EOD include *intrapartum* maternal fever, ruptures of membranes more than 18 hours before delivery, preterm labour or delivery before 37 weeks of gestation, and previous pregnancy with GBS-positive screening¹¹⁹.

LOD pathogenesis is not completely elucidated but is likely multifactorial. Vertical transmission from vaginal colonised mothers can be a source of GBS neonatal infection

and LOD cases^{120–123}. A few reports suggest breastmilk as a potential source for LOD, since no GBS was detected other than the breastmilk^{124,125}. However, it is unclear if LOD is related to a recent GBS infection from breastmilk or if it is a result of gut translocation from an already GBS-colonised infant¹²⁶. GBS horizontal transmission from non-maternal sources, such as hospitals or communities is also hypothesised^{127,128}. The intermittent or transient nature of GBS vaginal colonisation among a reduced percentage of pregnant women might explain the undetectable GBS in some LOD cases leading to the assumption of non-vaginal vertical transmission¹²⁹. Risk factors contributing to LOD include preterm birth, HIV co-infection, young maternal age, African ethnicity and infected siblings¹³⁰.

GBS meningitis

The serotype classification based on the CPS is currently composed of 10 groups (Ia, Ib and II-X), revealing serotypes Ia, Ib, II, III and V as the most prevalent in neonatal diseases, with serotype III responsible for up to 82% EOD and 84% LOD cases. Serotype III strains, particularly strains belonging to CC17, are referred to as hypervirulent strains, associated with 80% of GBS neonatal meningitis^{108,131,132}.

Although the mechanisms of GBS-induced meningitis are not completely understood, significant progress has been made in the past two decades (Figure 2). To cause meningitis, GBS must reach the bloodstream, by crossing lungs or gut barriers, and reach the CNS, where it needs to cross the last barrier, the BBB, to reach the meninges and the brain parenchyma. GBS can adhere to mucosal epithelia, such as lungs and gut, and cross the layers paracellularly, by transiently opening the tight junctions between epithelial cells¹³³. Additionally, GBS conserved toxin β -haemolysin injures the gut and lung epithelial cells and microvasculature, facilitating GBS leakage to the blood^{134,135}. Moreover, the host immune system response to GBS may exacerbate tissue damage¹¹⁸. The immaturity of the intestinal microbiota and the incomplete epithelial polarisation favour GBS traverse to the blood¹³⁶.

The predominant meningitis-associated GBS strain, CC17, can cross the intestinal barrier transcellularly using different virulence factors (Figure 2). The hypervirulent GBS adhesin (HvgA) efficiently adheres to intestinal epithelial cells¹³⁷. Intestinal translocation through M cells is another route explored by GBS CC17. This is mediated by the surface serine-rich repeat protein (Srr2), which binds to epithelial fibronectin, and is favoured by the perinatal hormones oestradiol and progesterone^{138,139} (Figure 2).

Once in the bloodstream, the CPS plays a critical role in avoiding bacterial killing. GBS isogenic mutants lacking CPSs show increased susceptibility to neutrophil-mediated killing and decreased virulence, proving the importance of CPS for blood survival^{140,141}. In addition, GBS expresses sialic acid on its surface, which has evolved to mimic host self-antigens and to avoid the deposition of complement molecules, thereby dampening host recognition and phagocytic and killing abilities¹⁴². Upon circulation and replication in the blood, GBS reaches the CNS, where several virulence factors contribute to BBB translocation (Figure 2). During the perinatal period, the BBB and BCSFB overexpress integrins on their membrane surface, which function as ligands for Srr2, facilitating GBS CC17 adhesion and internalisation¹⁴³ (Figure 2). Complementarily, GBS CC17 crosses the BBB and BCSFB transcellularly using

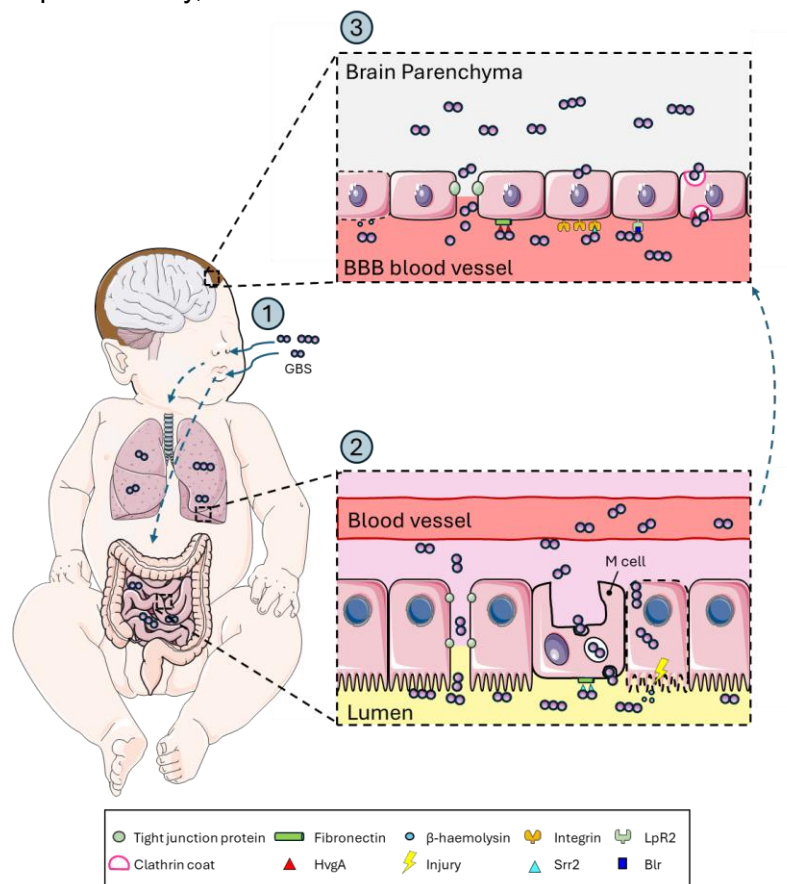


Figure 2. Group B *Streptococcus* needs to overcome several human barriers to cause meningitis. 1.

Group B *Streptococcus* (GBS) enters the host by ingestion or inhalation of maternal-contaminated fluids during birth, primarily colonising the lungs and the gut. 2. Inside these tissues, GBS crosses the mucosal barriers using different virulence factors. GBS can disrupt tight junction connections, traversing the barrier paracellularly. GBS toxin β-haemolysin/cytolysin injures the epithelial and microvascular barriers allowing bacterial traversal. GBS uses HvgA to adhere to fibronectin and cross M cells in the mucosal barriers. 3. The endothelial cells from the blood-brain barrier (BBB) are enriched in integrins. GBS can bind through the Srr2 protein to transcellularly cross the epithelium. Moreover, HvgA binds to clathrin-coated vesicles, promoting GBS endocytosis and BBB cross. The lipoprotein Blr is also a virulence factor, as it binds LpR2 in endothelial cells and GBS can invade the parenchyma. The illustration is for explanatory purposes, and it is not drawn to scale. HvgA, hypervirulent GBS adhesin; Srr2, surface serine-rich repeat protein; LpR2, lipophorin receptor 2; Blr, B leucine-rich lipoprotein.

HvgA adhesin via a clathrin-dependent mechanism^{137,144} (Figure 2). Furthermore, protein analysis of infected brains showed that GBS alters the host proteomic signature, leading to the downregulation of BBB proteins important for integrity, including decreased expression of tight junction proteins^{145,146} (Figure 2). Two additional routes facilitate GBS traverse to the brain. The bacterial surface lipoprotein B leucine-rich (Blr) interacts with the host lipophorin receptor 2 (LpR2), allowing GBS brain infiltration¹⁴⁷ (Figure 2). GBS modulation of host cell signalling involving sphingosine 1-phosphate receptor 2 (S1P2), epidermal growth factor receptor (EGFR) and cysteinyl leukotrienes (CysLTs) facilitate transcellular penetration of the BBB¹⁴⁸.

Altogether, GBS is equipped with multiple mechanisms to cross the different host barriers, complicating the development of new and efficient therapeutics to prevent infection or, at least, minimise the neurological burden in surviving infants.

GBS treatment guidelines

Currently, the guidelines from the American College of Obstetricians and Gynecologists and the American Academy of Pediatrics towards GBS infection prevention rely on culture-based antenatal GBS screening using vaginal and/or rectal cultures in pregnant women between 36 and 37 weeks of gestation. For positive colonised pregnant women, the available treatment is *intrapartum* antibiotic prophylaxis (IAP)^{149,150}. Penicillin is the preferred antibiotic administered¹⁵¹. In the case of penicillin allergy, individuals receive other antibiotics, like vancomycin, cefazolin or clindamycin, depending on the allergy severity and GBS antimicrobial susceptibility¹⁵¹. Antibiotic administration reduced GBS colonisation, especially if administered 4 h before delivery, helping to decrease deaths due to EOD by up to 70%^{114,152}. However, these are guidelines followed by the United States and are not mandatory worldwide. While numerous nations adhere to these guidelines, it is important to recognise that prenatal screening remains controversial and differs across countries^{153–156}. Moreover, IAP has not diminished LOD incidence nor LOD-associated deaths^{157,158}. Infants with LOD were shown to be more exposed to antibiotics than uninfected infants, posing the question of the relation between IAP and later infections leading to LOD¹⁵⁹.

The widespread use of antibiotics has contributed to the emergence of new antibiotic-resistant strains of GBS, becoming a significant concern within the scientific community^{114,160}. Numerous studies have also highlighted the negative effects of antibiotics on the gut microbiota of newborns, delaying the colonisation of beneficial bacteria and/or hindering the complete recovery of the microbiota following antibiotic treatment^{161–163}. Alterations in the gut microbiota can have serious health implications for neonates, as they

play a crucial role in the proper postnatal development of the brain, gut, and metabolic and immune systems¹⁶⁴.

GBS continues to be a major pathogen causing meningitis and permanent neurologic impairment. There is currently no specific and efficient treatment; management remains to treat symptomatically and with broad-spectrum antibiotics, with undesired side effects. All efforts by the scientific and medical communities must align with the World Health Organisation's (WHO) 2030 goal of defeating meningitis, including reducing mortality by 70% and disability towards an improved life quality¹⁶⁵.

II. THE MENINGES: AN UNDERRATED BARRIER FOR A (TOO) LONG TIME

This topic contains part of the paper “Meningeal immunity: anatomy, function and neonatal unique features”, submitted for publication in the European Journal of Immunology. Unrevised version of the article. Under revision at the European Journal of Immunology. The integral paper is in Annex 1.

Introducing meninges

The CNS is a highly complex, sensitive, and dynamic system responsible for controlling the entire body's actions. The meninges were long thought to be only a protective barrier, surrounding the brain and spinal cord, conferring physical protection from impacts¹⁶⁶. Despite being first reported in an Ancient Egyptian medical text around ca. 1600 BCE¹⁶⁷, it has only been over the last two decades, particularly with the (re)discovery of meningeal lymphatic vessels, that the meninges have gained attention and their role has been redefined beyond a mere physical barrier. The meninges are strategically located at the interface between the periphery and the brain, and their known functions have been continuously expanding. The meninges regulate the development of the forebrain and hindbrain, skull and brain blood vessels^{168–175}. Additionally, they contribute to neural survival, migration, and position in the brain, and in the maintenance of the pial basement membrane^{171,172,176,177}. Notably, a stem cell niche with the ability of neural differentiation was also discovered to reside in the meninges, being already present during the embryonic period. These cells can be triggered by an injury, such as tissue damage and inflammatory signals, proliferating and migrating to the CNS to activate parenchymal response^{169,175,178}. Furthermore, a vast immune cell repertoire was found in the meninges. Meningeal immunity has been linked to central roles in CNS immunosurveillance and repairment, lymphatic drainage and brain homeostasis¹⁷⁹. Meningeal inflammation has also been found to precede the onset of neurological disease, even in non-infectious conditions¹⁸⁰. Thus, the meninges remind an immunological barrier, comparable to those found in the skin and intestine¹⁷⁹. Indeed, a disruption in the meningeal immune homeostasis may result in inflammation or cytokine milieu dysregulation, potentially leading to brain dysfunction and/or pathology. While increasing knowledge of meningeal immunity has been accumulating in understanding meningeal immunity in adults, very little is known regarding the neonatal period.

Meningeal structure

The meninges are classically composed of three cellular layers: the dura mater (adjacent to the skull), the arachnoid mater and the pia mater (the closest to the parenchyma) (Figure 3)¹⁸¹. The arachnoid and pia mater form the leptomeninges. Between these two layers is the subarachnoid space (SAS), where the CSF produced by the choroid plexus flows, being extremely important in protecting the brain from physical trauma¹⁸¹. Recently, a novel meningeal layer, the subarachnoid lymphatic-like membrane (SLYM) was proposed¹⁸², in line with last century studies stating the SAS is subdivided by thin membranes^{183,184}. The SLYM is located between the arachnoid and the pia mater, dividing the SAS into outer (near the dura) and inner (closer to the pia) compartments^{182,185}. This layer is, however, controversial in the meningeal field, as other authors contest it as the inner arachnoid instead of a fourth meningeal layer^{186,187}. These results also align with studies from the second half of the last century^{188–194}.

The dura mater is a thick, dense, collagenous membrane, composed of two layers: the periosteal layer, closest to the skull, and the meningeal layer. Both layers are composed of

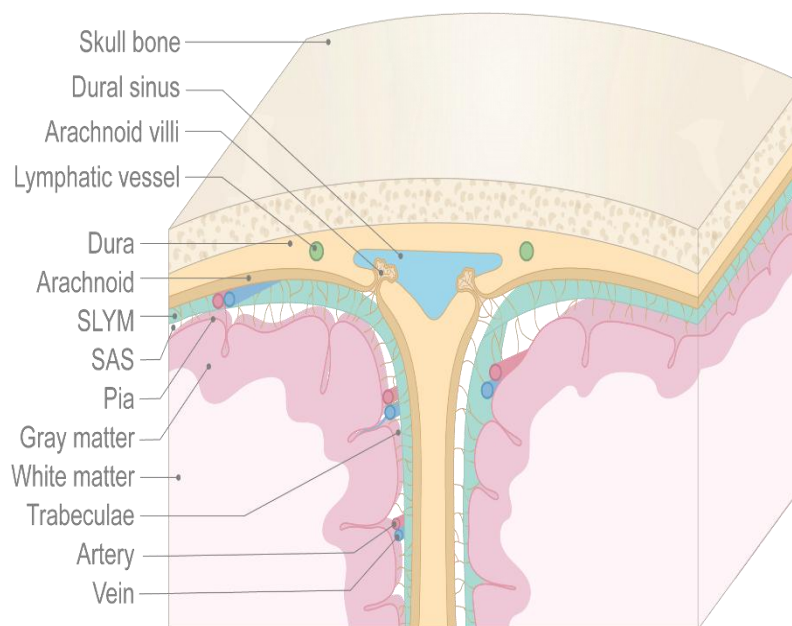


Figure 3. Steady-state meninges. The meninges consist of the following layers: the dura mater (adjacent to the skull), the arachnoid mater and the pia mater. A fourth meningeal layer has been proposed, the Subarachnoid Lymphatic-like Membrane (SLYM), dividing the subarachnoid space (SAS). The dura mater contains blood and lymphatic vessels. The arachnoid is avascular and composed of cells connected by tight junctions, representing an impermeable barrier. The arachnoid fibroblasts form trabeculae connecting the arachnoid and pia mater. The SLYM is composed of a mesothelium membrane covering the vessels in the subarachnoid space (SAS) and creates a permeability barrier in this space. The pia mater covers the brain parenchyma and forms sheaths around the vessels that run from the subarachnoid space to the deep brain parenchyma (Virchow-Robin spaces), important for the clearance of brain waste products and cellular debris. The SAS is settled between the arachnoid and pia mater and is where the cerebrospinal fluid flows. The major arteries are also present in this space.

collagen fibres and fibroblasts, being these cells flattened in the inner layer of the dura mater¹⁶⁶. These layers are mostly fused, widening to harbour the dural vessels and separating at the longitudinal fissure that defines the hemispheres. Here, only the meningeal layer enters the brain to form the falx cerebri, whereas the periosteal layer remains attached to the skull^{166,181}. The dura mater is highly innervated with sympathetic, parasympathetic and sensory fibres¹⁸¹. Dural vascularisation is also extensive, being its blood vessels fenestrated lacking tight junctions, allowing the passage of relatively large molecules (~45 kDa)¹⁹⁵. Moreover, the dura mater harbours conventional and functional lymphatic vessels (LVs) unevenly distributed throughout the brain, in both mice and humans^{196–199}. While the superior portions scarcely present LVs, the base of the skull harbours a large LV network¹⁹⁸. Recent studies have revealed a role for the dural LVs, located parallel to the venous sinus (Figure 3), in continuous drainage and clearance of CSF, macromolecules and immune cells from the brain into the cervical lymph nodes (CLN)^{198,200}.

The central meningeal membrane is the arachnoid layer. This thin, avascular structure is composed of cells connected by tight junctions, forming an impermeable layer, important to regulate the entry of dural blood-derived molecules into the CSF^{166,181}. The arachnoid fibroblasts, together with the connective intercellular tissue, protrude the subarachnoid space forming the trabeculae, a vast spiderweb-like network connecting the arachnoid and pia mater²⁰¹. The subarachnoid space is filled with the CSF and the most important brain arteries¹⁶⁶.

The recently proposed SLYM is composed of a mesothelium membrane covering the vessels in the SAS. The SLYM divides the SAS, creating a barrier between the two compartments for solutes of or bigger than 3 kDa, guiding and selecting the CSF that reaches the brain parenchyma¹⁸².

The pia mater, the innermost layer, consists of two sub-layers: the epipial layer and the intima pia. The epipial layer is composed of collagen fibres attached to the arachnoid by the trabeculae, while the inner layer comprises reticular fibres and is connected to the external layer of the parenchyma, the glial membrane¹⁶⁶. As in the dura mater, the pial membrane is highly innervated¹⁸¹. The pia also harbours arteries, arterioles, veins, and venules, forming sheaths around the vessels that run from the subarachnoid space into the deep brain parenchyma, creating an interstitial fluid (ISF)/CSF-filled space, the Virchow-Robin space, important for the brain waste clearance^{166,181,202}. Both subarachnoid and pial vessels are composed of epithelial cells connected by tight junctions, forming the impermeable blood-meningeal barrier¹⁹⁵.

Structurally, the neonatal meninges resemble those of adults. The three-layered structure (as the SLYM remains to be confirmed in the neonatal period) starts to develop during the foetal stage, more precisely at gestation week 5 in humans and embryonic day 9.5 in mice. At birth, the three layers, as well as the SAS and arachnoid trabeculae are already developed¹⁷⁵. Regarding vascularisation, blood vessels are already established at birth²⁰³. However, recent research in mice reveals that the blood vessel network in the dura mater changes during the neonatal period²⁰⁴. During the first week of life, the outer layer of the meninges presents a large number of fine and complex branching points. As the neonate matures, these vessels become larger and the vascular network simpler²⁰⁴. The lymphatic system, however, only starts to develop at birth (Figure 4)^{197,203}. Studies using whole-mounts of mouse meninges show that the lymphatic system develops alongside blood vasculature and cranial and spinal nerves during the first month of life¹⁹⁷. Lymphatic vessels first appear just before birth at the base of the skull, near the foramen magnum (FM) and the

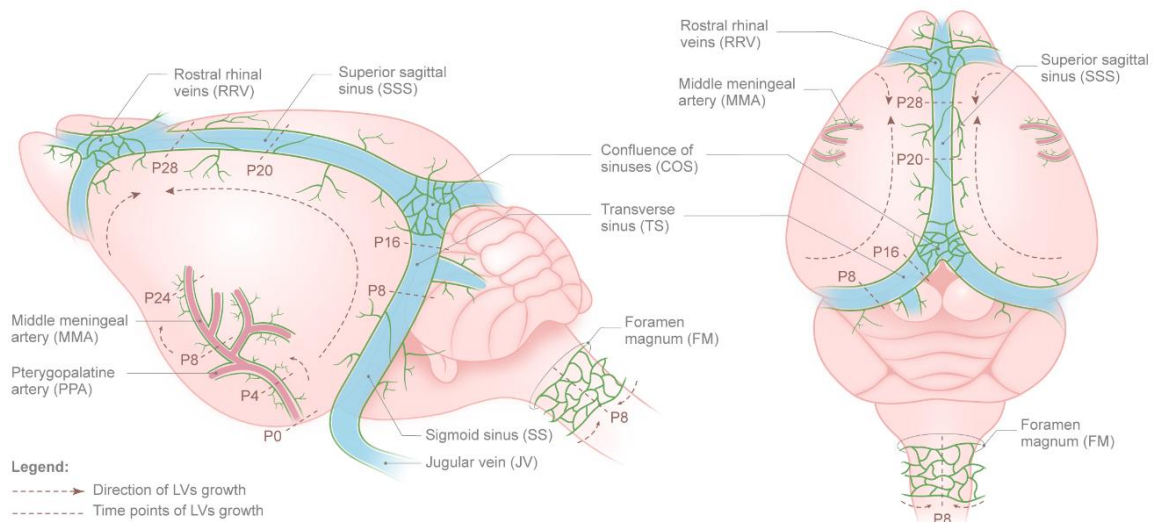


Figure 4. Neonatal mice development of the lymphatic vessels. Dorsal, caudal-rostral turn (a) and top (b) view of mouse brain. The lymphatic network (green) develops postnatally, starting from the pterygopalatine artery (PPA), foramen magnum (FM) and meningeal artery (MMA), around birth (P0). Two weeks after birth, the lymphatic vasculature is fully developed around the FM and reach the superior sagittal sinus (SSS). From P16 to P24 the LVs were observed overlying the olfactory bulb and besides the rostral rhinal veins (RRV). The lymphatic vasculature is fully developed around a month after birth. JV, jugular vein; SS, sagittal sinus; TS, transversal sinus; COS, confluence of the sinuses.

pterygopalatine artery (PPA)¹⁹⁷. At birth, LVs also started developing at the base of the middle meningeal artery (MMA)²⁰³. Lymphatic valves are also observed at birth, increasing until postnatal day (P) 20¹⁹⁷. LVs develop all around the FM during the first two weeks of life¹⁹⁷. LVs at PPA extend along the MMA, after P4, and reach the top of the skull, more precisely the sagittal suture, at around P24¹⁹⁷. Parasinusoidal LVs develop alongside the jugular vein, through sagittal, transverse and superior sagittal sinuses^{197,203}. The LVs reach the transverse sinus by P8 and the superior sagittal sinus by P16^{197,203}. From this day, LVs

are also observed covering the cerebellum, alongside veins branching from the transversal sinus¹⁹⁷. From P16 to P24, the LVs extend over the olfactory bulb and beside the rostral rhinal veins¹⁹⁷. Under the olfactory bulb, LVs are detectable as early as P2 and connect to nasal mucosal LVs, draining into the dCLN¹⁹⁷. Currently, no corresponding studies exist for humans. Overall, the lymphatic system develops from the base of the skull to the top, consistent with the unevenly distributed meningeal vasculature already described in this review, being completely developed one month after birth^{197,203}.

Neonatal meningeal immunity

Little is known about the neonatal meningeal immune repertoire and function. As with other resident macrophages, these cells are seeded in the meninges before birth, more precisely at embryonic day 9.5 in mice²⁰⁵. Immunohistochemistry analyses of whole-mount meninges show that macrophages are already present alongside the sinuses at birth^{203,204}. Their number and morphology change over time, with the highest density and the most rounded morphology observed at birth²⁰⁴. At this early stage, meningeal macrophages are mainly MHC-II negative, potentially leading to increased pathogenic burden at the borders of the brain^{206,207}. As the neonate matures, macrophage density significantly decreases, and from week two after birth, their shape shifts from ameboid/round to a more rod-like form²⁰⁴. Moreover, by P14, macrophages show significant alignment along blood vessels²⁰⁴, in accordance with their patrolling and antigen presentation functions²⁰⁸. Using a mouse model of human neonatal *E. coli* meningitis, with pups inoculated intravenously at P5, bacteria were found in the leptomeninges and dura mater 22 h after infection²⁰⁹. Moreover, single nucleus RNA sequencing showed that infection leads to altered transcriptome in the most abundant meningeal cell types, including endothelial cells, macrophages, and fibroblasts²⁰⁹.

B cells appear in neonatal meninges as soon as one day after birth, predominantly comprising the B-1a subset²⁰⁸. These cells can infiltrate the brain parenchyma, through a CXCL13–CXCR5 dependent signalling, where they promote oligodendrogenesis²⁰⁸. Neutralisation of the receptor for the Fc region of IgM secreted by B-1a cells results in impaired oligodendrocyte precursor cells (OPCs) proliferation and reduced myelinated axons in the developing brain. These data suggest that meningeal B-1a infiltration into the brain parenchyma promotes oligodendrogenesis and axon myelination by stimulating OPCs proliferation via IgM-Fc receptor signalling²⁰⁸. Moreover, and similarly to the adult meninges, B cells in the developing mouse and non-human primate are replenished by skull marrow developing B cells, undergoing CNS antigen-dependent checkpoints to prevent self-reactivity²¹⁰.

T cells are also found in the neonatal meninges at birth, with different kinetics regarding their subsets^{211,212}. While $\gamma\delta$ T cells peak one week after birth and decrease over time, $\alpha\beta$ T cells tend to accumulate after weaning^{211,212}. During the neonatal period, $\gamma\delta$ T cells exhibit high proliferative activity, and parabiotic studies indicate that they possess a tissue-resident phenotype²¹¹. Their function during the neonatal period is, however, still unclear. Postmortem studies of preterm human infant brains with periventricular leukomalacia (PVL), the most common preterm brain injury affecting the white matter, showed the presence of both T and B cells in the meninges, contrary to the control cases²¹³. These cells seem to be involved in the induction of brain injury, as a mouse model of infection using RAG1^{-/-} mice showed protection against white matter loss²¹³. $\gamma\delta$ T cells were also found in the meninges of neonatal humans and mice with PVL, and a mouse model of preterm brain injury showed a significant increase of these cells 6 h upon infection²¹⁴. Depletion of $\gamma\delta$ T cells conferred protection against brain injury²¹⁴, suggesting their potential role in driving in brain damage.

Meningeal infection and immunity

As the meninges harbour both blood and lymphatic vessels, they may serve as a gateway for microorganisms' entrance with concomitant inflammation, disease, and later neurological impairment. A few studies already reported the presence of neurotropic pathogenic microbes in the meninges upon infection^{215–225}. A phylogenetic study showed that the meninges are the primary transport tissue for HIV-1 infection, with the virus initially migrating from the meninges to the brain within monocytes/macrophages, then freely migrating to the brain, as the BBB disrupts. In addition, the virus may traffic from the meninges back to the periphery²²². Therefore, not only do meninges can act as an entry point for pathogens, but they are also a gateway out into the periphery²²². Given the recent findings of a complete lymphatic system at the meninges and its connection to the periphery through the dCLN, this is likely the exit route viruses use to travel from the meninges to the periphery.

Further supporting the role of the meninges as an entry point for pathogens, several studies have observed microbes and inflammatory cells in the meninges even without or prior to brain parenchyma infiltration^{217,219,224}. For example, the Zika virus was found in the meninges of human neonates without evidence of parenchyma invasion²¹⁷. Inflammatory lymphocytes and macrophages were also observed in the meninges upon infection, particularly in the Virchow-Robin space²²⁶. The lymphocytic choriomeningitis virus (LCMV), a natural pathogen in humans and mice that causes meningitis, predominantly infects the meninges with minimal parenchymal invasion²¹⁹. In this case, fibroblast-like meningeal cells

and resident macrophages are the primary infected cells^{227,228}. Within two days of infection, one-third of meningeal macrophages are infected, triggering the upregulation of antiviral cytokines²²⁸. Moreover, these macrophages present upregulated genes associated with their activation (phagocytosis, endocytosis, and APCs and antiviral activities), engaging in programmed cell death around a week after infection, being cleaned and replaced after the onset of meningitis²²⁸. The virus also spreads into the periphery, inducing strong CD8⁺ T cell responses²¹⁹. These cells, along with myelomonocytic cells (LysM⁺ cells, including monocytes, macrophages and granulocytes^{229–231}), infiltrate the meninges via meningeal blood vessels around six days post-infection, causing immune-mediated pathology and brain oedema^{219,220,228}. Depletion of antiviral CD8⁺ T cells prevented fatal disease and the recruitment of myelomonocytic cells²²⁷, suggesting a role of CD8⁺ T cells in recruiting myelomonocytic cells, either by releasing chemoattractants or by inducing other cells to do so²²⁷. Notably, mice lacking CD8⁺ T cell classical effector pathways (TNF- α , IFN- γ , Fas, perforin, granzymes and degranulation) succumb to LCMV-induced meningitis, suggesting an alternative unknown pathway of CD8⁺ T cell-induced disease²²⁷. Intravital imaging of the meninges during disease onset showed myelomonocytic cells extravasating from meningeal blood vessels, causing severe vascular injury, including vasculature rips and endothelial leakage²²⁷. The detailed mechanism of vascular injury by myelomonocytic cells is still unknown. Moreover, engrafted monocytes become refractory to secondary microbial challenge and present a reduced ability to control inflammation²²⁸. Thus, CD8⁺ T cell-mediated disease is dependent, at least in part, on the short- and long-term effects of myelomonocytic cells recruited to the meninges.

Parasites, such as *Trypanosoma brucei*, were also found in the meninges before invading the parenchyma²²⁴. Injecting *T. brucei* directly into the brain does not result in infection, while intraperitoneal injection rapidly leads to the appearance of this parasite in the CSF and meninges²²³. Moreover, a kinetic analysis shows that the parasite accumulates in the meninges approximately two weeks before entering the parenchyma²³². These results suggest that *T. brucei* likely enters the brain parenchyma through the meninges. After infection, T cells infiltrate the cortical meninges, particularly the dura mater, where the parasites are mainly confined²¹⁸. APCs, mainly DCs, also increase in the meninges²¹⁸, in accordance with the observed T cell infiltration dependent on antigen presentation. Recruited macrophages to the parenchyma outnumber the resident ones and exhibit a different phenotype, by expressing antimicrobial genes²³². In contrast, *Toxoplasma gondii* only invades the brain parenchyma but still requires meningeal immunity and even brings a new player to the game: the regulatory T cells²³³. While effector CD4⁺ T cells infiltrate the parenchyma to encounter and fight this pathogen, recruited T_{regs} are less migratory,

remaining in the meninges where they perform stable interactions with DCs. Reducing meningeal CD11c⁺ cells using anti-LFA-1 antibodies increases the motility of T_{regs}, suggesting a role of T_{regs}-DCs interaction in regulating their local behaviour²³³. T_{regs} are known to regulate CNS inflammation during infections^{234–236}, likely by suppressing APC function, downregulating the costimulatory signals or their cytokine production, thus fine-tuning the effector T cells response²³³. Alternatively, DCs may provide survival signals to maintain Tregs in the brain²³⁷.

The fungus *Candida albicans* was also found localised within the dural sinus walls before spreading to the brain parenchyma when mice were intravenously inoculated. In this context, meningeal IgA⁺ cells act as an immunological shield, playing a decisive role in fungal entrapment in the peri-sinus region²¹⁵.

Regarding bacterial infection and meningeal immunity, our knowledge is also scarce. *Salmonella enterica*, *L. monocytogenes* and *Neisseria meningitidis* are a few bacteria already found to be present in the meninges^{216,221,225}. During oral infection with *S. enterica*, bacteria were detected in the meninges with little or no parenchymal invasion²¹⁶. Mild meningeal inflammation due to monocyte infiltration was also detected by immunofluorescence in *S. enterica* infected mice²¹⁶. *L. monocytogenes* is also capable of infecting both the meninges and the brain parenchyma²²¹. Moreover, following intracisternal infection, meningeal infiltration, vascular inflammation and subarachnoid haemorrhages were observed within 24 hours²²¹. However, the mechanisms through which these bacteria reach the meninges and eventually invade brain parenchyma, how they interact with meningeal cells and how these cells respond to this stimulus and signal to others are still poorly understood. Regarding *L. monocytogenes*, one study recently uncovered a mechanism through which it can invade the CNS. The bacterial surface protein InlB prevents monocyte infected cells from CD8⁺ T cell Fas-mediated death²³⁸. Increasing monocytes lifespan render increased survival to the bacteria itself favouring parenchymal invasion²³⁸.

N. meningitidis, a human-specific pathogen, has been studied mainly through *in vitro* models^{239–241}. Using human brain sections and meningioma cell cultures, researchers demonstrated that *N. meningitidis* preferentially adheres to the leptomeninges rather than the brain parenchyma²⁴¹. Upon challenge, meningeal cells increased the secretion of pro-inflammatory cytokines and chemokines, including IL-6, IL-8, monocyte chemoattractant protein-1, RANTES and granulocyte-macrophage colony-stimulating factor (GM-CSF), suggesting an innate immune response upon infection^{239,240}. Although no direct infection of meningeal cells was detected, prolonged *N. meningitidis* infection led to meningioma cell necrosis, likely mediated by external molecules produced and secreted by the bacteria²⁴⁰.

An *in vivo* mouse model of intranasal infection demonstrated that 20% of challenged mice developed lethal meningitis without detectable bacteria in the blood²²⁵. The olfactory epithelium, which is connected to the CNS, was disrupted, with detectable bacteria along the olfactory nerve and bulb and, finally, in the meninges, particularly the SAS²²⁵. How the bacteria induce mucosal epithelium damage is still unclear, as well as the mechanisms of immune cell activation leading to the secretion of pro-inflammatory cytokines and chemokines.

Several other pathogens are known to cause meningitis, such as *Streptococcus pneumoniae*, *E.coli* and *S. agalactiae*²⁴². However, to our knowledge, detailed information on the immune mechanisms triggered by these pathogens in the meninges remains limited. Intravenous infection with *S. pneumoniae* and *S. agalactiae* revealed the presence of bacteria in the meninges as soon as 6 and 12 h after infection, respectively²⁴³. The presence of bacteria in this tissue is dependent on meningeal macrophages, as the polarisation of its transcriptional profile led to reduced dural antimicrobial defences²⁴³. Nasal infection with *S. pneumoniae* also demonstrated the presence of bacteria in the dura mater up to at least 72 h post inhalation²⁴⁴. Interestingly, depletion of the nociceptor Nav1.8, present in a subset of sensory neurons, significantly reduced meningeal and brain bacterial invasion 24 h after infection with either species²⁴³. Both bacteria secrete toxins that activate nociceptors which, in turn, stimulate the release of calcitonin gene-related peptide (CGRP). *S. pneumoniae* infection ultimately led to meningeal immunosuppression, dampening host defences²⁴³. Additionally, intravenous infection with *S. pneumoniae* revealed that bacterial presence was predominantly restricted to the meningeal compartment²⁴⁵. In response, meningeal macrophages and monocytes are highly activated, with skull marrow presenting phenotypically different monocyte progenitors²⁴⁵. These are more immature for emergency myelopoiesis for high production of monocytes to fight the infection²⁴⁵.

In summary, the meninges are a unique structure with an immune niche from early life. The presence of the bloodstream may serve as a gateway for pathogens to invade the CNS and cause neuronal damage, representing a risk for neurological impairment. As a novelty topic in research, meningeal immunity must be further studied to deeply understand their contribution to (patho)physiology during this critical period of life.

III. NEUROIMMUNE INTERACTIONS IN TISSUE PHYSIOLOGY AND INFECTION

The immune system extends beyond host-pathogen interactions, playing critical roles in maintaining tissue physiology. Immune cells detect environmental cues from the uninfected organism and respond to dietary, metabolic, commensal and nervous system signals, promoting both intra- and inter-organ communication to maintain homeostasis^{246,247}.

Neuroimmune interactions have been known for decades, especially since the middle of the 20th century, when physicians started to dedicate time to understanding the link between psychosomatic medicine, i.e., the mind-body relationship, and health or disease^{248–250}. Since then, the intricate association between the nervous and immune systems has been extensively studied and implicated in numerous pathologies, including cancer, autism, neurodegenerative diseases, chronic inflammation and multiple sclerosis^{251–256}. These systems colocalise throughout the body, with neuroimmune cell units (NICUs) identified in mammals as anatomical locations where immune and neuronal cells physically interact and regulate tissue physiology²⁵⁷. In fact, the relationship between these systems is so profound that coevolution has already been proposed²⁵⁸.

Gut physiologic neuroimmune interactions

The gut-brain axis is perhaps the most studied neuroimmune inter-organ interaction. The gut comprises about 70- 80% of the body's immune cells, highlighting its critical role in host immunity²⁵⁹. On the other hand, the human enteric nervous system (ENS) is composed of up to 600 million sensory neurons, with the cell bodies located in the gastrointestinal tract wall²⁶⁰. The gut is also richly innervated by extrinsic neuronal pathways, including sympathetic, parasympathetic and sensory innervations originating from the spinal cord, brainstem and vagus nerve and dorsal root ganglia, respectively²⁶¹. The sympathetic neurons enable response to stressors and the parasympathetic neurons restore homeostasis²⁶². The sensory neurons, as the name implies, detect environment signals, transmitting inputs to the spinal cord²⁶³. While neurons express cytokine receptors for immune signals, immune cells express surface receptors for neuropeptides secreted by neurons, enabling bidirectional communication²⁶¹.

Numerous enteric innate immune cells interact with neuronal cells in response to danger signals. For instance, a specific population of enteric macrophages, muscularis macrophages, produce bone morphogenic protein 2 (BMP2) that signals to the respective receptor in the enteric neurons and regulates intestinal transit and motility. Reciprocally,

enteric neurons maintain this macrophage population by secreting macrophage survival factors such as macrophage colony-stimulating factor (M-CSF)²⁶⁴. Similarly, activated mast cells release inflammatory mediators such as histamine, serotonin, and tryptase to activate sensory neurons, causing visceral pain. In response, the neurons produce neuropeptides including substance P, CGRP, vasoactive intestinal peptide (VIP), and corticotropin-releasing hormone, which further activates mast cells²⁶⁵. Additionally, neurotransmitters from sympathetic and parasympathetic neurons can modulate mast cell degranulation²⁶⁵.

Adaptive immune responses also play a role in gut neuroimmunity. B cells colocalise with intestinal nerves in mice, suggesting a role of neurons to B function and/or vice-versa^{265,266}. *In vitro* studies also showed that the neuropeptide substance P and VIP promote B cell expansion and IgA production, respectively^{267,268}.

The gut microbiota significantly influences neuroimmune interactions via three primary mechanisms. First, neuronal cells, including those in the ENS, peripheral nervous system, or CNS, can directly sense microorganisms – the so-called gut-brain axis. For example, dysbiosis caused by antibiotic treatment significantly decreases the secretion of macrophage BMP2 and the respective neuronal activation²⁶⁴. Antibiotic-treated mice also exhibited impaired intestinal structure and function with concomitant enteric neuron loss²⁶⁹. Second, bacterial metabolites can also be recognised by neurons or cross the BBB and be perceived by parenchymal neurons. Short-chain fatty acids (SCFA) restore the number of enteric neurons lost upon antibiotic treatment²⁶⁹. SCFA also promote microglia maturation in germ-free (GF) mice, while their receptor depletion recapitulates impaired microglia maturation²⁷⁰. In contrast, long-chain fatty acids promote the differentiation of Th1 and IL-17-producing Th17 cells in the gut, contributing to CNS autoimmune pathologies²⁷¹. Third, immune cells can detect bacteria and relay signals or migrate from the gut to the brain, where they play neuromodulatory functions. Studies in mice have shown that immune cells produce cytokines in response to bacteria further impacting brain development or neuroinflammation, as well as synapse formation, neural functions and behaviour^{272–275}. Alternatively, systemic immune cells translocate to the brain. *In vivo* cell tracking experiments showed gut-residing $\gamma\delta$ T cells migrating to the meninges following a stroke^{276,277}. Moreover, increased CNS levels of IL-17-producing $\gamma\delta$ T cells were found in children and mouse models of epilepsy, and their number proportionally correlated with the disease severity²⁷⁸. Contrarily, the number of Tregs negatively correlated with disease severity²⁷⁸.

Interestingly, gut neuroimmune interactions are not restricted to the individual and can rather be intergenerational. Previous studies have shown the maternal microbiota's

importance to foetal brain development. For example, maternal high-fat diets during gestation alter commensals, leading to social deficits and reduced oxytocin levels in the offspring²⁷⁹. These alterations are reversible upon administration of *Lactobacillus reuteri*²⁷⁹. The same social phenotype was observed in the offspring of dams receiving late gestation antibiotic treatment²⁸⁰. Moreover, in this study, the pups showed increased levels of cytokines in the frontal cortex and increased BBB permeability²⁸⁰. Transient colonisation of germ-free pregnant females showed that maternal gut microbiota enhances the offspring's immune system by increasing type III innate lymphoid cells (ILC3) and macrophages²⁸¹.

Neuroimmune interactions in lung physiology

The lungs are a critical barrier constantly exposed to microorganisms and allergens. Therefore, they are equipped with robust immune cells network and neuronal innervations to maintain homeostasis and respond to pathogenic stimuli. Similar to the gut, the lungs present extrinsic sympathetic, parasympathetic and sensory neurons²⁸². These neurons regulate physiological processes after birth²⁸². Moreover, the lungs feature intrinsic parasympathetic neurons residing in the trachea and major bronchi, which play a pivotal role in early stages of lung development²⁸². Specialised epithelial cells called pulmonary neuroendocrine cells (PNECs) are present in the lungs²⁸³. They closely interact with the sensory neurons and produce hormones and neuropeptides in response to neuronal stimuli²⁸³. For instance, bronchoconstriction in asthma was found to be present only in mice with intact innervation and in a serotonin-dependent manner²⁸⁴. Moreover, mice lacking mast cells did not develop bronchoconstriction due to the absence of serotonin, revealing the implication of the neuroimmune interaction in this mechanism²⁸⁴. In addition, neuropeptides such as VIP and substance P induce mast cell to produce histamine, leukotriene C4, prostaglandin D2, IL-6 and TNF- α , contributing to allergic reaction^{284–286}. Type 2 innate lymphoid cells (ILC2s) express VIP receptors and secrete IL-5, which drives eosinophilia²⁸⁷. Reciprocally, IL-5 is sensed by pulmonary neurons that release VIP, further activating ILC2s, in a positive feedback loop²⁸⁸. In addition, in both asthmatic murine models and human asthma patients the levels of nucleotide adenosine triphosphate (ATP) are increased^{289,290}. Sensory neurons can detect ATP through P2X receptors, triggering coughing²⁸⁹. Additionally, extrinsic neurons contribute to the amelioration of pulmonary fibrosis, as vagotomy decreases the production of transforming growth factor (TGF)- β and IL-4, reducing the macrophage number and collagen deposition in the lungs²⁹¹.

Pulmonary neurons can also directly control eosinophil recruitment through the expression of intercellular adhesion molecule 1 (ICAM1) and C-C motif chemokine ligand 11

(CCL11)^{292–294}. Eosinophils cluster and adhere to parasympathetic nerves²⁹². When this adhesion is blocked antigen induced-hyperreactivity is prevented²⁹². Additionally, *in vitro* studies show that parasympathetic nerves exposed to IL-4 or IL-13 increased the expression of eotaxins (small proteins that attract eosinophils)²⁹⁴. Finally, PNECs are also involved in neuroimmune interactions, regulating goblet cell hyperplasia and ILC2s via γ -aminobutyric acid (GABA) and CGRP production, respectively²⁹⁵.

Neuroimmune interactions in tissue infection

During infection, the immune system is the major contributor to eliminating the pathogen and restoring homeostasis. However, much like in tissue physiology, immune cells do not work solitarily in the host but interact with other systems, including the nervous one.

During *Salmonella typhimurium* infection, gut extrinsic sympathetic nerves are activated and release noradrenaline, which acts on muscularis macrophages driving a tissue-protective phenotype with anti-inflammatory activity²⁹⁶. In turn, these macrophages secrete polyamines that protect enteric neurons from pathology and death induced by the infection²⁹⁷.

Neuron-ILC crosstalk is also critical for host defence against infections. Enteric neurons secrete the neuropeptide neuromedin U (NMU), which activates ILC2s to produce IL-5 and IL-13, mediating protection against oral *Nippostrongylus brasiliensis* infection²⁹⁸. On the contrary, CGRP secretion by ENS, extrinsic neurons, or by ILC2s, suppresses ILC2-derived IL-13²⁹⁹, suggesting a role for ILC2s regulation. Nonetheless, β 2-adrenergic receptor (β 2AR) expressed on ILC2s is activated by the neurotransmitter epinephrine, resulting in reduced ILC2s proliferation and increased worm burden during *N. brasiliensis* infection³⁰⁰. These findings highlight the importance of synchronised neuroimmune communication during infection to efficiently contribute to host defence. ILC3s exhibit similar contradictory findings. While enteric glial cells, also abundant in the gut, enhance ILC3-derived IL-22 production through glial-derived neurotrophic factor (GDNF), helping host defence against *Citrobacter rodentium*³⁰¹, VIP production by enteric neurons decrease IL-22 produced by ILC3s rendering susceptibility to the bacteria³⁰².

Adaptive immune T cells also participate in neuroimmune signalling during infection. Neuronal production of acetylcholine (ACh) enhances T cell production of IL-13 and IFN- γ , facilitating the resolution of *N. brasiliensis* and *S. typhimurium* infections³⁰³. Additionally, acetylcholine-producing T cells recruited to the colon during enteric *C. rodentium* infection produce IFN- γ , IL-17A, and IL-22³⁰⁴. Further studies are required to assess the role of CD4⁺

T cell producing Ach during these specific infections. Nevertheless, these cells were found to signal splenic macrophages to promote anti-inflammatory responses during infection³⁰⁵. Moreover, they are also critical for vasodilatation in response to LMCV infection³⁰⁶. However, CD8⁺ T cells negatively impact enteric neuronal survival in viral infections including Chikungunya, West Nile, and Zika virus, causing enteric neuronal death^{307,308}.

Neuroimmune crosstalk is also observed during pneumonia. For example, Ach signalling in alveolar macrophages and neutrophils prevents acute lung injury caused by *E. coli* by reducing the production and pro-inflammatory cytokines and chemokines, as well as reducing transalveolar neutrophil migration to the airspaces and diminishing transalveolar permeability³⁰⁹. Lung sympathetic norepinephrine-producing innervations using a model of LPS lung injury proved to be critical in tempering inflammation³¹⁰. Pneumonia caused by *Staphylococcus aureus* showed that sensory neurons release CGRP that suppresses neutrophil and $\gamma\delta$ T cell immune responses, with lethal consequences for the host³¹¹. These neurons are also triggered in lung tuberculosis. *Mycobacterium tuberculosis* releases molecules such as glycolipid sulfolipid-1 (SL-1), which is perceived by sensory neurons and leads to the cough reflex, a well-known symptom of human tuberculosis³¹².

Respiratory viral infections are also linked to inflammation and neuroimmune interactions. Influenza A virus (IAV) activates sympathetic neurons and exacerbates inflammation and mortality³¹³. Further studies are required to enlighten the mechanisms involved in this process. Nonetheless, Ach was shown to be required for anti-inflammatory response to IAV infection³¹⁴. RNA sequencing showed that bronchopulmonary sensory neurons, in response to IAV infection, increase the expression of numerous genes associated with an antiviral and pro-inflammatory response, with a concomitant increase in inflammatory cell recruitment³¹⁵. Ach-producing lymphocytes are recruited and directly communicate with macrophages towards dampening inflammation and promoting tissue repair³¹⁶.

As in tissue physiology, neuroimmune interactions upon inflammation can occur intergenerationally. In mouse models of maternal immune activation (MIA), the adult offspring presented long-term behaviour deficits, including abnormal communication, reduced sociability, repetitive behaviours and increased anxiety levels, symptoms of human neurological disorders such as autism^{317,318}. Abnormal behaviours upon MIA are caused by Th17 cells and IL-17 production. Genetic ablation of Th17, CNS IL-17RA deficiency or Th17 blockage all showed amelioration of behaviour deficits in the offspring MIA mice^{319,320}. Moreover, these behaviour phenotypes are associated with maternal intestinal microbiota. Pregnant mice colonised with commensal bacteria that induce Th17 development, either mouse segmented filamentous bacteria or human commensal bacteria, exhibit an increased

likelihood of neurodevelopmental disorders in their offspring following infection or autoinflammatory syndromes^{321,322}.

Our body is delicately regulated by the synchrony of the different systems, in a magnificent, impressive dance where the host must lead with different partners, beneficial and detrimental, and where one wrong footstep leads to severe consequences and the end of the ball.

CHAPTER II

MANUSCRIPT 1

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DEVELOPMENT OF BIOLUMINESCENT GROUP B STREPTOCOCCAL STRAINS FOR NON-INVASIVE LONGITUDINAL INFECTION STUDIES

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Abstract

Group B *Streptococcus* (GBS) remains the leading bacterial cause of invasive neonatal disease, resulting in substantial morbidity and mortality. New therapeutic approaches beyond antibacterial treatment to prevent neonatal disease outcomes are urgent. One significant limitation in studying GBS disease and progression is the lack of non-invasive technologies for longitudinal studies. Here, we develop and compare three bioluminescent GBS strains for *in vivo* pathogenic analysis. Bioluminescence is based on the *luxABCDE* operon on a replicative vector (*lux*GBS-CC17), and the red-shifted firefly luciferase on a replicative vector (*ffluc*GBS-CC17) or integrated in the genome (*gluc*GBS-CC17). We show that *lux*GBS-CC17 is suitable for *in vitro* analysis but does not produce a significant bioluminescent signal in infected pups. In contrast, the *ffluc*GBS-CC17 results in a strong bioluminescent signal proportional to the organ colonisation level. However, the stability of the replicative vector depends on the route of infection, especially when pups acquire the bacteria from infected vaginal mucosa. Stable chromosomal integration of luciferase in *gluc*GBS-CC17 leads to significant bioluminescence in both haematological and vertical infection models associated with high systemic colonisation. These strains will allow the preclinical evaluation of treatment efficacy against GBS invasive disease using whole-mouse bioluminescence imaging.

Introduction

Group B *Streptococcus* (GBS) remains the leading bacterial pathogen responsible for invasive disease and death in infants under two months old¹. GBS colonises the lower genital tract of pregnant women and can be transmitted vertically to the newborn *in utero* or during birth, leading to early-onset disease (EOD), mostly associated with sepsis and pneumonia, or late-onset disease (LOD), frequently presented as meningitis². Invasive GBS disease results in a mortality rate of 10-15% and life-long neurodevelopment impairment (NDI) in up to 50% of survivors³. GBS meningitis is particularly concerning, as its incidence has been increasing over the last decade⁴⁻⁶. Moreover, no neuroprotective treatment is currently available, and intrapartum antibiotic prophylaxis is ineffective to prevent LOD meningitis⁷, highlighting the necessity for developing new preventive and therapeutic solutions.

Over the past decades, significant efforts have been made to elucidate the pathophysiology and long-term NDI resulting from GBS neonatal disease, leading to the establishment of several animal models, including hematogenous, oral and direct brain infections⁸⁻¹¹. Recently, our group developed an animal mouse model that mimics the natural human route

of infection, *i.e.* mother-to-progeny transmission, through intra-vaginal colonisation of pregnant females¹². However, these models still do not provide a complete understanding of the disease establishment and progression^{2,12}. Traditional colony-forming unit (CFU) counting methods have limitations, including the need to euthanise animals to assess organ colonisation¹³. Using non-invasive bioluminescence imaging (BLI) would enable longitudinal *in vivo* studies, enhancing our understanding of the pathophysiology of GBS neonatal disease. Additionally, BLI would facilitate testing the efficacy of novel prophylactic and therapeutic treatments. Moreover, this technique aligns with the ethical principles of the 3Rs in animal experiments by reducing and refining animal usage, allowing each animal to be studied across multiple time points and organs, rather than sacrificing one animal per time point for limited organ studies^{13,14}.

Bioluminescent reporter systems have been developed in several pathogenic streptococcal species^{15–18}. In GBS, luminescence has been obtained with a replicative vector containing the luciferase gene (*luc*) from the firefly *Photinus pyralis*¹⁵. The firefly luciferase converts D-luciferin, its substrate, to oxyluciferin, emitting light with a maximum intensity of 560 nm¹⁹. The replicative vector is stabilised by antibiotic selection and a toxin-antitoxin (TA) system plasmid stability for up to 10 days *in vitro* even without the antibiotic selective pressure¹⁵. However, this system was not evaluated *in vivo*, in animal models of infection. Group A *Streptococcus* (GAS) has been the most studied streptococcal species with bioluminescent reporters^{15–18}. Initially, the bacterial luciferin-luciferase operon (*luxABCDE*) was used, which has the advantage of not requiring an external luciferin substrate for bioluminescent light emission (maximum intensity at 490 nm)¹⁸. Nevertheless, this system produces a weaker signal, and the vector is less stable¹⁸. To improve sensitivity *in vivo*, a variant using the red-shifted firefly luciferase (*ffluc*), which emits light at 610 nm, was created in GAS¹⁸. Bioluminescent streptococcal strains have been used for studying disease progression, antimicrobial compounds, vaccine efficacy or even pathogenic synergism^{20–23}, highlighting the importance and wide-range applicability of BLI for enhanced *in vivo* studies.

In the present study, we aimed to develop bioluminescent GBS strains to further improve our knowledge of neonatal GBS invasive disease. We found that vector-based approaches are unstable and inefficient for longitudinal studies with GBS, even with a stable *ffluc* plasmid carrying a TA system. However, genomic insertion of the red-shifted firefly luciferase gene into GBS creates a stable bioluminescent strain suitable for evaluating severe GBS disease using whole-mouse live imaging.

Methods

Bacterial strains, plasmids and growth conditions

Enterococcus faecalis containing the plasmid pSL101P32 carrying the *luxABCDE* cassette (Table 1) was kindly provided by Dzung B. Diep²⁴. The pLZ12Km2-P23R:TA:fflucRT plasmid was a gift from Thomas Proft (Addgene, #88901)¹⁸. *E. faecalis* and *Escherichia coli* DH5 α (Table 1) were grown in Brain Heart Infusion (BHI) broth or agar (Difco Laboratories) and Luria Bertani (LB) broth or agar (GRiSP, Lda), respectively, at 37 °C. The wildtype GBS strain BM110, a CC-17 clinical isolate, was cultured at 37 °C in Todd-Hewitt (TH) broth or agar (Difco Laboratories). For strains containing vectors (Table 1), antibiotics were added as appropriate at the following concentrations: spectinomycin (EMD Millipore) at 500 μ g/mL (*E. faecalis*), 70 μ g/mL (*E. coli*) or 200 μ g/mL (GBS); kanamycin (Kan) (Sigma Aldrich) at 50 μ g/mL (*E. coli*) or 200 μ g/mL (GBS); erythromycin (Erm) (Sigma Aldrich) at 150 μ g/mL (*E. coli*) or 5 μ g/mL (GBS).

Table 1. Bacterial strains and plasmids nomenclature.

Bacterial strain	Plasmid	Selective antibiotic	Nomenclature (if applicable)
<i>Enterococcus faecalis</i>	PSL101P32	Spectinomycin	
<i>Escherichia coli</i> DH5 α	PSL101P32	Spectinomycin	
<i>Escherichia coli</i>	pLZ12Km2-P23R:TA:fflucRT	Kanamycin	
<i>Escherichia coli</i> XL-1 blue	pG-oxi-luc-ldh	Erythromycin	
Group B <i>Streptococcus</i> BM110			GBS-CC17
Group B <i>Streptococcus</i> BM110	PSL101P32	Spectinomycin	<i>lux</i> GBS-CC17
Group B <i>Streptococcus</i> BM110	pLZ12Km2-P23R:TA:fflucRT	Kanamycin	<i>ffluc</i> GBS-CC17

Bacterial growth and *in vitro* luminescence

*Lux*GBS-CC17 was grown overnight (ON) and 1:100 subcultured in non-vented culture flasks in 30 mL of TH with 5 μ g/mL erythromycin at 37 °C, for 7 h. The optical density (OD) at 600 nm (OD_{600 nm}) was measured every 30-60 minutes using a spectrophotometer (Biochrom Libra S6+). The culture was plated at the same timepoints on TH agar with 5 μ g/mL erythromycin and incubated at 37 °C ON for CFU count. All the other strains were

1:100 subcultured in TH with the respective antibiotic, in transparent 96-well microplates. The cultures were maintained at 37 °C and measurements were performed every 10 minutes for 7 h, on Synergy 2 Plate reader (OD_{600 nm}).

For the luminescent signal, the strains were grown in non-vented culture flasks containing 30 mL of TH. An aliquot of the culture was taken at indicated timepoints and read on Synergy 2 Plate reader, using a white 96-well flat bottom microplate (settings at 37 °C, 3s shaking before reading and luminescence read with 150 of gain). In all experiments technical duplicates and biological triplicates were performed.

GBS transformation

Plasmids were extracted using commercial kits according to manufacturer's instructions. The pSL101P32 or pLZ12Km2-P23R:TA:fflucRT vectors were introduced in GBS by electroporation. Electrocompetent bacteria were prepared starting from an ON culture in TH at 37 °C used to inoculate (1:10) fresh medium. The culture was grown at 37 °C until exponential growth phase (OD 600nm around 0.9). Then, one volume of a 1 M sucrose solution and 0.05% of glycine were added, and the culture was incubated for 1 h at 37 °C with mild shaking. The culture was centrifuged at 2,000 g for 10 minutes at 4 °C, washed twice with electroporation buffer (7 mM sodium phosphate, 500 mM saccharose, 1 mM magnesium chloride, pH 7.4), and re-suspended in electroporation buffer with 15% glycerol. The culture was stored at -80 °C until electroporation was performed. For electroporation, 6 µL of plasmid was added to 100 µL of competent bacteria, and the mixture was electroporated at 200 Ω, 2500 V / cm electric field, 25 µF. Thereafter, 900 µL of TH was immediately added and the solution was incubated for 1 h 30 minutes, at 37 °C. The bacteria were then plated on TH plates containing spectinomycin or kanamycin at 37 °C. Isolated GBS spectinomycin-resistant transformant containing the vector pSL101P32 was referred to as *lux*GBS-C17 and the kanamycin-resistant transformant containing the vector pLZ12Km2-P23R:TA:fflucRT as *ffluc*GBS-CC17.

Polymerase chain reaction (PCR)

Colony PCR was performed using the following primers (Table 2) to target the *lux*ABCDE operon: *LuxA* sense (5'GCCTACCGATGATATTAAGTTG 3') and *LuxB* anti-sense (5'ACCACACTAATGGATCGCTCG 3'), *LuxD* sense (5' TGCGCGGATAGCTTATGCAA 3') and *LuxE* antisense (5'ACCACACTAATGGATCGCTCG 3'); and the following primers to target *Ffluc* gene: sense 5'AAGGTCCTGCCCCATTTTACC 3' and antisense

5'GCGGCAAATGACTGGTTACG 3'. The PCR reaction comprising NZYtaq 2x Green Master Mix (NZYTech) was run under the following conditions: an initial denaturation step at 95 °C for 5 minutes, 30 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 25 seconds, followed by a final extension step at 72 °C for 10 minutes. The PCR products were run in a 1 % agarose gel, containing Green Safe Premium (NZYTech). Gel images were acquired in a gel Imaging system (Gel-Doc XR+, Bio-Rad) using Image Lab (V6.0.1, Bio-Rad) software.

Table 2. Oligonucleotide sequences of the utilised primers

Primer ID	Oligonucleotide sequence
<i>LuxA</i> (sense)	GCCTACCGATGATATTAAGTTG
<i>LuxB</i> (anti-sense)	ACCACACTAATGGATCGCTCG
<i>LuxD</i> (sense)	TGCGCGGATAGCTTATGCAA
<i>LuxE</i> (anti-sense)	ACCACACTAATGGATCGCTCG
<i>Ffluc</i> (sense)	AAGGTCCTGCCCCATTTTACC
<i>Ffluc</i> (anti-sense)	GCGGCAAATGACTGGTTACG
879	GAATTCGTAATCATGTCATAGCTGT
880	GAATTCGTAATCATGTCATAGCTGT
1688	CCTGCAGGTCGACTCTAGAGGATCCGACAATTTGGGATAATTCTCGTG
1689	GAGCTTAAGAATTGCCGCTCTAGATCATCCTTTAACAAGGTCAAAAATG
1690	GTTAAGCGAAATGCTTAGCCTTTTACGAAAAGCCCTGACAACCCTTGTTG
1691	CATTTTTGACCTTGTTAAAGGATGATCTAGAGCGGCAATTCTTAAGCTCG
1692	CGAGCTTAAGAATTGCCGCTCTAGATCATCCTTTAACAAGGTCAAAAATG
1694	GTTGATATGGTGATCTTAGCTGTTG
1695	GTTATTGGTTCAGGTACTTCACTTG

Plasmid stability

GBS strains containing the luminescent plasmid (pSL101P32 or pLZ12Km2-P23R:TA:fflucRT) were grown in TH ON at 37 °C, with or without antibiotics and subsequently subcultured 1:1000 in fresh TH, with or without antibiotics, and incubated at 37 °C for 24 h. This step was repeated every 24 h for 8-10 days. Subcultures were serially diluted and plated on TH agar medium, with or without selection, every day and incubated ON at 37 °C. Plasmid stability was assessed given the difference in the CFU count in the presence and absence of antibiotic selection.

Construction of pG-oxi-luc-ldh

To construct the *gluc*GBS-CC17 strain with a chromosomal *ffluc* gene, we constructed the pG-oxi-luc-ldh integrative vector (Supplementary Figure 5). First, the *ffluc* gene was amplified from the pLZ12Km2-P23R:TA:*ffluc* vector using high-fidelity DNA polymerase (Phusion, New England Biolabs) and primers 1690 and 1691 (Table 2). Two contiguous 500 bp region of the BM110 genome were similarly amplified using primers 1688 and 1689 and 1692 and 1695 (Table 2). The three amplicons contain overlapping sequences and were purified (QIAquick PCR purification, Qiagen) and mixed for a final PCR with the external primers 1688 and 1693. The final amplicon was gel purified (QIAquick gel purification, Qiagen) and used for Gibson assembly into the pG1 thermosensitive vector previously amplified with the 879 and 880 primers (Table 2).

Integration of *ffluc* into the GBS CC17 genome

The pG-oxi-luc-ldh vector was introduced in *E. coli* XL-1 blue (Stratagene) and selected with erythromycin. After purification and sanger sequencing (Eurofins Genomics), the pG-oxi-luc-ldh was introduced in BM110 by electroporation with erythromycin selection at 30°C, the permissive temperature for vector replication. Chromosomal integration at the targeted locus was selected by growing isolated transformants at 37°C on TH yeast (THY) agar in the presence of erythromycin. Following a second round of isolation on THY with erythromycin, a single colony was inoculated into 10 ml of THY without antibiotics, grown at 30°C, and sub-cultured four times under the same conditions. Diluted cultures ($d = 10^{-5}$) were then spread on THY agar, grown overnight at 37°C, and single colonies ($n = 48$) were inoculated into 150 μ l of THY in microwell plates. After 4 hours of incubation at 37°C, replicas were made using a 96-pin microplate replicator (Boeckel) on THY with and without erythromycin to differentiate colonies that retained or lost the vector. Erythromycin-sensitive colonies were then tested by PCR with primers 1694 and 1695 (table 2) to determine whether they had restored a wild-type genomic sequence (indicating loss of the integrated *lux* gene) or whether the *lux* gene had precisely integrated at the targeted genomic position. The outcome (WT back or *lux* integration) depends on the side of homologous recombination (5' or 3') between the flanking genomic regions cloned around the *lux* gene. Colonies with the integrated *lux* gene were isolated and their genomic DNA were purified (DNeasy Blood and Tissue kit, Qiagen) and sequenced (Illumina, Novogen). High-quality reads were mapped against the BM110 genome (362x genome coverage) and analysed for SNP, indel, and genomic rearrangement (Genious Prime, Biomatters Ltd). The selected

*gluc*GBS-CC17 strain, with *luc* inserted at position 1,000,921 in the BM110 genome (NCBI RefSeq NZ_LT714196) and without any secondary mutation, was then stored in 20% glycerol at -80°C.

Adhesion and invasion assays

The human colonic epithelial cell line Caco-2²⁵, derived from a human colorectal adenocarcinoma (ATCC), was cultured at 37 °C in a 5% CO₂ atmosphere in DMEM high glucose with GlutaMax (HyClone) supplemented with 10% heat-inactivated FBS, 1% sodium pyruvate (Gibco) and 1% penicillin/streptomycin (6.0 µg/ mL / 10.0 µg/mL, Biowest). The cell line was grown until cells reached 80% confluence. For infection assays, cells were seeded on 12 well-standard plates at 3 x 10⁵ cells/well for 4 days at 37 °C under a 5% CO₂ atmosphere. Briefly, cells were washed thrice and infected with GBS in media without FBS or antibiotics at a multiplicity of infection (MOI) of 30. The number of adhered cells was counted to adjust the bacterial numbers. Infection was allowed for 3 h, followed by five washes to remove nonadherent bacteria. Subsequently, the cells were lysed with TrypLE Express (Gibco) containing 0.2% Triton X-100 for 10 minutes at 37 °C. The bacterial adhesion was quantified by subtracting the intracellular bacteria from the total cell-associated (intracellular plus surface-adherent bacteria).

For invasion assays, 3-hour-infected cell monolayers were further incubated with cellular culture media containing 100 µg/mL gentamycin (Lonza) for 1.5 h to kill the extracellular bacteria, before cell lysis. Appropriate dilutions were plated on TH agar plates, and CFU were counted. The percentage of invasion was calculated relative to the bacterial inoculum.

Animal handling and ethics statement

C57BL/6 mice were purchased from Charles River Laboratories and housed at the Instituto de Investigação e Inovação em Saúde (i3S) animal facility under a 12 h light/dark cycle and *ad libitum* access to food and water. All animal procedures were approved by the Ethics Committee of the i3S, and the Portuguese national authority the Direção Geral de Alimentação e Veterinária (DGAV). All animal experiments were performed in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and Directive 2010/63/EU and Portuguese rules (DL 113/2013). All animal experiments were planned to minimise mice suffering and number, and always handled by licensed investigators.

Additionally, all experiments were performed in accordance with relevant guidelines and regulations, including the ARRIVE guidelines for animal experiments.

In vivo mouse infection models

Overnight cultures of the different GBS strains were subcultured 1:100 on TH broth and grown until mid-log phase (OD_{600nm} around 1.000) at 37 °C. Bacteria were washed twice with PBS, and the OD_{600nm} was adjusted to $\approx 0.600 \pm 0.005$, corresponding approximately to 2.0×10^8 CFU / mL. Inocula were prepared by further dilution from this bacterial suspension.

Two-day-old C57BL/6 mice were intraperitoneally (i.p.) infected with the indicated CFU of WT GBS or luciferase-expressing strains in 40 μ L of total volume. Survival curves were determined over a 36 h experimental period. To assess bacterial colonisation, mice were euthanised by decapitation following isoflurane anaesthesia. The brain, lungs and liver were aseptically removed 18 h post-infection, homogenised, serially diluted in PBS, and plated on TH agar medium with or without antibiotic selection for CFU counts.

To vertically infect neonatal mice, we adapted our previously described mouse model of infection¹². Pregnant C57BL/6 mice were intravaginally inoculated, using a micropipette, with 40 μ L of GBS suspension containing 3×10^4 CFU, at the gestation (G) days 16 and 17. The presence of a vaginal plug was considered the day 1 of gestation. Females were allowed to deliver spontaneously, and infected pups were kept with their mothers throughout the experimental time. Organs were collected and plated as previously described.

Bioluminescence imaging

For the *in vitro* correlation between the bioluminescence signal of luciferase-expressing GBS strains, the CFU and the OD, bacteria were grown as described above and serially diluted 1:2 in white opaque flat clear bottom or transparent flat bottom 96-well plates. The luminescence and the absorbance at 600 nm were quantified on Synergy 2 (BioTek). For *ffluc*GBS BM110, 1:1 D-Luciferin potassium salt (0.5 mg / mL, Perkin Elmer) was added to the wells 5 minutes before luminescence quantification. Bioluminescence imaging was assessed on the IVIS Lumina LT system (Perkin Elmer). According to the strain, bacterial dilutions were plated on TH agar, with or without selection, for CFU enumeration.

For *in vivo* studies, C57BL/6 mice infected with the different bioluminescent strains were anaesthetised with 2.5% isoflurane (O_2 flow of 1.0 L/minutes). In the case of mice infected with *ffluc*GBS, 20 μ L of the indicated concentrations of D-Luciferin or CycLuc1 sodium salt

(AOBIOUS, AOB6377) were administered s.c.. The substrate was allowed to be distributed in the body of the anaesthetised animal for 5 minutes, unless stated otherwise, before image acquisition. Pups were allowed to recover from the anaesthesia before returning to the respective cage. Bioluminescence imaging was acquired on the IVIS Lumina LT system (Perkin Elmer) or Newton 7.0 (Vilber).

Statistical Analysis

All data were analysed and graphically represented using GraphPad Prism software (v.9.0.1, GraphPad Software Inc. CA). Means and standard errors of the means (SEM) were calculated and correspond to the indicated independent experiments. Student's t-test was used to analyse differences between two experimental groups. The differences between multiple groups were performed by one-way or two-way ANOVA, whenever appropriate, with Tukey's Multiple Comparison Test. The normality of the data was verified by the Shapiro-Wilk normality test. Survival curves were plotted using Kaplan-Meier plot, and statistical significance was determined using the log-rank (Mantel-Cox) test. CFU data were $\log_{10}$ transformed. Differences were considered significant for $P \leq 0.05$ and represented by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. The number of samples and independent experiments is expressed in the respective legend.

Results

Development and assessment of a *lux* bioluminescent reporter system in GBS

To generate a GBS bioluminescent reporter strain, we used the BM110 strain, of capsular serotype III and belonging to the CC-17 hypervirulent lineage responsible for more than 80% of meningitis cases^{12,26,27}. We first tested an available bioluminescent vector-based system containing the *luxABCDE* operon, which encodes the luciferase and its aldehyde substrate, thereby eliminating the need for an exogenous substrate^{13,28}. To this end, we introduced the replicative vector pSL101P₃₂, containing the constitutively active P32 promoter^{24,29} to generate the GBS strain *lux*GBS-CC17.

To evaluate whether light emission could be expressed as a function of cell growth, bacteria from the mid-log phase were serially diluted, and the luminescence (emission 490_{nm}), optical density (OD_{600nm}), and cell counts (CFU/mL) were determined. The bioluminescent signal of *lux*GBS-CC17 positively correlated with both OD_{600nm} and the number of CFU, indicating that the bioluminescent signal reliably predicts bacterial count (Figure 1a). Moreover, the

pSL101P₃₂ vector does not have a fitness cost since the growth curve was equivalent to the one of wild-type (WT) GBS (GBS-CC17) under standard conditions in TH (Figure 1b). To assess the stability of the pSL101P₃₂ vector, we grew serial cultures of *lux*GBS-CC17 with or without the selective antibiotic (spectinomycin) for 8 days. Bacteria were quantified every 24 hours, by evaluating CFU on selective and non-selective media (total *versus* spectinomycin resistant bacteria). No significant differences were found between total and

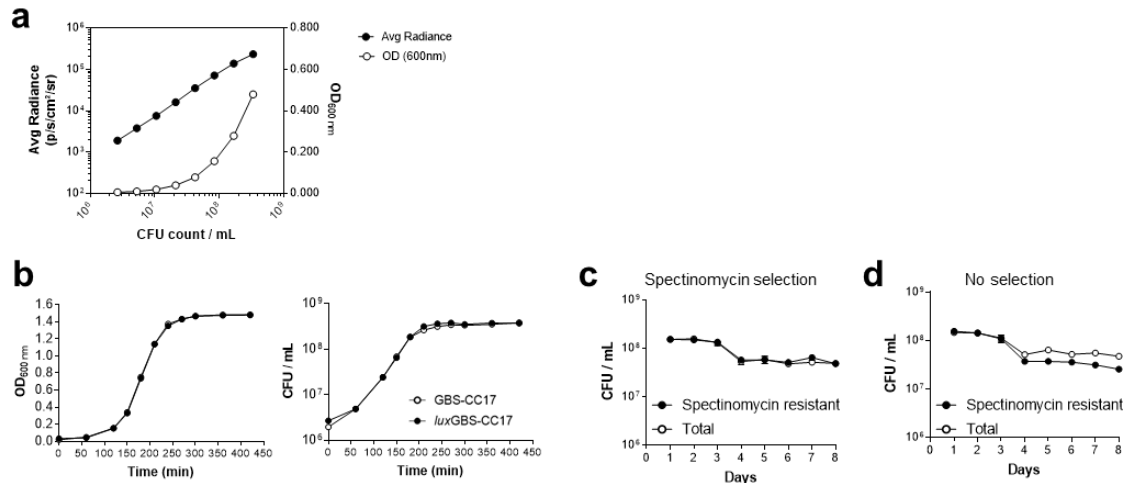


Figure 1. *In vitro* bioluminescence with the pSL101P₃₂ replicative vector in GBS. The pSL101P₃₂ vector was introduced in the BM110 wild-type strain (WT) to give origin to the strain *lux*GBS-CC-17. **(a)** Correlation of bioluminescent signal, optical density (OD) and CFU count. *lux*GBS-CC17 was grown until the mid-log phase and serially diluted in 96-well plate. Bioluminescence, OD and CFU quantified. Field symbols represent radiance, whereas open symbols OD. Bioluminescent signal plotted as radiance (photons/seconds/cm²/sr). Results represent data from 3 independent experiments. **(b)** Growth curves of WT and *lux*GBS-CC-17 strains. Optical density (left) measured every 30 or 60 minutes for 7 h and the corresponding GBS numbers (right). Mean values $\pm$ SEM data pooled from 3 independent experiments. Comparisons were performed by two-way ANOVA. **(c-d)** The *lux*GBS-CC-17 strain was daily diluted 1:1000 into fresh TH medium (with or without spectinomycin) and grown for 24 h, for 8 consecutive days. **(c)** CFU enumeration of both strains plated on TH medium with or **(d)** without selection for CFU count. Mean values $\pm$ SEM data pooled from 3 independent experiments. Comparisons were performed by two-way ANOVA. infection (hpi). The organs from WT infected pups were plated on TH medium, while those from *lux*GBS-CC17 were plated on TH with and without spectinomycin.

spectinomycin resistant GBS strains, even when grown without selective pressure, indicating that the plasmid was stable *in vitro* (Figure 1c and d).

To evaluate the infectivity of the bioluminescent strain, we tested its adhesion and invasion properties on human Caco-2 cells. No significant differences were observed between *lux*GBS-CC17 and WT GBS-infected cells for their ability to adhere to and invade the Caco-2 enterocytes (Figure 2a). To test the virulence of *lux*GBS-CC17, newborn mice at postnatal day (P) 2 were intraperitoneally (i.p.) infected with 5x10⁵ CFU of *lux*GBS-CC17 or GBS-CC17. No differences were observed in the survival rates of the pups between groups

(Figure 2b). To investigate invasion and colonisation of internal organs, we next determined the number of CFU in the brain, lungs, and liver 18 h post-infection (hpi). No differences were observed between groups, showing that the replicative vector did not affect bacterial virulence (Figure 2c). Moreover, all *lux*GBS-CC17 colonies recovered from infected organs are spectinomycin-resistant, as assayed by CFU counting on media with and without antibiotics, indicating that the vector is stable for the duration of the experiment (Figure 2c). To determine whether this strain generates a robust bioluminescent signal, mice infected with lower (5×10^4 CFU) or higher (1×10^5 CFU) doses of *lux*GBS-CC17 were imaged using

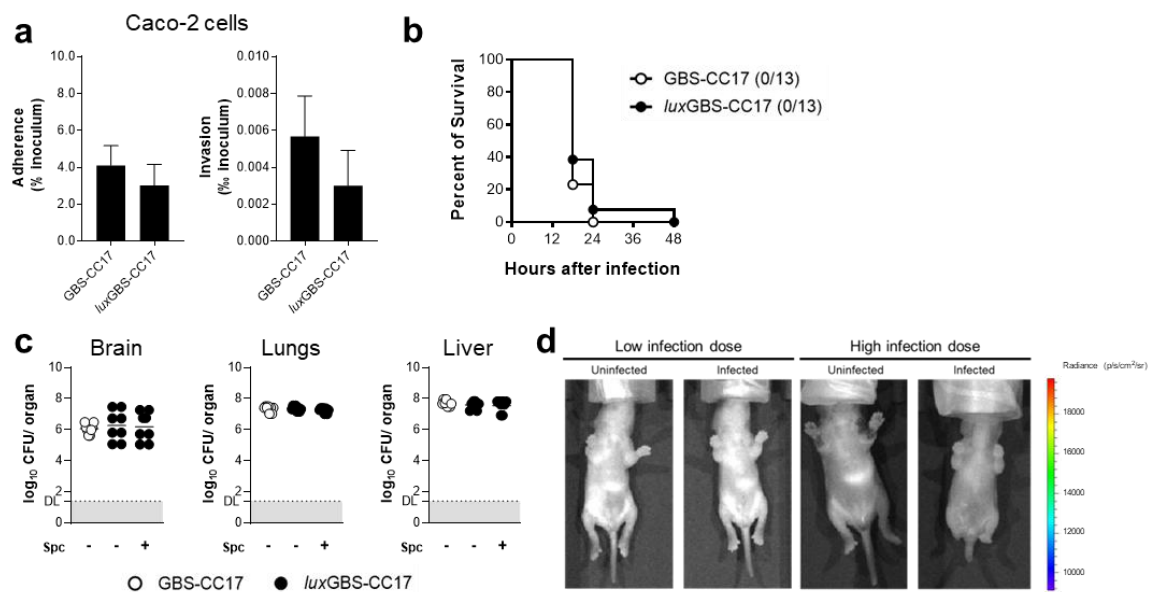


Figure 2. Infectivity and *in vivo* bioluminescence of *lux*GBS-CC-17. (a) Caco-2 cells were infected with WT or *lux*GBS-CC17 strains at a multiplicity of infection of 30, for 3 h, in triplicates. Bacteria adhesion and invasion were determined. Results are expressed relative to the initial inoculum. Data are shown as mean + SEM pool from 3 independent experiments. (b-c) Neonatal mice were inoculated intraperitoneally with 5×10^4 CFU of WT or *lux*GBS-CC17. (b) Kaplan-Meier survival curve monitored for 48 h. Numbers in parenthesis indicate the number of animals that survived versus the total number of infected animals ($n = 13$). Results represent data pooled from 2 independent experiments. Comparisons with the log-rank Mantel-Cox test. (c) Bacterial load in the brain, lungs and liver 18 hours post-infection (hpi). The organs from WT infected pups were plated on TH medium, while those from *lux*GBS-CC17 were plated on TH with and without spectinomycin. Data are shown as means, and each symbol represents data from individual pups ($n = 8$). (d) Neonatal mice were inoculated intraperitoneally with 5×10^4 (low dose) or 1×10^5 (high dose) CFU of *lux*GBS-CC17 and euthanised 18 hpi. Representative images of uninfected or infected pups resulting from the superimposition of the bioluminescence signal map and a grey-scale photograph of the mice in the ventral position.

an IVIS Lumina LT (Figure 2d). Unexpectedly, as shown in Figure 2d, no signal was detected on any tested pup regardless of the infection dose, suggesting that the individual luminescence signal was not high enough to be detected. Overall, these results show that the bioluminescent *luxABCDE*-based GBS strain is not efficient for *in vivo* studies.

Development and evaluation of a firefly luciferase-expressing GBS strain for *in vivo* short-term infection studies

Aiming at developing a bioluminescent GBS strain for longitudinal studies, we next used a replicative vector containing the red-shifted firefly luciferase, pLZ12Km2-P23R:TA:*ffluc*^{15,18} and the constitutively active P23 promotor³⁰. The red-shifted firefly *ffluc* luciferase system has a peak of light emission with a longer wavelength (610 nm) which enhances tissue penetration compared to the *lux* reporter system^{18,31}. Therefore, we introduced the pLZ12Km2-P23R:TA:*ffluc* vector in BM110 by electroporation, creating the *ffluc*GBS-CC17 strain. Similarly to *lux*GBS-CC17, the bioluminescent signal of *ffluc*GBS-CC17 positively correlated with the OD_{600nm} and the number of CFU (Figure 3a). The presence of the plasmid

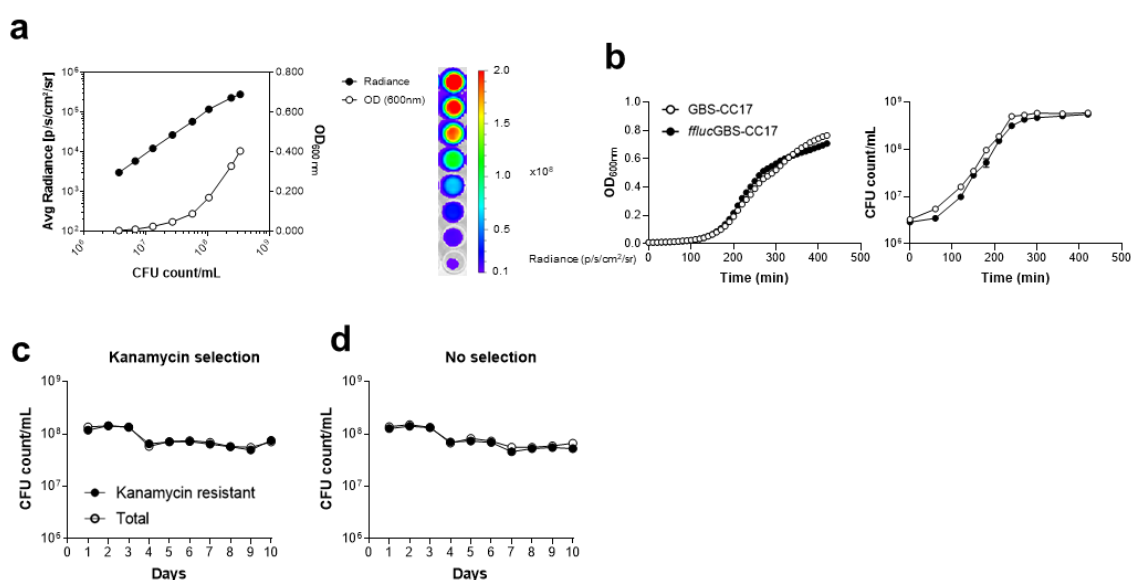


Figure 3. *In vitro* bioluminescence with the pLZ12Km2-P23R:TA:*ffluc*RT replicative vector in GBS. The WT strain was transformed with the pLZ12Km2-P23R:TA:*ffluc*RT vector to give the *ffluc*GBS-CC17 strain. **(a)** Correlation of bioluminescent signal, optical density (OD) and CFU count. The *ffluc*GBS-CC17 strain was grown until the mid-log phase and serially diluted on a 96-well plate. Left: Bioluminescence (Radiance, 0.5 mg/mL of Luciferin) and OD quantification on a plate reader (Synergy 2), and CFU counts. Field symbols represent radiance, whereas open symbols represent OD. Right: Bioluminescence signal imaged using IVIS Lumina LT. Areas with high photon emission correspond to the red colour and the areas with lower emission are displayed in blue. The detection of bioluminescent signals by the system resulted in the generation of signal maps overlaid on a grey-scale photograph of the 96-well plate. Bioluminescent signal plotted as radiance (photons/seconds/cm²/sr). Results represent data pooled from 3 independent experiments. **(b)** Growth curves of WT and *ffluc*GBS-CC17 strains. Optical density (left) measured every 10 minutes for 7 h on Synergy 2. GBS numbers were counted every 30-60 minutes (right). Mean values $\pm$ SEM data pooled from 3 independent experiments. Comparisons were performed by two-way ANOVA. **(c-d)** WT and *ffluc*GBS-CC17 strains were daily diluted 1:1000 into fresh TH medium (with or without kanamycin) and grown for 24 h, for 10 consecutive days. Numbers of kanamycin resistant GBS and total GBS cells grown in medium with **(c)** or without **(d)** kanamycin selection were counted. Mean values $\pm$ SEM data pooled from 3 independent experiments. Comparisons were performed by two-way ANOVA.

did not affect bacterial growth, as indicated by overlapping growth curves of the WT strain and *ffluc*GBS-CC17 (Figure 3b). Moreover, the plasmid remained stable *in vitro* for up to 10 days without antibiotic selective pressure (Figure 3c and d). Using the human enterocytes cell line Caco-2, we confirmed that the *ffluc*GBS-CC17 strain adheres to and invades cells similarly to the WT strain (Figure 4a). Moreover, when assessing the infectivity *in vivo* using a neonatal mouse model of infection, no differences in survival were observed between pups infected with *ffluc*GBS-CC17 or with the WT strain (Figure 4b). Bacterial loads in the brain, lungs and liver were comparable between groups 18 hpi (Figure 4c). Additionally, no differences were found when enumerating *ffluc*GBS-CC17 CFU in organs plated on selective and non-selective media, confirming plasmid stability *in vivo* (Figure 4c).

To validate the strain for BLI, P2 pups were infected with a low (5×10^4 CFU) and high (1×10^5 CFU) doses of *ffluc*GBS-CC17. At 18 hpi, pups were administered D-Luciferin subcutaneously (s.c.) 5 minutes before BLI acquisition, and images were captured in both dorsal and ventral positions (Figure 4d). As expected, high inoculum levels resulted in significantly ($P = 0.0003$) stronger bioluminescent signals, measured by average radiance (photons/second/cm²/steradian) (Figure 4d-e). However, 7 out of 12 pups infected with the low dose did not exhibit bioluminescent signal (Figure 4e). Thus, to determine whether these differences were correlated with different bacterial colonisation, GBS burden in the brain, lungs and liver were evaluated. Pups infected with the low dose presented significantly ($P = 0.0003$) lower bacterial loads than those infected with the high dose (Figure 4f). Among the 7 pups without bioluminescent signal, only 2 had CFU counts below the detection level, suggesting that this inoculum led to colonisation below the detection threshold of the IVIS system. Importantly, a positive correlation was observed when plotting the whole-body radiance with the sum of bacterial colonisation in the lungs, liver, and brain (Figure 4g and h). Taken together, these data show that the overall lack of bioluminescent signal intensity was due to reduced number of GBS in these organs.

To determine whether the bioluminescent *ffluc*GBS-CC17 strain could be used as a reliable bacterial quantification method in a mouse model mimicking the pathophysiology of GBS disease, newborn mice were infected vertically using our mouse model of infection¹², adapted for C57BL/6 mice. Briefly, pregnant mice were colonised with GBS-CC17 (WT or *ffluc*) in their vaginal mucosa at gestational days 16 and 17. No selective antibiotic was given to the mother or their progeny. Three-day-old pups were given D-luciferin, anaesthetised, subjected to BLI, and sacrificed for organ colonisation assessment. Unexpectedly, no bioluminescent signal was detected in any of the tested pups (data not shown). As expected, the organ colonisation in the brain, lungs and liver is lower than in the systemic infection, as our infection model usually results in decreased bacterial load (Figure

4c and i). Contrary to what was found in the i.p. model, CFU counts in the brain differed when plated on agar-medium with and without antibiotic selection (Figure 4i). In the brain, only 1 out of 7 pups had detectable GBS CFU on kanamycin-containing medium, while 5 out of 7 pups had detectable CFU when using non-selective medium (Figure 4i). Regarding the lungs, only 1 pup presented colonisation without selection, and none with antibiotic selection (Figure 4i). To investigate if this result was due to plasmid loss, we performed a colony PCR of brain CFU using *ffluc* plasmid-specific primers (Figure 4j and Supplementary figure 2). Only 1 out of 11 colonies tested retained the plasmid, indicating that GBS loses the plasmid *in vivo* over time, especially under conditions requiring extended analysis periods after infection. Overall, these data show that while the firefly luciferase-expressing GBS with the pLZ12Km2-P23R:TA:ffluc plasmid is a useful tool for models of infection leading to high organ colonisation, it is unsuitable for long-term studies due to plasmid instability over time *in vivo*.

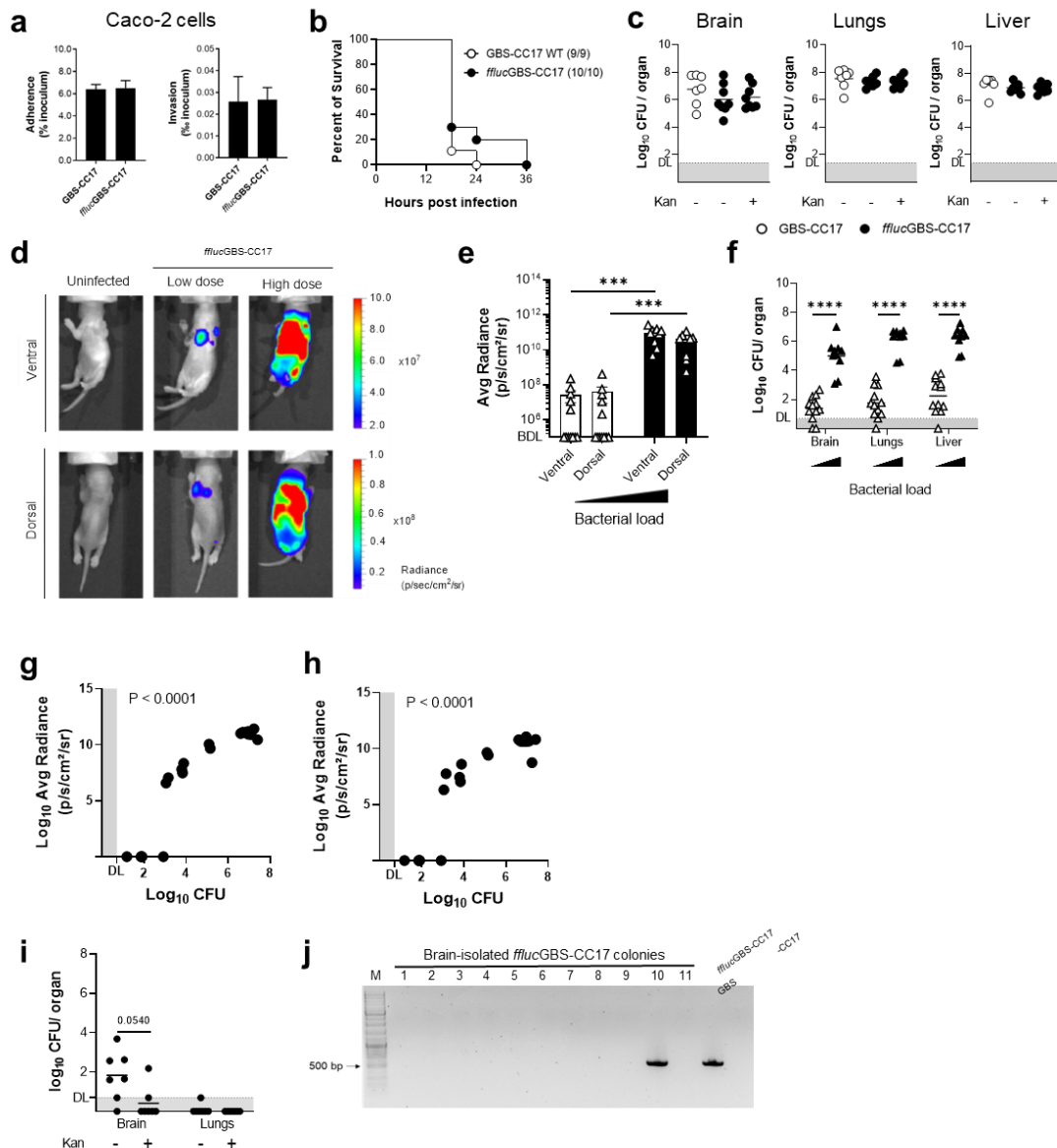


Figure 4. Infectivity and *in vivo* bioluminescence of fflucGBS-CC17. (a) Caco-2 cells were infected with WT or fflucGBS-CC17 for 3 h. Bacteria adhesion and invasion were determined. Results are expressed relative to the initial inoculum. Data shown as mean + SEM from 3 independent experiments. (b-c) Neonatal mice were inoculated intraperitoneally with 5x10⁴ CFU of WT or fflucGBS-CC17. Data pooled from 3 independent experiments. (b) Kaplan-Meier survival curve monitored for 36 h. Numbers in parenthesis: survivors versus total (n = 9 WT; n = 10 fflucGBS-CC17). Log-rank Mantel-Cox test comparisons. (c) Bacterial load in indicated organs at 18 hours post-infection (hpi), plated on TH with or without kanamycin. Data shown as mean, each symbol represents one pup (n = 7 WT; n = 8 fflucGBS-CC17). (d-h) Pups were inoculated intraperitoneally with 5x10⁴ (low dose) or 1x10⁵ (high dose) CFU of fflucGBS-ST17 and analysed 18 hpi. (d) Representative images by superimposition of the bioluminescence signal map and a grey-scale photograph. (e) The regions of interest (ROIs) were used to quantify the whole mouse bioluminescence signal and expressed as average radiance (photons/seconds/cm²/steradian). Mean ± SEM, each symbol represents one individual. BDL, below detection limit. (f) Bacterial load in the indicated organs. Data shown as mean, each symbol represents one animal (n = 12 Low dose; n = 10 High dose). Statistical analysis using unpaired Student's t-test. *** P<0.001; ****P < 0.0001. DL, detection limit. (g-h) Correlation between colonisation and average radiance in infected pups in the (g) ventral and (h) dorsal positions. Each symbol represents one animal. DL, detection limit. *P<0.0001. (i-j) Pregnant mice were intra-vaginally inoculated with 8x10⁴ fflucGBS-CC17. Three-day-old pups were analysed. (i) Bacterial load in the indicated organs. Data shown as mean pooled from 2 independent experiments. Each symbol represents one animal (n = 7). Statistical analysis by unpaired Student's t-test. * P<0.05. DL, detection limit. (j) Representative agarose gel showing colony PCR for screening fflucGBS-CC17 collected from pups' brains, using plasmid-targeted primers (495 bp amplicon). M, molecular weight marker (10 kb GeneRuler DNA ladder mix); 1-11, bacteria isolates; fflucGBS-CC17 and WT GBS-CC17 as controls.

A stable luciferase reporter for *in vivo* imaging in GBS infections

Integration of the firefly luciferase gene into the bacterial chromosome ensures stability and transmissibility of the reporter. Thus, we next sought to insert the red-shifted firefly luciferase into the BM110 genome. We first amplified the *ffluc* gene from the pLZ12Km2-P23R:TA:*ffluc* vector, including the P23 promoter³⁰, as well as two contiguous chromosomal GBS regions of 500 bp in a conserved intergenic region between the BQ8897_RS05445 and BQ8897_RS05450 genes, which encode a FAD-dependent oxidoreductase and L-lactate dehydrogenase, respectively (Supplementary Figure 1). The three amplicons were combined and cloned into the pG1 integrative vector, with *ffluc* inserted into the middle of the 1 kb chromosomal sequence. Following GBS transformation, selection for homologous recombination at the targeted locus, derecombination, and vector loss, we isolated the *gluc*GBS-CC17 strain. Whole-genome sequencing of *gluc*GBS-CC17 confirmed the integration of *ffluc* at the targeted locus, with no remaining traces of the integrative vector and no other secondary mutations in the genome.

Similar to the previously developed reporter models, we started by assessing the *in vitro* luminescence signal and fitness. The optical density and CFU of *gluc*GBS-CC17 cultures

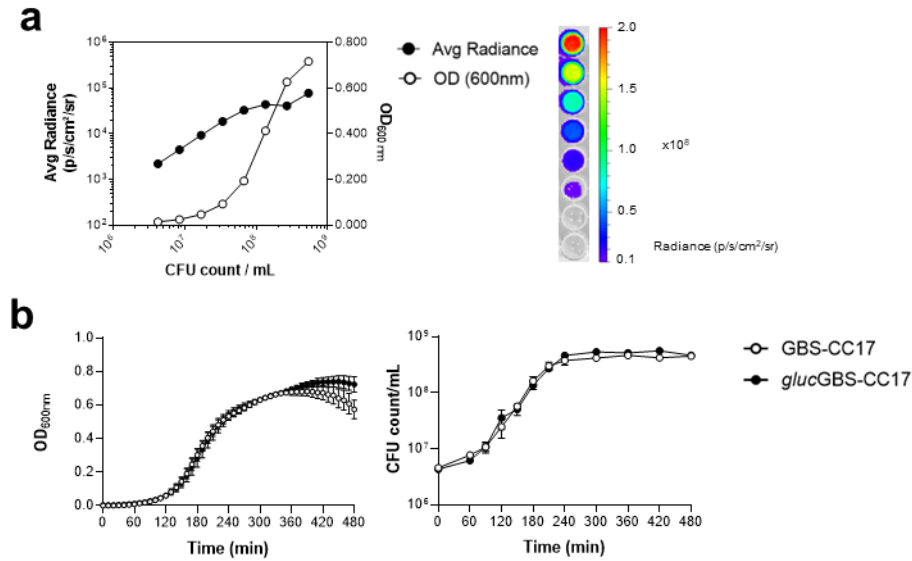


Figure 5. *In vitro* bioluminescence of the *glucGBS-CC17* with a chromosomally integrated luciferase.

The red-shifted firefly luciferase gene was integrated into the genome of the WT strain to give the *glucGBS-CC17* strain. **(a)** Correlation of bioluminescent signal, optical density (OD) and CFU count. The *glucGBS-CC17* strain was grown until the mid-log phase and serially diluted in a 96-well plate. Left: Bioluminescence (Radiance, 0.5 mg/mL of Luciferin) and OD quantification on a plate reader (Synergy 2), and corresponding CFU counts. Field symbols represent radiance, whereas open symbols represent OD. Right: Bioluminescence signal imaged using IVIS Lumina LT. The detection of bioluminescent signals by the system resulted in the generation of signal maps overlaid on a grey-scale photograph of the 96-well plate. Bioluminescent signal plotted as radiance (photons/seconds/cm²/sr). Results represent data from 3 independent experiments. **(b)** Growth curves of WT and *glucGBS-CC17* strains. Optical density (left) measured every 10 minutes for 7 h on Synergy 2. GBS numbers counted every 30-60 minutes (right). Mean values $\pm$ SEM pooled from 3 independent experiments. Comparisons by two-way ANOVA.

reliably correlate with the radiance signal (Figure 5a) and no differences in bacterial growth were found between the *glucGBS-CC17* and the WT strains (Figure 5b). Next, we tested the infectivity and virulence *in vivo* using a haematological model of infection. No significant differences in organ colonisation 18 hpi was observed between the *glucGBS-CC17* and the WT strain (Figure 6a), confirming that the integrated *ffluc* does not impact bacterial load in all tested organs. To test the *in vivo* bioluminescence, we infected mice using low (5×10^4 CFU) and high (1×10^5 CFU) inocula of *glucGBS-CC17*. The radiance values were significantly ($P = 0.0292$ ventral position, $P = 0.0103$ dorsal position) different and proportional to the infection doses (Figure 6b-d). As expected, bacterial colonisation in the brain, lungs, and liver was significantly ($P < 0.0001$) higher in pups infected with the higher dose (Figure 6d). These results indicate that this bioluminescent GBS strain is suitable for studying systemic infections. However, the *glucGBS-CC17* strain contains a single copy of

the luciferase gene, resulting in a lower signal when compared to the *ffluc*GBS-CC17 strain containing the pLZ12Km2-P23R:TA:*ffluc* replicative vector (Figure 4e and 6c).

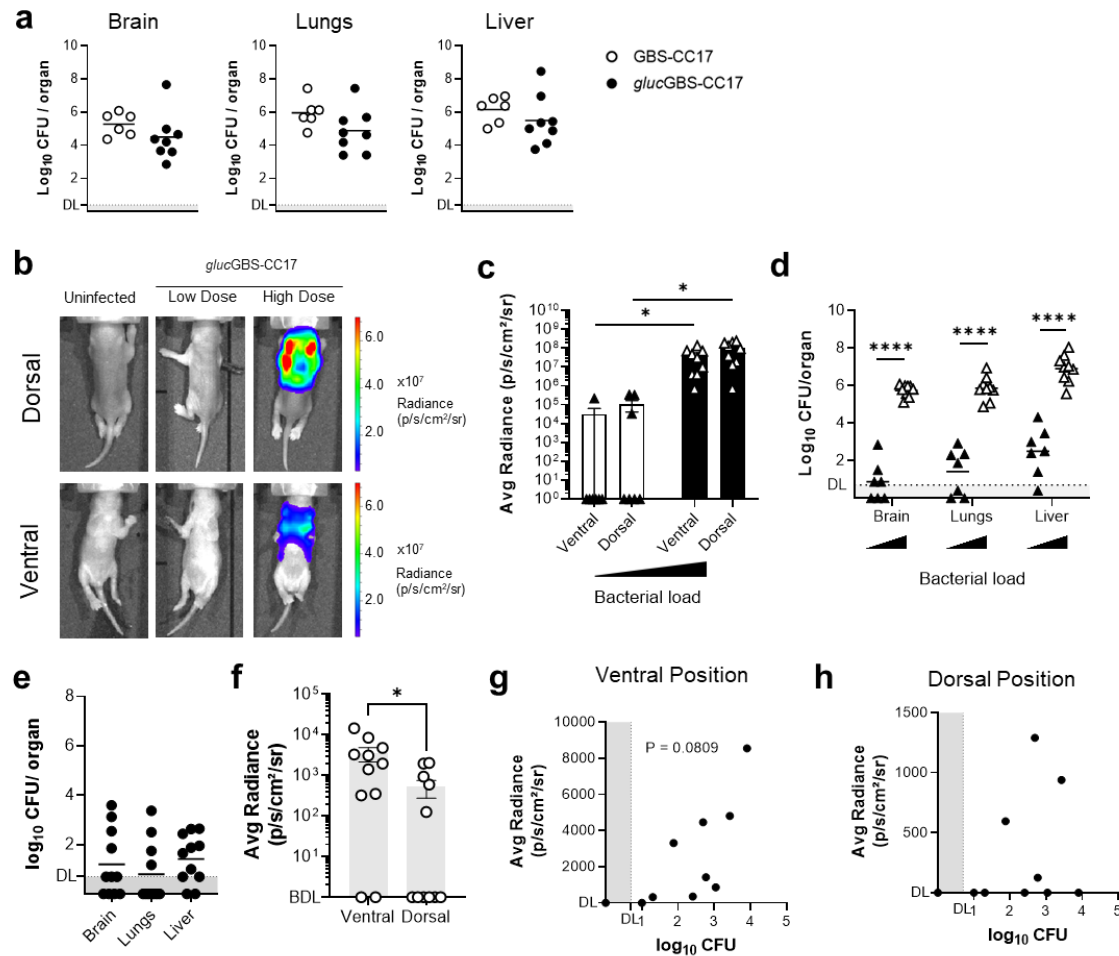


Figure 6. Infectivity and *in vivo* bioluminescence of *gluc*GBS-CC17. (a) Neonatal mice were inoculated intraperitoneally with 5×10^4 CFU of *gluc*GBS-CC17. Bacterial load in the indicated organs at 18 hpi. Data as mean, each symbol represents one pup ($n = 6$ WT; $n = 8$ *gluc*GBS-CC17). (b-d) Neonatal mice were inoculated intraperitoneally with 5×10^4 (low dose), 1×10^5 (high dose) CFU of *gluc*GBS-CC17 or left uninfected. (b) Representative images of pups through the superimposition of the bioluminescence signal and a grey-scale photograph in the indicated positions. (c) The regions of interest (ROIs) were used to quantify the bioluminescence signal, expressed as average radiance (photons/s/cm²/steradian). Bars represent the mean $\pm$ SEM. Each symbol represents one animal ($n = 6$ Uninfected; $n = 7$ Low dose $n = 9$ High dose). (d) Bacterial load in the indicated organs and infection doses. Data as mean, each symbol represents one animal ($n = 7$ Low dose; $n = 9$ High dose). Statistical analysis using unpaired Student's t-test. * $P < 0.05$, **** $P < 0.0001$. DL, detection limit. (e-h) Pregnant mice were intra-vaginally inoculated with 5×10^4 CFU of *gluc*GBS-CC17 during gestational. Three-day-old pups were analysed. (e) Bacterial load in the indicated organs. Data as mean, each symbol represents one animal ($n = 11$). DL, detection limit. (f-h) The ROIs were used to quantify the bioluminescence signal from the whole mouse, expressed as average radiance (photons/s/cm²/steradian). (f) Whole body average radiance in ventral and dorsal position. Mean $\pm$ SEM, each symbol represents one individual ($n = 11$). BDL, below detection limit. (g-h) Correlation between colonisation and the average radiance in the infected pups in the (g) ventral and (h) dorsal positions. Each symbol represents one individual ($n = 10$). BDL, below detection limit. * $P < 0.05$

The stable chromosomal integration of *ffluc* allows testing the *gluc*GBS-CC17 strain in our intra-vaginal (i.vag.) infection model of mother-to-pup transmission during delivery. At P3, the brain and liver of almost all pups were colonised, while only 4 out of 11 pups showed colonisation in the lungs (Figure 6e). Despite the relatively low bacterial burden, significant luminescence signals were detectable in the pups (Figure 6f). To further evaluate whether the radiance values could be correlated with bacterial brain colonisation a square region (ROIs) was drawn in the anatomical area of the brain and the bioluminescent signal obtained was plotted against the respective bacterial levels. No positive correlation was found regardless of ventral or dorsal mouse position (Supplementary Figure 3a and b). However, close to statistical significance ($P = 0.0809$) was obtained when plotting the whole-body radiance obtained in the ventral position with the sum of bacterial colonisation in the lungs, liver, and brain (Figure 6g), sustaining the validity of this strain to study moderate and severe GBS disease. Finally, aiming to improve sensitivity, we also tested a synthetic analogue of D-luciferin, cyclic alkylamino luciferin (CycLuc1), reported to produce a higher signal than D-luciferin^{32,33}. Pups were i.p. infected with *gluc*GBS-CC17, and at 18 hpi different concentrations of CycLuc1 and time points were tested (Supplementary figure 4). However, no differences in the bioluminescent signal were observed compared to standard D-luciferin, regardless of concentration or time post-injection (Supplementary figure 4a and b). Altogether, our results show a bioluminescent GBS strain constitutively expressing the luciferase gene stably integrated in the chromosome suitable for studying GBS invasive disease.

Discussion

Group B *Streptococcus* neonatal infections remain a major clinical problem. One limitation in studying GBS neonatal infection is that colonisation assessment entails animal euthanasia and, therefore, no longitudinal studies can be performed to study the kinetics of the infection, the disease progression, and the long-term sequelae. Here, we developed three bioluminescent GBS strains: one with the luciferase gene contained in the *lux* operon on a replicative vector, another with the *ffluc* gene also encoded on a replicative vector, and a third with the *ffluc* gene inserted into the GBS genome. Each bioluminescent strains are advantageous for specific applications: *in vitro* (*lux*GBS-CC17), *in vivo* for short term infections (*ffluc*GBS-CC17), or *in vivo* for complex infection models (*gluc*GBS-CC17). The choice between strains is based on the sensitivity (single or multi-copy) and the stability (vector-based or chromosomal integration) of the luciferase gene.

Our results suggest that the *lux*GBS-CC17 containing the *luxABCDE* operon on a replicative vector is appropriate for *in vitro* studies and not for invasive GBS infection models. *In vitro* luminescence is proportional to bacterial burden, consistent with results in other bioluminescent bacterial species¹³. The replicative vector is stable even in the absence of the antibiotic selective pressure, and no difference in growth with the WT strain is observed under standard conditions in TH broth. The main advantage is that this system does not require an exogenous substrate^{13,28}. However, no luminescence signal was detected in infected animals, even when using a high infection dose. This unexpected result might be due to the bacteria spreading throughout the body, resulting in a dispersed, undetectable signal, or being located deeper within organs. The short wavelength (490 nm) of the *lux* operon limits tissue penetration^{34,35}, especially due to absorption by hemoglobin^{34,36,37}. Miller and colleagues³¹ showed that tissue penetration for a *Staphylococcus aureus* strain transformed with the same *lux* operon is limited up to 2 cm. Although 3-day-old C57BL/6 mice are thinner than 2 cm, their organ density is high due to their small size³⁸, which could contribute to the limited detection. While it is unlikely that the P32 promoter is inactive during infection, and thus resulting in no bioluminescence signal, the optimisation of promoters should be explored in the future.

The second *ffluc*GBS-CC17 strain used contains the red-shifted firefly luciferase (*ffluc*) on a replicative vector, a *luc* reporter derivative validated for multiple streptococcal species¹⁵ overcoming the main limit of the *luxABCDE* system. The *ffluc*GBS-CC17 strain is therefore adapted to follow invasive infection in specific models. The *ffluc* has a longer wavelength (610 nm) than *lux* (490 nm)^{18,34}, and improved bioluminescent signal and tissue penetration to detect bacteria in deeper tissues¹⁸. The replicative vector was previously used in GBS for *in vitro* bioluminescence analysis¹⁵. Here, we confirmed the *in vitro* stability of the replicative vector and validated the *in vivo* stability in GBS, as previously shown in *Streptococcus pyogenes*²³. We also show that the pLZ12Km2-P23R:TA:*ffluc* vector did not impact on *in vitro* growth under standard conditions in TH broth and does not alter GBS virulence. The *ffluc*GBS-CC17 strain demonstrated robust bioluminescence, allowing visualisation and quantification of bacterial colonisation in the brain, lungs, and liver. The two infectious doses tested are the minimum for detecting luminescent signals associated with low and high bacterial loads, respectively. The lower dose of 5×10^4 CFU is the minimum needed to visualize colonisation through bioluminescence, while doubling the dose to 10^5 CFU produced higher, more uniform colonisation across all animals. Even when using a low inoculum to ensure increased pups' survival, the BLI signal was proportional to the CFU colonisation in the organs, in both ventral and dorsal mice positions. Both radiance and CFU values were statistically significant between the lower and higher infection dose, confirming

that the *ffluc*GBS-CC17 strain provides a reliable and sensitive method for tracking bacterial infection in live animals, even under non-selective antibiotic conditions.

The main limit of the *ffluc*GBS-CC17 strain emerged when tested in the vertical transmission model. Despite initial success in establishing infection, we observed plasmid loss over time, as evidenced by a lack of bioluminescent signal in later stages of infection. In this model, pregnant females are colonised in the vaginal mucosa at the gestational days 16 and 17, and allowed to deliver spontaneously¹². During birth, the pups cross the vaginal canal and may inhale or ingest contaminated fluids, becoming infected¹². As the pups are only studied after birth, the *ffluc*-containing plasmid should be stable for at least 3 to 7 days to detect luminescence during postnatal life. However, the stabilisation of the vector by the encoded toxin-antitoxin system is insufficient *in vivo* without an additional antibiotic selective pressure. Adding antibiotics to drinking water could stabilise the vector but would alter the microbiota. This plasmid instability under *in vivo* conditions suggests that while the *ffluc*GBS-CC17 strain is suitable for short-term studies and high-dose infections, it may not be ideal for long-term studies or infections acquired through natural routes.

The integration of the *ffluc* gene into the GBS genome resolves the stability issue. The *gluc*GBS-CC17 strain has a single copy of *ffluc* constitutively transcribed and integrated in a neutral intergenic region at the 3' ends of two divergent transcribed genes. The genome of the *gluc*GBS-CC17 strain is identical to the genome of the WT parental strain, except for the integrated *ffluc* which does not affect bacterial growth and virulence. The stable integration ensures the maintenance of the reporter gene over extended periods in complex infection model of infection. When tested in the haematogenous model, *gluc*GBS-CC17 allowed to visualise and quantify bacterial colonisation of key organs such as the brain, lungs, and liver. The bioluminescent signal was proportional to CFU counts, providing a reliable measure of bacterial load. Importantly, the use of the *gluc*GBS-CC17 strain facilitated the detection of bioluminescence in the pups' whole-body, even at lower infection doses.

The main limitation of the *gluc*GBS-CC17 strain is its weaker luminescence signals, linked to the presence of a single copy of the *ffluc* gene. This is especially limiting for brain colonisation in the vertical mouse model of infection. The low level of colonisation and the properties of the skull and blood-brain barrier (BBB), which impact and limit the access of D-luciferin to the parenchyma³², severely impacts the detection of the *gluc*GBS-CC17 strain. The luciferase substrate cyclic alkylamino luciferin (CycLuc1) has higher photon emission than D-luciferin at lower concentrations^{32,33} and increased BBB diffusion as it has increased lipophilicity^{32,39}. Previous findings showed that CycLuc1 allows the detection of

bioluminescence signals in cardiovascular brain regions, including the circumventricular subfornical organ (SFO) and paraventricular nucleus of the hypothalamus (PVN)³². Nevertheless, in our study CycLuc1 did not show a stronger luminescent signal regardless of the administered concentration or acquisition waiting time after injection. A possible explanation for this may be the lower bacterial load and the fact that it is widespread in the pup's body, reducing the signal per area. While the introduction of CycLuc1 as a luciferase substrate initially showed promise for enhanced CNS imaging, our results highlight the complexities and limitations in achieving consistent bioluminescent signals in neonatal mice, necessitating further exploration of alternative strategies. Optimisation of the *ffluc* gene and promoter, or the use of alternative luminescence genes, could improve bioluminescent sensitivity to obtain a universal bioluminescent GBS strain.

In conclusion, we generated three GBS bioluminescent strains for pathogenic analysis, among which two containing the red-shifted firefly luciferase (*ffluc*) suitable to follow short-term and long-term invasive infections. This strain enables detailed tracking of bacterial colonisation and disease progression *in vivo*, providing better understanding of GBS pathophysiology. Moreover, it will pave the way for discovering novel approaches against neonatal GBS infections, while also allowing animal reduction and experiment refinement, implementing the ethical animal experiments 3R's.

References

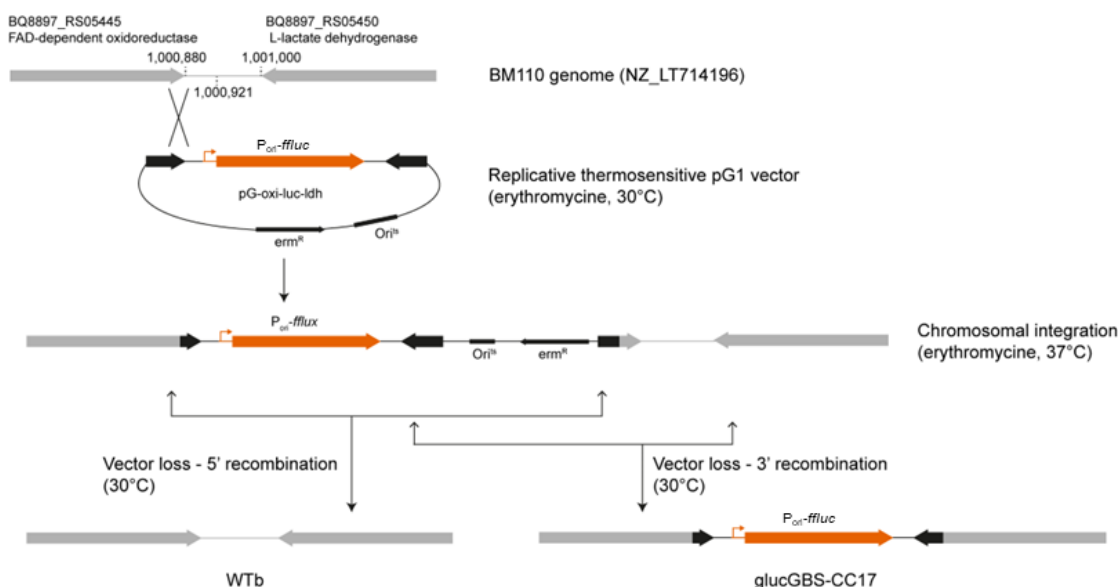
1. Thigpen Michael C. *et al.* Bacterial Meningitis in the United States, 1998–2007. *N. Engl. J. Med.* 364, 2016–2025 (2011).
2. Tavares, T., Pinho, L. & Bonifácio Andrade, E. Group B Streptococcal Neonatal Meningitis. *Clin. Microbiol. Rev.* 35, e00079-21 (2022).
3. Wilson, C. B., Nizet, V., Maldonado, Y., Remington, J. S. & Klein, J. O. *Remington and Klein's Infectious Diseases of the Fetus and Newborn Infant*. (Elsevier Health Sciences, 2015).
4. Nanduri, S. A. *et al.* Epidemiology of Invasive Early-Onset and Late-Onset Group B Streptococcal Disease in the United States, 2006 to 2015: Multistate Laboratory and Population-Based Surveillance. *JAMA Pediatr.* 173, 224–233 (2019).
5. Jamrozy, D. *et al.* Increasing incidence of group B streptococcus neonatal infections in the Netherlands is associated with clonal expansion of CC17 and CC23. *Sci. Rep.* 10, 9539 (2020).

6. Plainvert, C. *et al.* Multidrug-Resistant Hypervirulent Group B Streptococcus in Neonatal Invasive Infections, France, 2007-2019. *Emerg. Infect. Dis.* 26, 2721–2724 (2020).
7. Verani, J. R., McGee, L., Schrag, S. J., & Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention (CDC). Prevention of perinatal group B streptococcal disease--revised guidelines from CDC, 2010. *MMWR Recomm. Rep. Morb. Mortal. Wkly. Rep. Recomm. Rep.* 59, 1–36 (2010).
8. Tazi, A. *et al.* The surface protein HvgA mediates group B streptococcus hypervirulence and meningeal tropism in neonates. *J. Exp. Med.* 207, 2313–2322 (2010).
9. Ferrieri, P., Burke, B. & Nelson, J. Production of bacteremia and meningitis in infant rats with group B streptococcal serotypes. *Infect. Immun.* 27, 1023–1032 (1980).
10. Mancuso, G. *et al.* Beneficial effects of interleukin-6 in neonatal mouse models of group B streptococcal disease. *Infect. Immun.* 62, 4997–5002 (1994).
11. Barichello, T. *et al.* Evaluation of the brain-derived neurotrophic factor, nerve growth factor and memory in adult rats survivors of the neonatal meningitis by *Streptococcus agalactiae*. *Brain Res. Bull.* 92, 56–59 (2013).
12. Andrade, E. B. *et al.* A mouse model reproducing the pathophysiology of neonatal group B streptococcal infection. *Nat. Commun.* 9, 3138 (2018).
13. Avci, P. *et al.* In-vivo monitoring of infectious diseases in living animals using bioluminescence imaging. *Virulence* 9, 28–63 (2018).
14. Russel, W. & Burch, R. The principles of humane experimental technique [Internet]. *Lond. Ufaw* (1992).
15. Loh, J. M. S. & Proft, T. Stabilized plasmid-based system for bioluminescent labeling of multiple streptococcal species. *Biotechnol. Lett.* 38, 139–143 (2016).
16. Loh, J. M. S. & Proft, T. Comparison of firefly luciferase and NanoLuc luciferase for biophotonic labeling of group A Streptococcus. *Biotechnol. Lett.* 36, 829–834 (2014).
17. Sheel, M., Pandey, M., Good, M. F. & Batzloff, M. R. Correlation between Bioluminescence and Bacterial Burden in Passively Protected Mice Challenged with a Recombinant Bioluminescent M49 Group A Streptococcus Strain. *Clin. Vaccine Immunol.* 17, 127–133 (2010).
18. Loh, J. M. S. & Proft, T. Toxin–antitoxin-stabilized reporter plasmids for biophotonic imaging of Group A streptococcus. *Appl. Microbiol. Biotechnol.* 97, 9737–9745 (2013).
19. Marques, S. M. & Esteves da Silva, J. C. G. Firefly bioluminescence: a mechanistic approach of luciferase catalyzed reactions. *IUBMB Life* 61, 6–17 (2009).

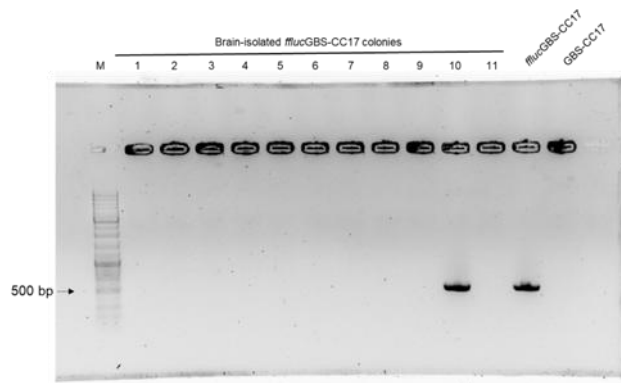
20. Loimaranta, V., Tenovuo, J., Koivisto, L. & Karp, M. Generation of Bioluminescent *Streptococcus mutans* and Its Usage in Rapid Analysis of the Efficacy of Antimicrobial Compounds. *Antimicrob. Agents Chemother.* 42, 1906–1910 (1998).
21. Short, K. R. *et al.* Using Bioluminescent Imaging to Investigate Synergism Between *Streptococcus pneumoniae* and Influenza A Virus in Infant Mice. *JoVE J. Vis. Exp.* e2357 (2011) doi:10.3791/2357.
22. Lamb, L. E. *et al.* Modelling invasive group A streptococcal disease using bioluminescence. *BMC Microbiol.* 18, 60 (2018).
23. Alam, F. M., Bateman, C., Turner, C. E., Wiles, S. & Sriskandan, S. Non-Invasive Monitoring of *Streptococcus pyogenes* Vaccine Efficacy Using Biophotonic Imaging. *PLOS ONE* 8, e82123 (2013).
24. La Rosa, S. L., Diep, D. B., Nes, I. F. & Brede, D. A. Construction and application of a luxABCDE reporter system for real-time monitoring of *Enterococcus faecalis* gene expression and growth. *Appl. Environ. Microbiol.* 78, 7003–7011 (2012).
25. Fogh, J., Fogh, J. M. & Orfeo, T. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J. Natl. Cancer Inst.* 59, 221–226 (1977).
26. Bellais, S. *et al.* Capsular switching in group B *Streptococcus* CC17 hypervirulent clone: a future challenge for polysaccharide vaccine development. *J. Infect. Dis.* 206, 1745–1752 (2012).
27. Landwehr-Kenzel, S. & Henneke, P. Interaction of *Streptococcus agalactiae* and Cellular Innate Immunity in Colonization and Disease. *Front. Immunol.* 5, 519 (2014).
28. Baldwin, T. O. Firefly luciferase: the structure is known, but the mystery remains. *Structure* 4, 223–228 (1996).
29. Gutekunst, H., Eikmanns, B. J. & Reinscheid, D. J. The Novel Fibrinogen-Binding Protein FbsB Promotes *Streptococcus agalactiae* Invasion into Epithelial Cells. *Infect. Immun.* 72, 3495–3504 (2004).
30. Que, Y. A., Haeffliger, J. A., Francioli, P. & Moreillon, P. Expression of *Staphylococcus aureus* clumping factor A in *Lactococcus lactis* subsp. *cremoris* using a new shuttle vector. *Infect. Immun.* 68, 3516–3522 (2000).
31. Miller, R. J. *et al.* Development of a *Staphylococcus aureus* reporter strain with click beetle red luciferase for enhanced in vivo imaging of experimental bacteremia and mixed infections. *Sci. Rep.* 9, 16663 (2019).
32. Simonyan, H., Hurr, C. & Young, C. N. A synthetic luciferin improves in vivo bioluminescence imaging of gene expression in cardiovascular brain regions. *Physiol. Genomics* 48, 762–770 (2016).
33. Evans, M. S. *et al.* A synthetic luciferin improves bioluminescence imaging in live mice. *Nat. Methods* 11, 393–395 (2014).

34. Archer, N. K., Francis, K. P. & Miller, L. S. Optical imaging. in *Imaging Infections: From Bench to Bedside* 43–76 (Springer International Publishing, 2017). doi:10.1007/978-3-319-54592-9_3.
35. Close, D. M., Xu, T., Sayler, G. S. & Ripp, S. In vivo bioluminescent imaging (BLI): noninvasive visualization and interrogation of biological processes in living animals. *Sensors* 11, 180–206 (2011).
36. Zhao, H. *et al.* Emission spectra of bioluminescent reporters and interaction with mammalian tissue determine the sensitivity of detection in vivo. *J. Biomed. Opt.* 10, 41210 (2005).
37. Hutchens, M. & Luker, G. D. Applications of bioluminescence imaging to the study of infectious diseases. *Cell. Microbiol.* 9, 2315–2322 (2007).
38. Petiet, A. E. *et al.* High-resolution magnetic resonance histology of the embryonic and neonatal mouse: A 4D atlas and morphologic database. *Proc. Natl. Acad. Sci.* 105, 12331–12336 (2008).
39. Mofford, D. M. & Miller, S. C. Luciferins behave like drugs. *ACS Chem. Neurosci.* 6, 1273–1275 (2015).

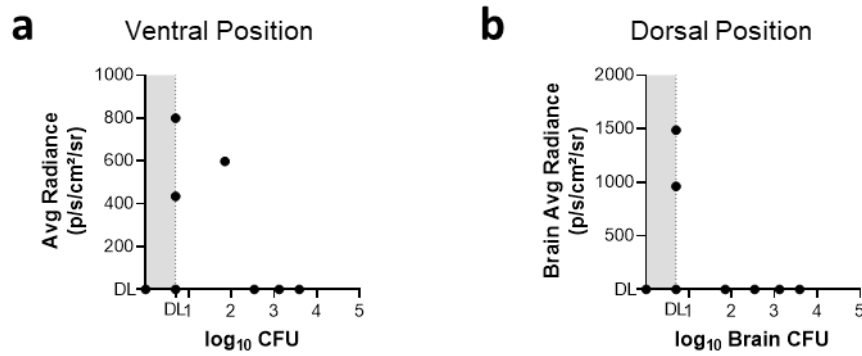
Supplementary Data



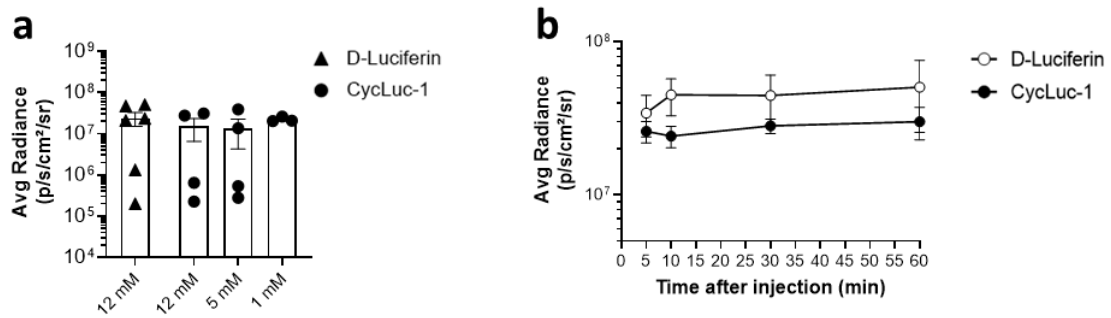
Supplementary Figure 1. Insertion of the *ffluc* gene into the genome of *BM110*. The pG-oxi-luc-ldh integrative vector was constructed by the insertion of the *ffluc* gene (orange) flanked by 500 bp of chromosome sequences (large black arrows) into the pG1 vector. The pG-oxi-luc-ldh vector was introduced in *GBS BM110* by electroporation with erythromycin selection at 30 °C, the permissive temperature for vector replication. Chromosomal integration by single crossing-over at the targeted locus (black cross) was selected by growing isolated transformants at 37 °C in the presence of erythromycin. De-recombination and vector loss were selected at 30 °C without antibiotic and can lead to the restoration of a WT sequence (left: single crossing-over between homologous 5' sequences) or to the precise integration of the *ffluc* gene into the chromosome (of the vector (right: single crossing-over between homologous 3' sequences). For simplicity, only homologous recombination at the 5' end of *P_{ori-ffluc}* is shown for the chromosomal integration step, although single crossing-over can also occur at the 3' end.



Supplementary Figure 2. Colony PCR of brain-isolated *ffluc*GBS-CC17. Pregnant mice were intra-vaginally inoculated with 8×10^4 *ffluc*GBS-CC17. Three-day-old pups were analysed. Agarose gel showing colony PCR for screening *ffluc*GBS-CC17 collected from pups' brains, using plasmid-targeted primers (495 bp amplicon). M, molecular weight marker (10 kb GeneRuler DNA ladder mix); 1-11, bacteria isolates; *ffluc*GBS-CC17 and WT GBS-CC17 as positive and negative controls, respectively.



Supplementary Figure 3. Evaluation of the brain bioluminescent signal in the vertical model of infection with the *glucGBS-CC17*. Pregnant C57Bl/6 mice were intra-vaginally inoculated with 5×10^4 CFU of *glucGBS-CC17* at gestational days 16 and 17. Three-day-old pups were analysed. **(a-b)** The ROIs were used to quantify the bioluminescence signal from the mouse head, expressed as average radiance (photons/s/cm²/steradian). Correlation between the average radiance in the infected pups' head and the brain colonisation, in the **(a)** ventral and **(b)** dorsal positions. Each symbol represents one individual (n = 8).



Supplementary Figure 4. Evaluation of the synthetic luciferase CycLuc1. Two-day-old C57BL/6 pups were infected intraperitoneally with 8×10^4 CFU of the bioluminescent strain (*gluc*GBS-CC17) or left uninfected. Analyses were performed 18 hours post infection **(a)** Average radiance (photons/s/cm²/steradian) of the whole body of infected pups. Data shown as mean $\pm$ SEM are represented in bars pooled from 2 independent experiments. Each symbol represents one animal ($n = 3$ for 1mM CycLuc; $n = 4$ for the remaining conditions). **(b)** Kinetics of the average radiance (photons/s/cm²/steradian) of the whole body of pups infected with the *gluc*GBS, injected with D-Luciferin or CycLuc-1. Data shown as mean $\pm$ SEM pooled from 3 independent experiments ($n = 6$ D-Luciferin; $n = 8$ CycLuc-1).

MANUSCRIPT 2

MENINGEAL GAMMA DELTA T CELLS DISRUPT THE BLOOD-BRAIN BARRIER DURING NEONATAL MENINGITIS TRIGGERING LONG-TERM NEUROLOGICAL SEQUELAE

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Abstract

Neonatal bacterial meningitis is a life-threatening condition and a leading cause of neurodevelopmental impairment among survivors. Despite its prevalence, the role of meningeal immunity to disease pathology during early life remains largely unexplored. Using a clinically relevant mouse model of neonatal meningitis and single-cell RNA sequencing, we identify an accumulation of IL-17 producing $\gamma\delta$ T cells ($\gamma\delta$ 17 T cells) in the meninges during the acute phase of Group B *Streptococcus* (GBS) meningitis, which persisted throughout the lifespan. The absence of $\gamma\delta$ T cells or IL-17 significantly reduces bacterial colonisation in the brain parenchyma. Importantly, IL-17 neutralisation into the cerebrospinal fluid leads to similar results. Reduced blood-brain barrier permeability in the absence of $\gamma\delta$ T cells results in decreased bacterial invasion and diminished microglia activation. During early adulthood, WT survivors exhibit increased attention deficit and hyperactivity disorder (ADHD)-like behaviours, a phenotype absent in *Tcrd*^{-/-} mice. Together, our findings establish a pathogenic role for meningeal $\gamma\delta$ 17 T cells in early life, uncovering a previously unknown mechanism driving long-term sequelae in GBS neonatal meningitis.

Introduction

Group B *Streptococcus* (GBS) remains the leading agent causing bacterial meningitis in newborns¹. GBS, a commensal microorganism of the genitourinary and gastrointestinal tracts of adult humans, colonises the vagina or rectum of 15-40% of pregnant women¹. Maternal vaginal colonisation is the primary risk factor for neonatal infection, which can progress to meningitis¹. Globally, this disease affects an infant every 3 minutes and kills one baby every 6-9 minutes^{2,3}. GBS neonatal meningitis has an estimated mortality rate of 10-15% and results in life-long neurodevelopment impairment (NDI) in up to 50% of survivors¹. Particular concern has arisen with some countries reporting a rising incidence of neonatal meningitis over the last decade⁴⁻⁷. The antibiotic prophylaxis administered to pregnant women vaginally colonised with GBS to prevent vertical transmission to the foetus is inefficient for meningitis prevention⁸, and no effective neuroprotective therapies are available to mitigate the long-term neurological sequelae.

The pathophysiology of GBS-induced meningitis and the mechanisms underlying its long-term NDI remain poorly understood. In the past decades, efforts have been made to understand GBS neonatal meningitis. However, by focusing on one particularity of the GBS pathophysiology, meningitis studies in animal models, such as hematogenous⁹⁻¹¹ and direct brain infection models¹²⁻¹⁵, have historically faced challenges generating misleading data as

they inadequately replicate clinical phenotypes. Consequently, it has delayed the development of novel therapeutic interventions aiming at improving brain injury outcomes. To address these limitations, our group developed a mouse model of mother-to-progeny GBS transmission, mirroring human infection pathogenesis¹⁶. Interestingly, this model revealed no leukocyte infiltration into the brain parenchyma or increased levels of inflammatory cytokines in the developing brain¹⁶. This contradicts previous assumptions that immune cells' recruitment into the parenchyma directly causes brain damage and NDI in GBS meningitis^{17–20}.

In the last 20 years, the meninges have gained protagonism as an immunological site rather than merely a physical protective barrier. The meninges harbour a wide repertoire of immune cells patrolling the existence of pathogens and tissue damage²¹. Additionally, meningeal immunity has been increasingly emphasised as crucial for maintaining steady-state brain function, particularly through the T cell compartment^{22–30}. Cognitive functions, such as spatial learning and memory, hippocampal neurogenesis and long-term potentiation depend on meningeal CD4⁺ and $\gamma\delta$ T cells^{22–27}. These cells are also crucial in regulating emotional behaviour, compulsive behaviour, nest building, anxiety and sociability^{27–30}. Additionally, emerging evidence suggests that the meninges may play essential roles in brain development³¹. However, studies of neonatal meningitis have largely overlooked the meningeal spaces.

In this study, we uncovered for the first time the role of meningeal immunity during neonatal meningitis and its short- and long-term sequelae. We found that interleukin-17A (IL-17)-producing $\gamma\delta$ T cells ($\gamma\delta 17$ T cells) accumulate in the meninges of neonatal mice during GBS meningitis, with their presence persisting into adulthood. Importantly, our data show that meningeal $\gamma\delta 17$ T cells contribute to blood-brain barrier (BBB) permeability during infection, facilitating bacterial invasion of the central nervous system (CNS) and microglia activation. This cascade ultimately results in attention deficit hyperactivity disorder (ADHD)-like behaviours in survivors. Together, we identify a novel mechanism underlying long-term sequelae of neonatal meningitis, that could be harnessed therapeutically to alleviate brain damage in surviving infants.

Methods

Bacterial strains and growth conditions

GBS BM110 strain (here referred to as GBS) is a bacterium belonging to the hyper-virulent clonal complex 17 (CC17), a well-known isolate from humans with invasive disease³². The

isogenic non-haemolytic (β H/C-deficient) Δ cylE mutant (here referred to as CC17 Δ cylE) was constructed by in-frame cylE gene depletion as previously described³³. The GBS NEM316 strain (here referred to as non-CC17) is also a capsular serotype III strain, belonging to the CC23. Overnight (ON) cultures at 37 °C in Todd-Hewitt (TH) broth (Difco Laboratories) were 1:100 diluted in the same medium and bacteria were grown until mid-log phase ($OD_{600\text{ nm}} \sim 0.900$), pellet and washed twice with phosphate-buffered saline (PBS). The $OD_{600\text{ nm}}$ was adjusted to $\approx 0.600 \pm 0.005$, corresponding approximately to 2.0×10^8 CFU/mL. Inocula were prepared by further dilution from this bacterial suspension. Inocula and organs were plated and ON grown in TH agar (Difco Laboratories) for bacterial count.

Animals and ethics statement

C57BL/6 and Tcrd^{-/-} mice (originally obtained from The Jackson Laboratory) were bred and housed at the Instituto de Investigação e Inovação em Saúde (i3S) animal facility. IL-17^{-/-} mice were housed and bred at the Gulbenkian Institute for Molecular Medicine (GiMM Faculdade de Medicina da Universidade de Lisboa). The genetically modified mouse strains used are in the C57BL/6J genetic background and have been backcrossed at least ten times. All mice were maintained in specific pathogen-free conditions. All experiments were conducted following the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and Directive 2010/63/EU and Portuguese rules (DL 113/2013). All experimental protocols including animals were approved by the competent national authority Direcção Geral de Alimentação e Veterinária (DGAV), and by the respective Institute's Animal Ethical Committee (ORBEA). People directly working with animals are all certified by DGAV. All animals were kept in a controlled environment (20°C, 45–55% relative humidity) under a 12 h light/dark cycle, with free access to water and food. Both male and female mice were used in experiments unless otherwise stated. All efforts were made to minimise animal suffering and to reduce the number of animals used. Since our experiments were designed to colonise progenitors during pregnancy, we did not randomise their litters. No blinding was carried out.

Mouse model of neonatal GBS meningitis

We adapted our intra-vaginal (i.vag.) mouse model of neonatal infection to the C57BL/6 mice¹⁶. Briefly, pregnant mice were i.vag. inoculated, using a micropipette, with 40 μ L of GBS suspension containing 3×10^4 cells, or with PBS, at the gestation days (G) 16 and 17.

The presence of a vaginal plug was considered G1. Females were allowed to deliver spontaneously, and infected pups were kept with their mothers throughout the experimental time. C57BL/6 WT, *Tcrd*^{-/-} and IL-17A^{-/-} were used. Both female and male pups were used unless otherwise stated.

Survival, clinical scoring and organ colonisation

Pups born from i.vag. infected or control dams were monitored every 12 h, during the first indicated days of life for survival studies. A neonatal longitudinal clinical scoring, adapted from Fehlhäber *et al.*³⁴, was used to evaluate the pup's disease progression and death prediction during the first 5 days of life.

For the bacterial dissemination and assessment studies, the indicated tissues were collected in PBS, homogenised, serially diluted and plated on TH agar for CFU count.

Flow cytometry

Animals were deeply anaesthetised by isoflurane inhalation, perfused with saline and tissues were aseptically removed. The meninges were collected in RPMI 1640 medium (HyClone GE Healthcare Life Sciences) with 2% FBS (heat-inactivated, Biowest) and 2.5 mg/ml collagenase D (Roche), and incubated at 37 °C for 30 min. The tissue is passed through a 1 mL syringe with a 25 G needle 5 times, washed twice with washing solution (RPMI 1640, 2% FBS, 2 mM EDTA), and passed through a 70 µm mesh filter. The meninges were centrifuged at 450 g, 5 min, at 4 °C, resuspended in RPMI 1640 with 2% FBS and counted. The brain was collected in RPMI 1640 medium, cut into small pieces (1–2 mm) and gently digested with 7.5 mg/mL collagenase D for 30 min. To aid dissociation, samples were pipetted up and down thoroughly every 10 min during the incubation period. Cells were then passed through a 100 µm cell strainer and washed. Stock isotonic Percoll (SIP, Cytiva) 90% was prepared with 10x HBSS without Ca²⁺ and Mg²⁺ (Gibco™), and further diluted to 70%, 37% and 30% in 1x HBSS. Cells suspensions were suspended in 70% SIP. Each dilution of Percoll was layered in a 15 mL conical tube, and Percoll gradients were centrifuged at 800 g, 20 min, at 20 °C. The enriched population of leucocytes/microglia were carefully collected at the 70–30% interphase washed and counted. The spleen and thymus were collected to RPMI 1640 with 2% FBS medium, gently dissociated in a 100 µm cell strainer to yield a single cell suspension, washed and counted.

A total of 5×10^5 (meninges) or 1×10^6 cells were plated in 96-well plates for staining. Suspensions were blocked with 5 $\mu\text{g/mL}$ rat anti-mouse CD16/32 (eBiosciences) and 0.5 mg/mL normal rat serum for 10 min on ice prior staining to reduce non-specific antibody binding. Cells were incubated with FVD eFluor 506 and then with CD45 FITC (Clone 30-F11), CD69 PE (H1.2F3), CD8 PerCP-Cy5.5 (53-6.7), CD3 Pe-Cy7 (17A2), TCR $\gamma\delta$ APC (GL3), TCR β eFluor 780 (H57-597, eBioscience), CD4 Pacific Blue (RM4-5), CD44 BV510 (IM7), Siglec-F PE (E50-2440, BD Biosciences), Ly6C PerCP-Cy5.5 (HK1.4), CD11b Pe-Cy7 (M1/70), Ly6G Pacific Blue (1A8), CD5 PE (53-7.3, BD Biosciences), TCR β PE (H57-597, Invitrogen), CD19 APC-Cy7 (6D5), IFN γ PE (XMG1.2), TCR $\gamma\delta$ Pe-Cy7 (GL3), IL-17 APC (TC11-18H10.1), CD3 APC-Cy7 (17A2), TCR $\gamma\delta$ APC (GL3), Ly6G AF647 (1A8), Ki67 Alexa Fluor 488 (16A8), TCRV γ 4 PE (UC3-10A6), CD45 PerCP-Cy5.5 (30-F11), TCRV γ 1 APC (2.11), TCR β eFluor 450 (H57-597, Invitrogen), CD62L PE (MEL-14), CD44 PerCP-Cy5.5 (IM7), PD-1 Alexa Fluor 647 (29F.1A12), CD69 BV421 (H1.2F3), CD4 BV421 (RM4-5), CD69 PE (H1.2F3), CD62L APC (MEL-14), TCRV γ 4 PerCP-Cy5.5 (UC3-10A6), TCRV γ 1 Pacific Blue (2.11), CD24 FITC (M1/69, Invitrogen), CD45RB Pacific Blue (C363-16A), CD69 Pe-Cy7 (H1.2F3), CD25 APC-Cy7 (PC61), Sirp α FITC (P84), CD200R PE (OX-110), MHC-II APC (M5/114.15.2), F4/80 APC-Cy7 (BM8) and/or CX3CR1 Pacific Blue (SA011F11), all from Biolegend unless otherwise mentioned, diluted in FACS buffer (2% FBS, 0.01% sodium azide, 2 mM EDTA). For intracellular staining, cells were stimulated in complete RPMI 1640 with PMA (Phorbol 12-Myristate 13-Acetate; 50 ng/ml, Sigma-Aldrich) and Ionomycin (1 $\mu\text{g/mL}$, Sigma-Aldrich) and Brefeldin A (BFA) (10 $\mu\text{g/mL}$, Sigma-Aldrich), for 4 h at 37 °C, 5% CO₂, before staining for extracellular markers. Cells were then permeabilised and stained using a Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Cells were acquired in FACS Canto II (BD Biosciences) or Aurora (Cytex) flow cytometers. Post-acquisition analysis was performed using FlowJo Software v10 (Tree Star). For flow cytometry cell sorting, cells were stained with FVD eFluor 780 (eBioscience) and CD45 FITC (Clone 30-F11, Biolegend), and a minimum of 20,000 live CD45⁺ cells were sorted (FACS Aria II) into microcentrifuge tubes containing 0.04% BSA in DPBS, that were previously coated with 1 mL FBS, for 24 h. Samples were always kept on ice.

Sample preparation for single-cell RNAseq (scRNAseq)

Male pups at P3 from infected and uninfected groups were deeply anaesthetised by isoflurane inhalation, perfused with ice-cold saline and the dural meninges were dissected and processed following adaptation from Scheyltjens and colleagues³⁵. Briefly, the meninges were collected in RPMI (and digested with 7.5 mg/mL of collagenase D for 35

min at 37 °C, with shaking at 300rpm. Every 10 min, the samples were pipetted up and down thoroughly at room temperature. The meninges were then passed through a 100 µm mesh filter and pooled into 15 mL falcon tubes, separated by group. Cells were washed, transferred to a 1.5 mL polystyrene round-bottom tube with cap, and rinsed thrice with RPMI 1640. The samples were finally washed, resuspended in DPBS and counted. After cell sorting, cells were centrifuged and resuspended in DPBS with 0.04% BSA at a concentration of 1,000 cells / µL for chip loading. The GEM (Gel Bead-In Emulsion) barcode generation was performed according to the 10x Genomics instructions.

scRNAseq Analysis

Raw sequenced files were processed in Cell Ranger (v7.1.0) using the GRCm38 mouse reference transcriptome (GENCODE vM23). This step aligns the sequences reads to the transcriptome and generates a count matrix table for each sample. Raw count data were then individually loaded into R (4.2.2) and analysed in Seurat v4.3.0. For each dataset, filtering was applied to exclude low-quality cells by removing those with low numbers of unique features and cells with a mitochondrial percentage > 10% were filtered to exclude dead cells. After the quality control step, we were left with 7142 control cells and 10418 Meningitis cells.

Samples were merged and normalised using the SCTransform normalisation function, a normalisation and variance stabilisation method which employs a regularised negative binomial regression. Then, a PCA was performed and the first 20 PCs were selected (based on inspection of PC elbow plot) as input for RunUMAP function for dimension reduction and visualization with the Uniform Manifold Approximation and Projection (UMAP) dimensional reduction technique. Finally, cells were clustered with the functions FindNeighbors and FindClusters, based on the default Seurat parameters with resolution parameter set to 0.3.

Cell-type marker genes for each cluster were identified using the function FindAllMarkers of Seurat. Clusters were annotated based on cell type-specific markers from recently published scRNA-seq datasets^{36,37}.

For gene set enrichment analysis, differentially expressed genes (DEGs) between gamma delta Control vs Meningitis were identified using FindMarkers function from MAST package. DEGs for gamma delta T cells ranked based on log2 Fold Change values. The gseGO function with ont="BP" from clusterProfiler³⁸ was used for the gene set enrichment analysis.

scRNAseq Analysis of IL-17R from adult mouse database

Previously published single-cell RNA sequencing data from the adult mouse brain³⁹ were download from <http://dropviz.org> and analysed using R. For our analysis, only the metadata and annotations referring to data derived from the indicated regions were used. The dataset was analysed using the original pipeline described by Jin et al., 2021, including the default method for the computation of the communication probability and the use of 10 cells as the threshold for the minimum number of cells needed expressing the ligand or receptor.

Bulk RNA sequencing

Male pups at P3 were deeply anaesthetised by isoflurane inhalation, perfused with saline, and euthanised by decapitation. The meninges were collected and frozen at -80 °C until RNA extraction. Two meningeal tissues were pooled prior to RNA isolation, and the pooled preparation constituted a single data point. Frozen tissues were retrieved on dry ice and RNA was extracted using the Pure link™ RNA Mini Kit (Ambion) with on-column DNase (Roche) treatment. Quantification and RNA integrity was assessed using an Agilent 2100 Bioanalyzer system. RNA integrity numbers (RIN) above 6.5 were used for library preparation and reverse transcription followed by sequencing using Illumina PE150 technology (Novogene).

Bulk RNA-seq data processing and analysis

RNA-seq reads were pre-processed in TrimGalore prior to alignment to remove low-quality and adapter sequences. Sequence alignment was carried in STAR v2.7.10a with the mouse GRCm39 reference using the Gencode vM33 gene models. Counts per gene were generated by STAR. A principal component analysis (PCA) was performed using the rlog-normalized count data to inform about the sample variability and structure across condition.

Differential expression analysis was accessed in DESeq2 (v.1.34). Log2 fold change results were shrunk using the apegglm package to remove noise (DE genes with low counts and/or high dispersion values) while preserving significant differences.

Gene set enrichment Pathway analysis was performed in ClusterProfiler (GSEA function) using a pre-ranked list of genes calculated using the signed log2 fold change times the -log10 of the p-value. Enrichment was calculated for the mouse hallmark gene sets that were retrieved from the Molecular Signatures Database (v2023.2.Mm).

Cytokine analysis

Meningeal and splenic supernatants, upon single cell suspension isolation and 24 h stimulation with 50 ng/mL phorbol myristate acetate (PMA) and 500 ng/mL ionomycin, were collected and stored at -80 °C until use. The cytokines IL-6, IL-10 (R&D Systems), IL-17, IFN- γ and TNF- α , through ELISA, following Invitrogen or R&D Systems kits' instructions, respectively.

Uninfected and Infected P3 splenic CD45^{high} cells were stimulated with GBS, at a multiplicity of infection (MOI) of 5 and 10, for 24 h. The cytokines IL-6, IL-10 (R&D Systems), IL-17 and TNF- α , through ELISA, following Invitrogen or R&D Systems kits' instructions, respectively.

To study $\gamma\delta$ T cell response to GBS, 2×10^4 of splenic irradiated antigen-presenting cells (APCs) per well were incubated for 6 h with heat-killed GBS BM110 MOI 100 or RPMI. Then, 5×10^3 $\gamma\delta$ T cell cells/well sorted from the lungs of P3 uninfected and infected pups were added and incubated for 72 h, with or without 10 ng/mL of recombinant mouse IL-1 β and IL-23 (Biolegend). The supernatants were collected and stored at -80 °C until use. IL-1 β , IL-23, IL-17 and IFN- γ were analysed through ELISA according to Invitrogen instructions.

For serum and CSF samples, blood from P3 mice was collected by pinching the submandibular vein and collecting the blood with heparin tubes. Blood samples were then centrifuged at 12,000g, for 15 min at 4 °C, and serum was collected. For CSF sampling, P3 mice were deeply anesthetised through hypothermia on ice. Disposable glass Pasteur pipettes were heated at the tip and stretched until a very thin capillary was obtained to be inserted in the cisterna magna for CSF collection. Serum and CSF samples were immediately stored at -80 °C until analysis. Cytokines were measured using the LEGENDplex Mouse Inflammation Panel (Biolegend), following the manufacturer's instructions. Samples were acquired on the BD Accuri C6 Plus cytometer. Data were analysed using the LEGENDplex data analysis software (Biolegend).

Intra-cisterna magna injections

P1 infected pups were wrapped in gauze and completely covered in ice for approximately 10 min. Newborns were injected via intra-cisterna magna with 1 mg/mL anti-mouse IL-17 or the IgG isotype control (InvivoMAb Biocell), diluted in 0.3 mg/mL of Fast Green, in a 2 μ L of total volume per pup. The injection was performed using a 10 μ L Hamilton syringe with a

30 G needle. The intra-cisterna magna was visually located through the thin clear skin, on the neck right below the skull, the needle was injected at 1.5 mm depth and the content was slowly injected. After injection, pups were allowed to completely recover on a heating pad under a red lamp before returning to the cage. CFU of meninges, brain, lungs and liver were analysed 24 h post-injection.

Blood-brain barrier permeability assessment

For *in vivo* BBB permeability assessment, 0.3 mM of 3-5 KDa FITC-dextran (FD4, Sigma-Aldrich), in a 40 μ L of total volume, were injected subcutaneously in P1 pups infected with GBS. Five minutes after injection mice were deeply anaesthetised with isoflurane. Blood was collected after the puncture of the right atrium and kept on ice. Blood was centrifuged at $10,000 \times g$ for 15 min at 4 °C and the serum was collected and stored at -80 °C until use. Mice were then perfused with PBS and the brain was extracted, divided into two hemibrains (hemispheres free of cerebellum and olfactory bulbs), weighted and immediately frozen on dry ice. The brains were stored at -80 °C until use. To measure the fluorescence intensity, the serum was diluted in PBS (20 μ L of serum in 30 μ L of PBS) and 100 μ L of PBS was added to the hemibrain to homogenise the tissue. The fluorescent intensity was measured using the SpectraMax iD3 (Molecular Devices). The degree of BBB disruption was described as the permeability index⁴².

Developmental milestones

Pups born from Uninfected and Infected C57Bl/6 WT and *Tcrd*^{-/-} mice were assessed daily for weight since P0 and the acquisition of developmental milestones since P1, as previously described⁴³. Pups were gently removed from their cages and placed on a heating pad and under a red lamp, while the litter was tested. Pups were weighted daily and visually examined for eye-opening. Each day the pups were evaluated through a battery of tests assessing the attainment of milestones appropriate for their age⁴³. After testing, the pups returned to their cages. The development milestones are as follows:

Surface righting (P1-P13): Pups were individually held on their backs (supine position), with paws facing upwards. The time pups took to flip over onto their abdomens (prone position) was noted. The test was terminated if the pup failed to turn over within 30 seconds or when it could turn itself in less than 1 second for two consecutive days.

Negative geotaxis (P1-P14): Pups were individually placed on a wire mesh set at a 45° angle with heads facing downwards. The time to turn around by 180° and move upwards was recorded. The test was repeated daily until pups passed in < 30 seconds for two consecutive days.

Cliff aversion (P1-P14): Pups were positioned on the edge of a pipette tip's box with forepaws and the snout hanging over the edge. The time taken to turn and crawl away from the edge was noted. The test was repeated daily until pups passed in < 30 seconds for two consecutive days.

Rooting (P1-P12): Pups were gently rubbed with the smooth tip of a brush along one side of the head. A positive response was noted if the pup moved its head toward the filament. The test was repeated daily until the pups responded correctly for two consecutive days.

Forelimb grasp (P4-P14): Pups were held with their forelimbs grasping a wire bar, while their hindlimbs were not supported on any surface. The time pups were able to remain suspended was noted. The test was repeated daily until pups could stay suspended for a minimum of 1s for two consecutive days.

Auditory startle (P7-P18): Pups were individually exposed to a hand clapping, at approximately 25 cm distance. The day the pups responded with a quick involuntary jump was noted.

Ear twitch (P7-P15): The fine filaments of a brush were gently touched against the tip of the pup's ear. Positive responses were noted if pups responded by flattening their ears. The test was repeated daily until the pups responded correctly for two consecutive days.

Open field traversal (P8-P21): Each pup was placed at the centre of a circular 1 cm diameter arena. The latency to move out of the arena was recorded. The test was repeated daily until pups walked out of the arena in < 30 seconds for two consecutive days.

Air righting (P8-P21): Pups were held on their backs (with four paws turning upwards), 10 cm above a cotton pad, and released. The test was repeated daily until pups landed on all four paws for two consecutive days.

Behaviour tests

Before the performance of the behavioural tests, P42 control or survivor mice were allowed a 2-week adaptation period to the behavioural test facilities. Tests were conducted in the dark (active) cycle. All materials were cleaned with a neutral detergent without smell between animals. The same groups of animals performed all tests (a 3R strategy). All tests

were videotaped with a camera positioned above the apparatus. Student's t-test was used to assess genotype and group effects, and the animals were separated by sex.

Elevated plus maze (EPM)

EPM apparatus was placed 50 cm above the ground and is composed of a cross form with two open arms (30 × 5 cm) and two closed arms (30 × 5 cm, surrounded by 15 cm-high opaque walls). Identical arms are in opposite positions to each other, with the arms emerging from a central platform (5 × 5 cm). The test was initiated by placing the mouse in the centre of the apparatus facing an open arm and allowed to move freely for 5 minutes. The lights were on during the test. The analyses were performed using a tracking system (Smart Video Tracking Software v 2.5, Panlab).

Social test

The apparatus consisted of a wide acrylic box divided into three compartments (two 33 × 33 cm side compartments and one 12 × 12 cm central compartment) connected by open doors. The social tests consisted of three phases: the habituation phase, where the animal is positioned in the central compartment and allowed to explore for 5 minutes; the target phase, where one side compartment has an empty cage (round and gridded) and the other side compartment has the same cage with an animal inside (of the same age and sex as the sample animal), and the animal is positioned in the central one and allowed to explore for 10 minutes; the novel vs familiar phase, where an animal is added to the empty cage and the sample animal is positioned in the central compartment and allowed to explore for another 10 minutes. Animal/cage exploration was defined as the mouse nose touching or directed towards the object at a distance shorter than 2 cm. The analyses were performed in the Observer 7 XT software (Noldus Information Technology, Wageningen, The Netherlands).

Open field

Each mouse was positioned in the centre of an opaque arena (43 × 43 cm) and allowed to move freely for 10 minutes. The total distance travelled, peripheral activity (locomotion along the walls) and centre activity (locomotion in the central zone) were automatically obtained through video tracking (Smart Video Tracking Software v 2.5, Panlab).

Novel object recognition

The NOR test was performed in an arena (43 × 43 cm) and consisted of three phases: the habituation phase, in which mice were allowed to explore the apparatus for 10 minutes; the acquisition/sample phase 24 h after the habituation, where mice were placed in the

apparatus with two identical objects (familiar object) for 10 minutes; and the retention/choice session, performed 4 h later, in which a novel object and a familiar object were used, and mice were allowed to explore the objects for 3 minutes. The objects chosen for this experiment were approximately the same height and weight, with different shapes, colours or materials. Object exploration was defined as mice nose touching or directed towards the object at a distance shorter than 2 cm, and the duration of time mice spent exploring each object was recorded by the observer, using Observer 7 XT software (Noldus Information Technology, Wageningen, The Netherlands). The recognition index was calculated as the ratio $T_{N/F} / (T_N + T_F)$ [T_N = time exploring the novel (N) object; T_F = time exploring the familiar (F) object]. As exclusion criteria, mice exploring the objects for periods lower than 10 s at the 3 min choice session were excluded from the analysis.

Brain Tissue Processing and Immunostaining

For immunohistochemistry analysis, animals were deeply anaesthetised and perfused with PBS. The brains were removed, post-fixed by immersion in 4% PFA for 24 h, at 4 °C, washed with PBS and then cryoprotected using sucrose 30%. Samples were thereafter washed in PBS and mounted in OCT (Thermo Scientific) embedding medium, frozen and cryosectioned in the CM3050S Cryostat (Leica Biosystems). Brain coronal sections (60 µm) were collected by free-floating, in PBS, transferred to cryoprotectant solution (30% sucrose and 30% ethylene glycol in 0.1 M phosphate buffer) and stored at -20 °C until staining. For immunolabeling, brain slides were permeabilised with 0.25% Triton for 10 min, blocked for 1 h in PBS with 0.1% Triton and 5% Bovine Serum Albumin (BSA) and incubated overnight (4 °C) with the primary antibodies: anti-Iba1 (FUJIFILM Wako; 1:500), anti-NeuN (Sigma-Aldrich; 1:500) and anti-CD68 (Bio-Rad; 1:500). The following secondary antibodies were used: goat anti-rat IgG F(ab')₂ Alexa Fluor 488 (Invitrogen; 1:1000), goat anti-rabbit Alexa Fluor 568 (Invitrogen; 1:1000) and goat anti-mouse Alexa Fluor 647 (Invitrogen; 1:1000) for 1h30 at RT. DAPI (Thermo Fisher Scientific; 1:100) was used to mark the nuclei. Sections were mounted using a Fluorescence mounting medium (Dako), sealed with nail polish and stored at -20 °C.

Primary Microglia Cell Cultures and Immunohistochemistry

Primary mixed glial cultures were obtained from newborn Wistar Han rat pups at P1 – P2. The pups' brains were dissected in Hank's Balanced Salt Solution (HBSS, Gibco™) with 1% Penicillin-Streptomycin (P/S) and the hippocampi were collected. After complete tissue

dissociation, enzymatic digestion was completed using DNase I (0.1 U/mL) (Irvine) and 0.25% trypsin-EDTA (Gibco™). The cells were suspended in Dulbecco's Modified Eagle Medium (DMEM) (1x)/Glutamax (Gibco™) supplemented with 10% of FBS and 1% of P/S. Cells were seeded in T75 flasks coated with 0.01 mg/mL of PDL at a density of two brains/flask. The cell cultures were maintained for 10 days at 37 °C in a humidified incubator with 5% CO₂. The medium was partially replaced at day 4 and then totally replaced every 2 days. MGCs were shaken at 200 rpm for 2 h at 37 °C to obtain purified microglia cells. The cell suspension obtained was centrifuged at 1200 rpm for 10 min and resuspended in DMEM/ Nutrient Mixture F-12 (DMEM/F-12) (Gibco™) supplemented with 10% of FBS and 1% of P/S. Purified microglia were plated at a density of 0.5×10^5 / cm in a 24-well plate with rounded coverslips in the wells.

For stimulation assays, microglia were cultured in medium alone or in the presence of heat-killed GBS in a multiplicity of infection (MOI) of 5. Each condition was set in duplicate, and cultures were maintained for 24 h at 37 °C with 5% of CO₂. Cells in coverslips were fixed with 4% PFA (Sigma-Aldrich) for 10 min at RT. Then, cells were permeabilised with 0.2% Triton X-100 (Sigma-Aldrich) for 10 min at RT and blocked with 3% BSA (Sigma- Aldrich) for 1h at RT. Fixed cells were incubated with primary antibodies iNOS (1:200, abcam) and CD68 (1:500, biorad) overnight at 4 °C. Cells were washed and incubated with secondary antibodies (1:1000, Invitrogen), for 1h at RT, and nuclei stained with 1 µg/mL Hoechst 33342 (Sigma-Aldrich) a10 min at RT. Coverslips were mounted with fluorescent mounting media (Dako, Agilent, Santa Clara, CA, USA).

Imaging acquisition and quantification

Brain images were acquired in a Leica TCS SP8 confocal microscope (Leica Microsystems), using a PL APO 63x /1.30 Glycerol immersion objective for the Iba-1 and CD68 quantification, and a PL APO 10x /0.40 CS2 objective for NeuN quantification. The prefrontal cortex, the motor cortex and hippocampus from both study groups were studied. A total of 3 slices per brain were acquired. In each slice, 5 images were taken of the cortex and hippocampus of P3 pups. In 6wk old animals, 5 images were taken of the cortex using each objective, and 6 and 12 images of hippocampus regions (CA1, CA2, CA3 and Dentate Gyrus) were acquired with the 10x and the 63x objective, respectively. For the analysis, Fiji software was used for microglia and neuron quantification. The quantification of neurons was automatically done with a custom-made ImageJ/Fiji script from the Advanced Light Microscopy platform at i3S. Three-dimensional microglia reconstruction was performed using Imaris software (version 9.1.1, Bitplane). For microglia cultures, imaging was

performed using a Zeiss Axiomager Z1 fluorescence microscope equipped with an Axiocam MR v3.0 camera, an HXP 120 light source, and a 40x/1.30 objective. The fluorescence intensity of microglial cells was quantified using ImageJ software.

Statistical analysis

All data were analysed with the GraphPad Prism software (v.9, GraphPad software Inc. CA). Means and standard errors of the means (SEM) were calculated and correspond to the indicated independent experiments. The log-rank (Mantel–Cox) test was used to analyse the survival curve. Differences between multiple groups were analysed by one-way or two-way ANOVA, whenever appropriate, with the Sidak's Multiple Comparison Test, for $\alpha = 0.05$. Image analysis of microglia morphology and activation was made using the Nested t-test. All other comparisons were done with an unpaired Student's t test. Normality was verified by the Shapiro–Wilk normality test. Homogeneity of variance was estimated by an F test. Assumptions of sphericity in ANOVA were also verified. The minimum sample size was determined using G*Power analysis for two-tail t-test comparing the mean and standard deviation of two independent groups with effect size $d = 7$, $\alpha = 0.05$ and power of 0.95. CFU data were log₁₀ transformed. Significance was represented by the following symbols: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Results

Clinical scoring does not predict neonatal death in the GBS meningitis model

We previously described a mouse model of GBS neonatal meningitis in the BALB/c mice¹⁶. Here, we established an adapted model for the C57BL/6 mice (Figure 1a). The gestational days (G) 16 and 17 were identified as the optimal window for vaginal colonisation of pregnant C57BL/6 females (Figure 1a). We found that doses ranging from 3×10^4 to 1×10^5 colony-forming units (CFU) lead to neonatal disease, with dose-dependent mortality (Figure 1b). The best-fitting model, compared to our previous results with the BALB/c model, was the colonisation with 3×10^4 CFU, with a mortality rate of approximately 40% (Figure 1b).

To assess disease progression and symptomatology appearance, as well as the predictability of death, in a non-invasive longitudinal and objectified sensitive manner, the offspring born from colonised and non-colonised dams were evaluated during their first five days of life for body weight and clinical scoring parameters (Figure 1c-g). As expected, infected animals presented significantly reduced body weight (Figure 1c). The observational

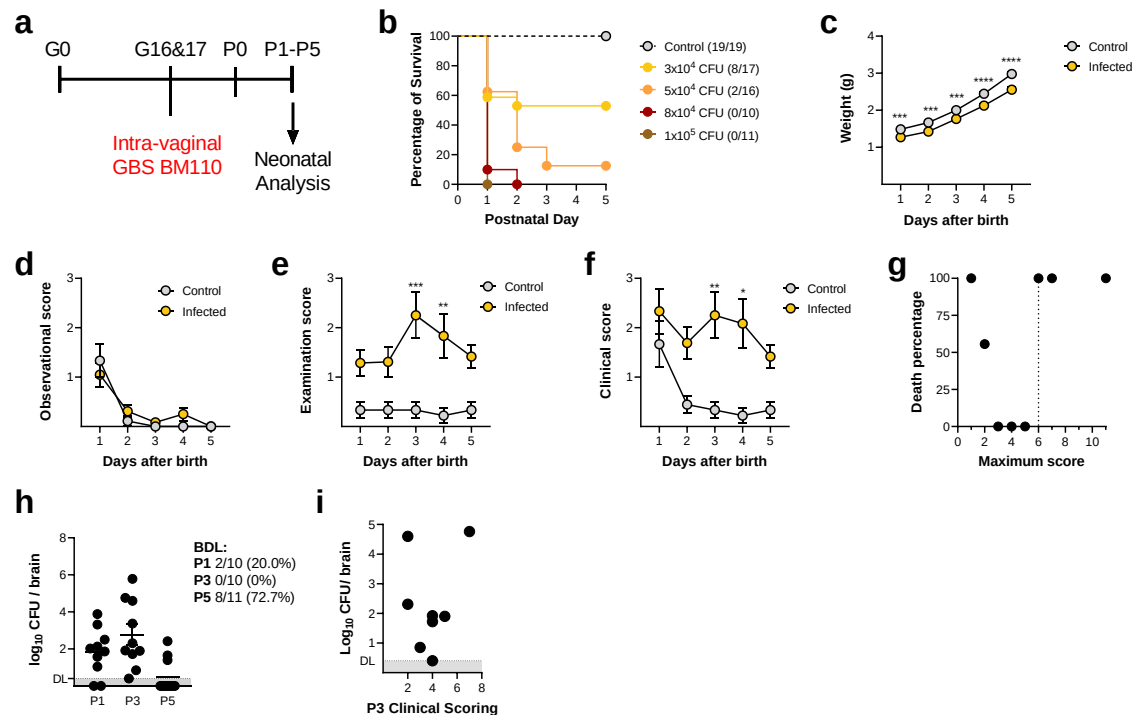


Figure 1. Clinical scoring does not predict neonatal death in experimental GBS meningitis. Pregnant C57BL/6 female mice were intravaginally colonised with 10^4 to 10^5 CFU GBS hypervirulent strain BM110, at gestational days 16 and 17. Control animals received PBS. **(a)** Schematic representation of the experimental protocol. **(b)** Kaplan-Meier survival curve of neonatal mice born from GBS-colonised dams, monitored for 5 days. The numbers in parentheses represent the number of animals that survived versus the total number of animals born. **(c)** Pups born from both groups were daily weighted until postnatal day (P) 5. Data is presented as mean values $\pm$ SEM ($n = 9$ Control, $n = 21$ Meningitis). Two-way ANOVA performed comparisons. **(d-f)** Observational (d), examination (e) and total (f) clinical scores of uninfected and infected pups, during the first 5 days of life. Total clinical scoring was calculated by summing up the points from all the observational and examination categories. Data are presented as mean $\pm$ SEM ($n = 9$ Control, $n = 21$ Meningitis). Comparisons were performed by two-way ANOVA. **(g)** Correlation between the percentage of death within the maximum clinical scores obtained from the infected pups. Data are presented as mean $\pm$ SEM ($n = 29$). Comparisons were performed by two-way ANOVA. **(h)** Newborn mice were sacrificed at P 1, 3, and 5 and the brain bacterial load was determined. Each symbol indicates data from a single pup ($n = 10$ P1, $n = 10$ P3, $n = 11$ P5). Data are presented as mean $\pm$ SEM. **(i)** Correlation between the clinical scoring and the bacterial load in the brain of infected P3 pups. Each symbol indicates data from a single pup ($n = 8$). Statistical differences (P values) between groups are indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. DL, detection limit.

score, composed of skin colour, presence of milk spot, spontaneous movement and head posture, showed no differences between groups (Figure 1d). In contrast, the examination score, comprising capillary refill time, dehydration, reaction to tactile stimuli, and abdominal palpation, was increased in the infected pups every day, significantly worsening on postnatal days (P) 3 and 4 (Figure 1e). The total clinical scoring, combining observational and examination scores, was significantly increased on these days (Figure 1f). Death predictability based on the clinical scoring was assessed by graphing the maximum

obtained score vs the percentage of animals that died within the respective score (Figure 1g). The maximum score obtained varied between 1 and 11 (Figure 1g). Importantly, while a score ≥ 6 predicted 100% mortality (Figure 1g), pups with lower scores (<3) also exhibited high mortality, limiting the scoring system's predictive utility for death. Analysis of brain colonisation revealed that bacterial levels were already present in 80% of the pups by P1, peaking at P3, and declining to nearly undetectable levels by P5 (Figure 1h). However, brain bacterial burden did not correlate with clinical scores, as animals exhibited variable brain colonisation at different clinical scores (Figure 1i), further emphasising the unpredictable nature of disease severity in this model. In conclusion, unless a severe scenario of septicaemia is present, a correlation between bacterial levels in the brain, symptom appearance, and disease progression is impossible to achieve in this animal model of neonatal meningitis.

Neonatal GBS meningitis leads to increased meningeal $\gamma\delta$ T cells

To define the immune landscape of the developing meninges during meningitis, we dissected the dura mater and performed single-cell RNA sequencing (scRNA-seq) analysis on sorted CD45⁺ immune cells from pups with neonatal meningitis and uninfected controls at P3 (Figure 2a). The dura was chosen given their vast repertoire of immune cells²¹. This time point was selected as it corresponds to the peak of infection. To exclude possible confounders between cases of meningitis and septicaemia, pups with a clinical score higher than 6 were excluded. As a control for meningitis, brain colonisation was confirmed in all except one pup, as well as a significant decrease in brain weight compared to the control group (Figure 2b and c). Clusters were identified based on the expression of known specific marker genes for the corresponding cell types^{36,37} (Figure 1d). As expected, uniform manifold approximation and projection (UMAP) analysis revealed a diverse repertoire of immune cells, identified in 21 distinct clusters, including border-associated macrophages (BAM), macrophages, monocytes, mast cells, basophils, neutrophils, dendritic cells, plasmacytoid dendritic cells, B cells, type 2 and type 3 innate lymphoid cells (ILC2 and ILC3), T cells, NK cells, and NK T cells (Figure 2d-f). BAM appeared as the main cluster, followed by classical monocytes and mast cells, while lymphocyte clusters were much smaller (Figure 1e and f). Due to the low abundance of neutrophils, classical mediators of the meningitis inflammatory response, and lymphoid cells, we further validated our findings by flow cytometry (Figure 3). At the peak of the infection, a significant decrease in the total number of immune cells was found in the meningitis pups, in agreement with their reduced body and brain weight (Figure 3b, kinetics shown in Figure S1a). Concomitant with this

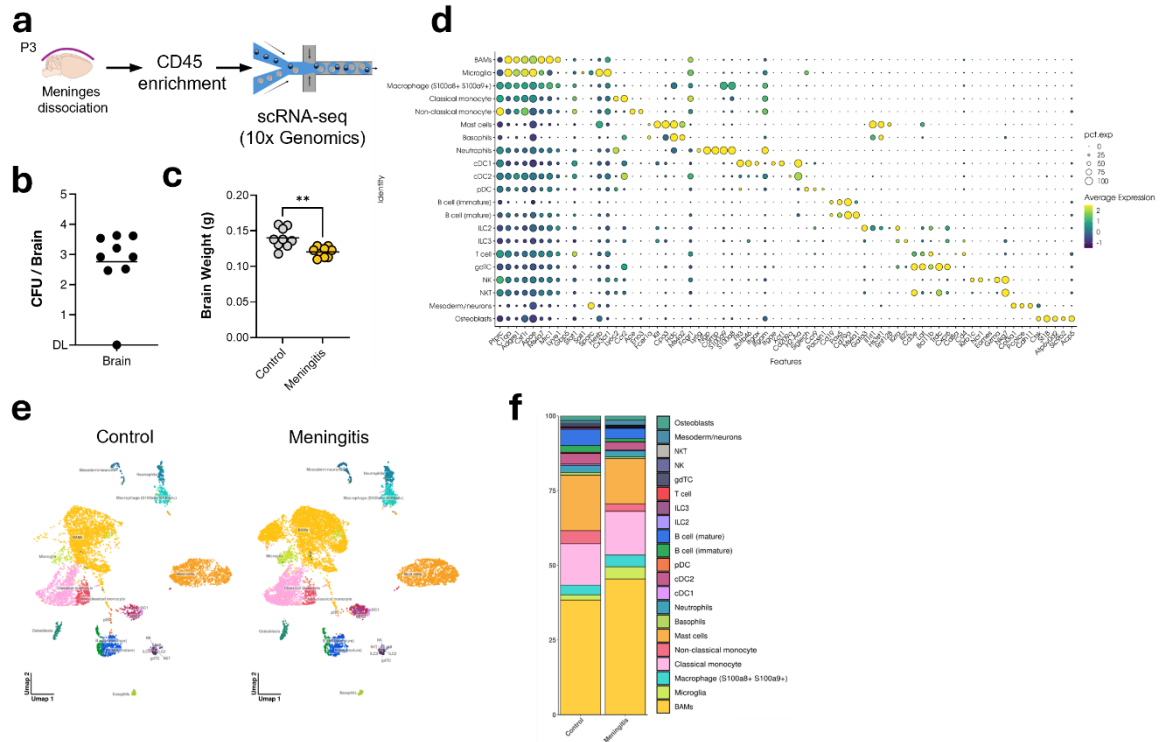


Figure 2. Composition of meningeal cell populations during neonatal meningitis by scRNAseq. Pregnant C57BL/6 female mice were intravaginally colonised with 3×10^4 CFU GBS hypervirulent strain BM110, at gestational days 16 and 17. Control animals received PBS. Newborn mice were sacrificed on postnatal day (P) 3. The meninges from 9 pups per group were harvested, pooled and processed for single-cell RNA sequencing (scRNA-seq). **(a)** Schematic illustration of P3 dural meninges isolation for scRNA-seq analysis. **(b-c)** The brain bacterial loads **(b)** and brain weight **(c)** were determined. Each symbol represents one pup ($n = 9$). Data are presented as mean. **(d)** Dot plot showing the average expression levels per cluster that most clearly discriminate among major meningeal cell types, as well as contaminating neurons and microglia. The colour and size of each dot represent the average expression and percentage of cells expressing each gene, respectively. **(e)** Uniform manifold approximation and projection (UMAP) visualisations of CD45⁺ cell types in the dural meninges at steady state and during GBS meningitis. Each data point in a UMAP plot represents a cell. **(f)** Bar graph depicting frequencies of cell populations in Control vs. Meningitis samples. Statistical differences (P values) between groups are indicated; ** $P < 0.01$.

finding, the frequency and number of B cells were significantly decreased in the infected animals (Figure 3c, kinetics shown in Figure S1b). No differences were observed in the conventional $\alpha\beta$ T cells (Figure 3c, kinetics shown in Figure S1c). Notably, the frequency of $\gamma\delta$ T cells at P3 was significantly increased in the meningitis pups, almost doubling those from the control counterpart (Figure 3c and d, kinetics shown in Figure S1d). Systemically, the same significant difference was observed in the spleen of pups with meningitis (Figure S1j). The kinetics of immune cells' frequency and number during the first 5 days of life can be observed in the supplementary Figure 1. No differences were observed neither in CD11b⁺Ly6G⁻ macrophages/monocytes nor in CD11b⁺Ly6G⁺ neutrophils (Figure 3 e-g, kinetics shown in Figure S1e and f). Moreover, no differences were observed in the main

lymphoid and myeloid cells in the brain parenchyma (Figure S2), supporting our previous observation that GBS meningitis does not lead to leukocyte infiltration¹⁶.

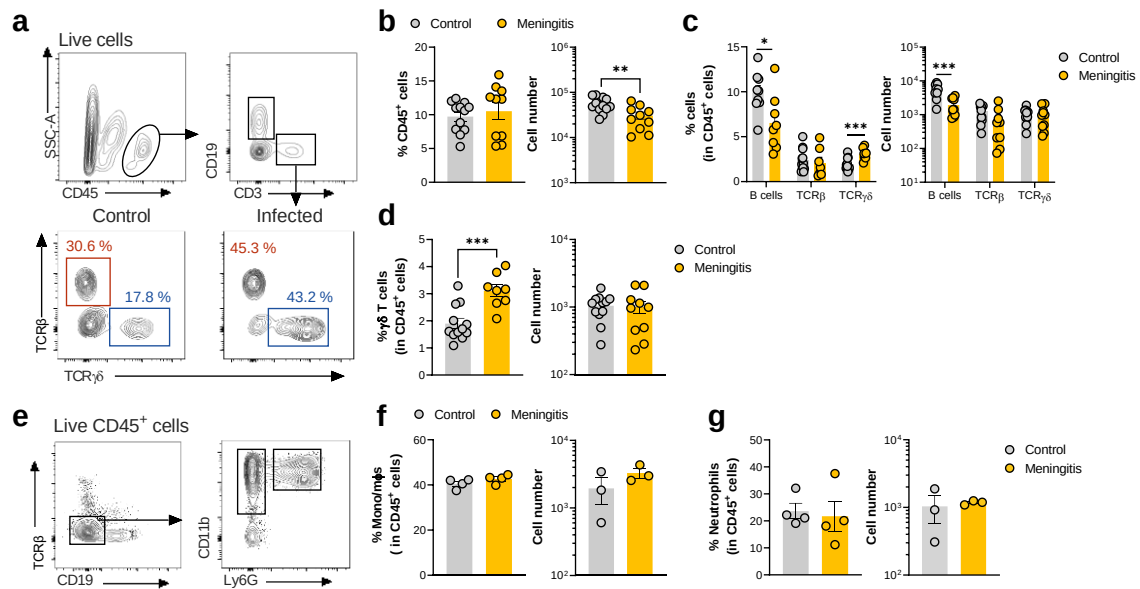


Figure 3. Increased frequency of $\gamma\delta$ T cells in the meninges of GBS-infected newborn mice. Pregnant C57BL/6 female mice were intravaginally colonised with 3×10^4 CFU GBS hypervirulent strain BM110, at gestational days 16 and 17. Control animals received PBS. Newborn mice were sacrificed at indicated postnatal days (P). The meninges were harvested and processed for flow cytometry analyses. **(a)** Representative flow cytometry scheme showing the lymphoid gating strategy. **(b-d)** Frequency and number of indicated populations. Each symbol represents a single pup ($n = 12$ Control, $n = 10$ Meningitis). Error bars indicate the mean $\pm$ SEM. Comparisons by students' t-test. **(e)** Representative flow cytometry scheme showing the myeloid gating strategy. **(f-g)** Frequency and number of indicated populations. Each symbol represents a single pup ($n = 4$). Error bars indicate the mean $\pm$ SEM. Comparisons by students' t-test. Statistical differences (P values) between groups are indicated; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Neonatal meningitis leads to increased meningeal $\gamma\delta 17$ T cells for life

It has been reported that male infants surviving invasive GBS disease are at higher risk of developing NDI⁴⁴. Thus, we next evaluated whether mouse sex could affect the frequency of $\gamma\delta$ T cells at P3. Analysis revealed an increased frequency of $\gamma\delta$ T cells in the meningitis group, regardless of pup sex, with males presenting a significantly increased frequency, and females almost reaching significance ($P = 0.051$). The same pattern was observed in the spleen, where infected animals exhibited an increased frequency of these cells (Figure S3a). Moreover, there were no sex-based differences in bacterial levels in the brain and meninges (Figure 4c). Interestingly, the frequency of $\gamma\delta$ T cells did not correlate with bacterial colonisation levels in the brain (Figure 4d).

After confirming the increased frequency of $\gamma\delta$ T cells in the meningitis group across sexes, we sought to understand the functional implications of this alteration. Under healthy conditions, meningeal $\gamma\delta$ T cells are bona fide IL-17 producers ($\gamma\delta$ 17 T cells), with a T cell receptor (TCR) repertoire mostly restricted to the gamma chain variable region 6 (V γ 6) (operationally defined by default as double negative for V γ 1 and V γ 4)^{26,29}. To assess whether neonatal meningitis leads to an altered phenotype of meningeal $\gamma\delta$ T cells, we further characterised this subset at P3. Flow cytometry analysis revealed that the TCR repertoire of $\gamma\delta$ T cells during meningitis remained biased toward the V γ 6 subset (Figure 4a and e). Accordingly, intracellular staining showed that meningeal $\gamma\delta$ T cells in both groups remained mostly IL-17 producers, with a negligible population of IFN- γ -producing $\gamma\delta$ T cells (Figure

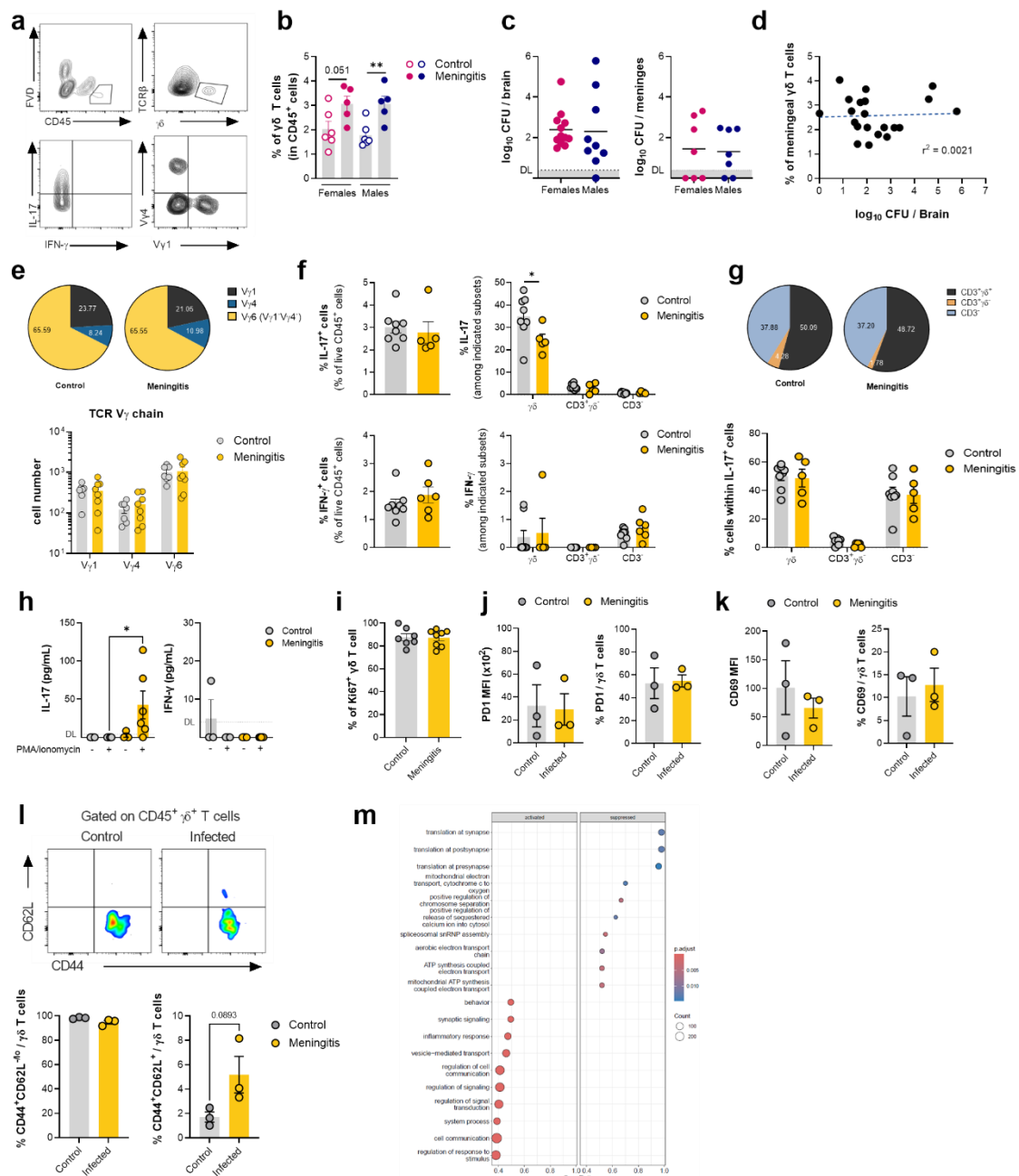


Figure 4. Neonatal meningitis alters $\gamma\delta$ T cells IL-17 production in the meninges. Pregnant C57BL/6 female mice were intravaginally colonised with 3×10^4 CFU GBS hypervirulent strain BM110, at gestational days 16 and 17. Control animals received PBS. Newborn mice were sacrificed at postnatal day (P) 3. **(a)** Representative gating strategy of the $\gamma\delta$ T cells analysis. $\gamma\delta$ T cells were defined as $CD45^+FVD-CD19-Tcr\beta-\gamma\delta^+$. **(b)** Frequency of $\gamma\delta$ T cells in the meninges of male and female pups at P3. Each symbol represents one pup (n = 6 Control, n = 5 Meningitis). Data are presented as mean $\pm$ SEM. Comparisons by Students' t-test within sexes. **(c)** Bacterial load in the brain (left, n = 12 Females, n = 9 Males) and meninges (right, n = 7) in male and female pups, at P3. Each symbol represents one pup. **(d)** Linear regression plots of the frequency of $\gamma\delta$ T cells and brain colonisation in P3 pups. Each symbol represents one newborn (n = 21). **(e)** Pie charts depicting the percentage of meningeal $V\gamma1^+$, $V\gamma4^+$ and $V\gamma6$ ($V\gamma1-V\gamma4^-$) $\gamma\delta$ T cells, at P3 (top panel) and columns represent their frequency by animal (bottom panel). Each symbol represents one pup (n = 7 Control, n = 8 (Meningitis). Error bars indicate mean $\pm$ SEM. Comparisons by Students' t-test with each clone. **(f)** Frequency of IL-17 (top panel) and IFN- γ (lower panel) produced by meningeal $\gamma\delta$ T cells at P3. Each symbol represents one pup (n = 8 Control, n = 6 Meningitis). Error bars indicate mean $\pm$ SEM. Comparisons by Students' t-test between groups. **(g)** Pie charts represent indicated cell populations within total $IL17^+CD45^+$ cells in the meninges (top panel) and column graph represent the mean frequency of the indicated populations (bottom panel). Each symbol represents one pup (n = 8 Control, n = 6 Meningitis). Error bars indicate mean $\pm$ SEM. Comparisons by Students' t-test within clones. **(h)** Supernatant concentrations of indicated cytokines from ex vivo cultured meningeal cells, isolated from P3 pups, after 24 h of PMA and ionomycin stimulation. Each symbol represents a pool of 2 pups (n = 4-12). Data are presented as mean $\pm$ SEM. Comparisons by Students' t-test within PMA absence or presence. **(i)** Frequency of Ki67 in meningeal $\gamma\delta$ T cells. Each symbol represents one pup (n = 7 Control, n = 8 Meningitis). Error bars indicate mean $\pm$ SEM. Comparisons by Students' t-test. **(j-l)** Thymic characterisation of $\gamma\delta$ T cells. **(j-k)** MFI and frequency of indicated surface markers in meningeal $\gamma\delta$ T cells. Each symbol represents one pup (n = 3). Error bars indicate mean $\pm$ SEM. Comparisons by Students' t-test. **(l)** Representative gating strategy (top panel) and frequency of indicated populations (bottom panel). Each symbol represents one pup (n = 3). Error bars indicate mean $\pm$ SEM. Comparisons by Students' t-test. **(m)** Gene Set Enrichment Analysis (GSEA) of Gene Ontology (GO) terms showing the top 10 activated and suppressed pathways by gamma delta T cells in Meningitis vs Control. Statistical differences (P values) between groups are indicated; *P < 0.05, **P < 0.01. DL, detection limit.

4f). However, we observed a mild but significant decrease in IL-17 transcription by meningeal $\gamma\delta$ T cells in pups with bacterial meningitis (Figure 4f). Nonetheless, gating on IL-17-positive cells confirmed that neonatal $\gamma\delta$ T cells remained the major IL-17-producing cells in both groups (Figure 4g). Increased IL-17 secretion by meningeal cells during meningitis was further validated by ex vivo stimulation with phorbol myristate acetate (PMA) and ionomycin (Figure 4h). IFN- γ levels were almost below the detection threshold, while only in the infected group was possible to detect measurable levels of IL-17, even without stimulation (Figure 4h). No differences were observed in the secretion of TNF- α , IL-6, or IL-10 (Figure S3b). Although no differences were found in the frequency of splenic IL-17-producing $\gamma\delta$ T cells, accumulation of this cytokine was also found in culture supernatants from stimulated cells in the meningitis group (Figure S3c-d). It is worth mentioning that cerebrospinal fluid (CSF) levels of IL-17, as well as other cytokines and chemokines, were below the technical detection limits (Figure S4a-e). However, although

the serum levels of IL-17 were also below detection, a significant increase in IL-10, type I Interferon (IFN)- β , TNF- α and C-C motif chemokine ligand-2 (CCL2) was observed in the meningitis group compared to uninfected animals, suggesting an ongoing inflammatory response (Figure S4c-g).

$\gamma\delta$ 17 T cell development is restricted to the embryonic and neonatal thymus, where they acquire the capacity to produce IL-17^{45,46}. Thus, we tested whether the development of $\gamma\delta$ 17 T cells is altered in the neonatal thymus. No differences were found in IL-17 committed $\gamma\delta$ T cells between groups (Figure S3e and f). Since $\gamma\delta$ T cells have been shown to produce IL-17 in response to bacterial antigens and pro-inflammatory signals, such as IL-1 β and IL-23⁴⁷, we next tested whether GBS could directly modulate IL-17 production by these cells. To this end, we sorted $\gamma\delta$ T cells from the neonatal lung, as they are equally enriched in the TCR-V γ 6 subset (Figure S5, and stimulated *in vitro* with heat-killed GBS (hkGBS) in the presence or absence of splenocytes as a source of antigen-presenting cells (APCs) (Figure S3g). The secretion of IL-17 and IFN- γ was measured in the supernatants by ELISA. The results showed that $\gamma\delta$ T cells stimulated with hkGBS increased the production of IL-17, in the absence ($P = 0.054$) or presence of inflammatory stimuli (Figure S3g). Moreover, APC presence potentiated cytokine production by $\gamma\delta$ T cells (Figure S3g). To confirm that GBS can lead to the secretion of IL-1 β and IL-23, splenic cells were stimulated with hkGBS alone. We found a significant increase in both cytokines, suggesting that *in vivo* GBS could further fuel $\gamma\delta$ T cell activation (Figure S3h).

$\gamma\delta$ T cells progressively seed the meninges from early life and display a particularly activated tissue-resident phenotype^{26,29}. GBS meningitis did not alter their phenotype, as Ki67 staining revealed that meningeal $\gamma\delta$ T cells exhibited high proliferation at P3 (Figure 4i). In support of their activated resident phenotype⁴⁸, a percentage of meningeal $\gamma\delta$ T cells expressed the tissue residency markers PD1 and CD69 in both control and meningitis pups (Figure 4j and k). Moreover, almost all $\gamma\delta$ T cells displayed an activated CD44⁺CD62L^{-/lo} phenotype in both groups, showing no alterations in their tissue-homing and activation states (Figure 4l). CD44⁺CD62L⁺ cells, an effector memory phenotype, showed a tendential but not significant increment in the meningitis group (Figure 4l). Next, we performed gene set enrichment analysis (GSEA) for Gene Ontology (GO) terms of biological processes differentially expressed between meningeal $\gamma\delta$ T cells from pups with meningitis or healthy controls. Our results demonstrated the enrichment of pathways related to the inflammatory response, cell communication, and response to stimuli, while ATP and synaptic translation-related pathways were significantly suppressed (Figure 4m).

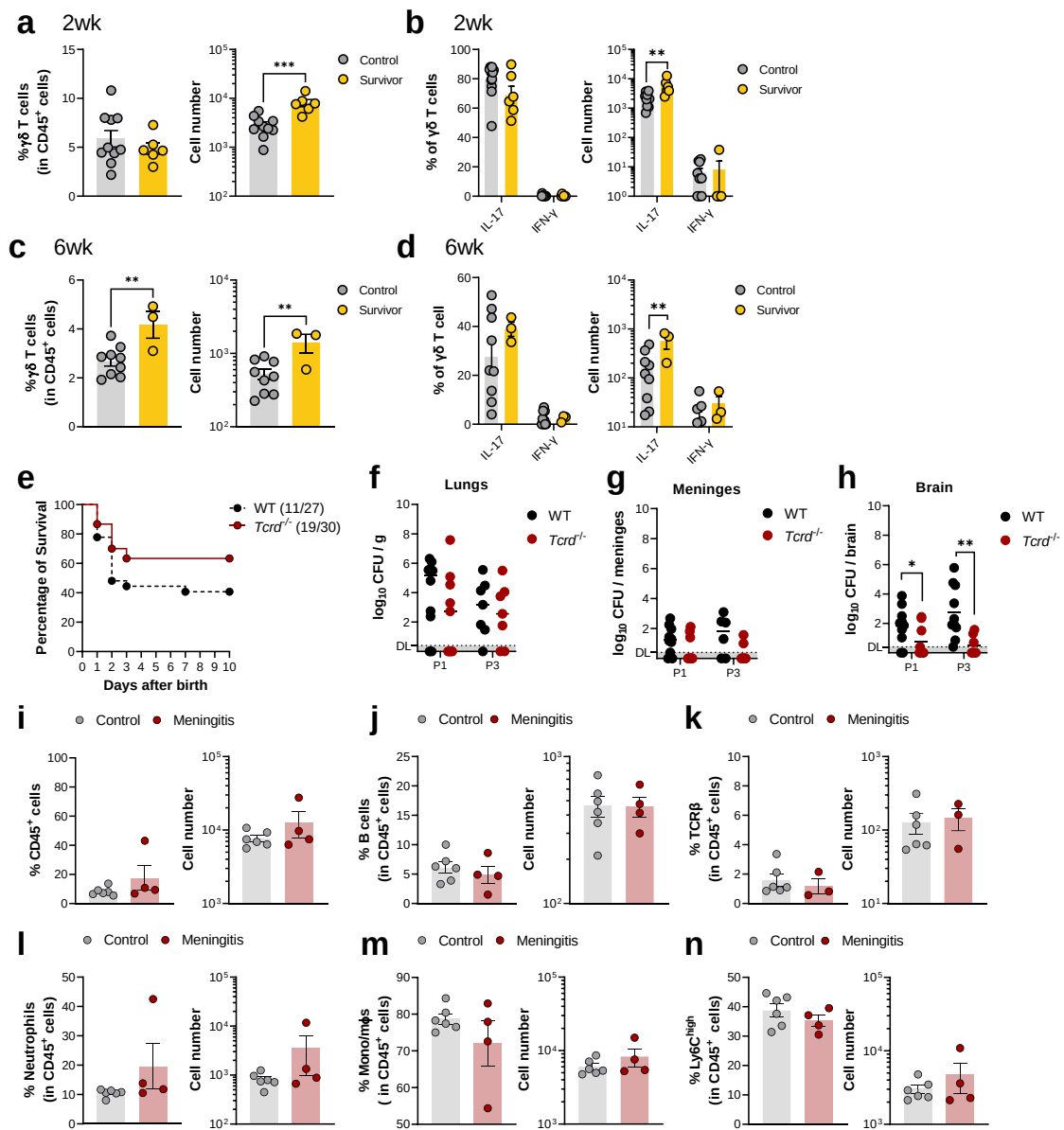


Figure 5. Meningeal $\gamma\delta$ 17 T cells are altered for life and may be prejudicial during meningitis. Pregnant C57BL/6 WT or *Tcrd*^{-/-} female mice were intravaginally colonised with 3×10^4 CFU GBS hypervirulent strain BM110, at gestational days 16 and 17. Control animals received PBS. **(a-d)** Frequency and number of $\gamma\delta$ T cells and IL-17 produced by them at 2 week- **(a-b)** and 6 week- **(c-d)** old control vs survivor WT animals. Each symbol represents one animal ($n = 10$ Control 2 wk, $n = 9$ Control 6 wk, $n = 6$ Meningitis 2 wk, $n = 3$ Meningitis 6 wk). Error bars indicate mean $\pm$ SEM. Comparisons by Students' t-test. **(e)** Kaplan-Meier survival curve of neonatal mice born from GBS-colonised WT and *Tcrd*^{-/-} dams, monitored for 10 days. The numbers in parentheses represent the number of animals that survived versus the total number of animals born. **(f-h)** Bacterial load in the lungs **(f)**, meninges **(g)** and brain **(h)** at postnatal day (P) 1 and 3 *Tcrd*^{+/+} vs *Tcrd*^{-/-} pups. Each symbol represents one pup ($n = 6-10$ Control, $n = 7-9$ Meningitis). Error bars indicate mean $\pm$ SEM. Students' t-test performed analysis. **(i-n)** Frequency and number of indicated populations. Each symbol represents a single pup ($n = 6$ Control, $n = 4$ Meningitis). Error bars indicate the mean $\pm$ SEM. Comparisons by students' t-test. Statistical differences (P values) between groups are indicated; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Finally, to evaluate whether $\gamma\delta$ T cell homeostasis is restored following neonatal meningitis,

flow cytometric analysis of 2-week and 6-week-old mice was performed (Figure 5a-d). Surprisingly, 2-week-old pups surviving neonatal meningitis still presented bacteria colonisation in the brain, which was no longer detected at 6 weeks (Figure S5). Interestingly, $\gamma\delta$ T cells remained significantly increased in meningitis survivors at both time points (Figure 5a and c). As expected, intracellular cytokine staining confirmed that the $\gamma\delta$ T cells increased in survivors are the IL-17 producers (Figure 5b and d). Altogether, these results show that GBS neonatal meningitis leads to $\gamma\delta$ 17 T cell accumulation in the meningeal microenvironment, persisting throughout life.

$\gamma\delta$ T cells drive GBS brain invasion in neonatal meningitis

To comprehend the contribution of $\gamma\delta$ T cells in host protection against neonatal meningitis, we next performed infectious studies in wild-type (WT) and $\gamma\delta$ T cell-deficient ($Tcrd^{-/-}$) pups. Interestingly, no significant differences were observed in the survival rate of infected $Tcrd^{-/-}$ neonates compared to WT pups (Figure 5e). Moreover, there were no significant differences in bacterial load in the lungs or meninges, at P1 and P3 (Figure 5f and g). However, $Tcrd^{-/-}$ pups showed significantly decreased infection in the brain at both time points, indicating that the absence of $\gamma\delta$ T cells protects the brain from bacterial colonisation (Figure 5h). We next hypothesised that infection could regulate meningeal immune cell response. Flow cytometric analysis of immune cell populations in the meninges showed no differences in lymphoid (B cells and T cells) and myeloid (neutrophils, $Ly6C^{hi}$ monocytes and macrophages) populations between infected and uninfected $Tcrd^{-/-}$ pups at P3 (Figure 5i-n). Similarly, immune populations in the spleen did not differ between groups (Figure S7).

As we found that meningeal $\gamma\delta$ T cells are mostly IL-17 producers during neonatal meningitis, we next sought to determine the role of this cytokine in the pathogenesis of GBS infection. To this end, we performed infection studies in mice deficient in IL-17A ($IL-17A^{-/-}$) or WT controls. Surprisingly, no significant differences were observed in the survival of $IL-17A^{-/-}$ infected pups compared to the WT ones (Figure 6a). However, $IL-17A^{-/-}$ pups proved to be protected against GBS colonisation, exhibiting a significant decrease in bacterial burden in all tested organs, namely meninges, brain, lungs and liver, with the majority of $IL-17A^{-/-}$ pups having bacterial levels below detection limits (Figure 6b). Although unlikely, $IL-17A^{-/-}$ progenitors could have increased control of GBS from their vaginal mucosa, leading to decreased vertical transmission. Vaginal lavages performed one day after delivery confirmed comparable vaginal colonisation levels between $IL-17A^{-/-}$ and WT dams (Figure 6c). Collectively, these data suggest that IL-17 contributes to GBS pathogenesis rather than host protection in neonates.

We next asked whether locally targeting meningeal IL-17 could affect the outcomes of

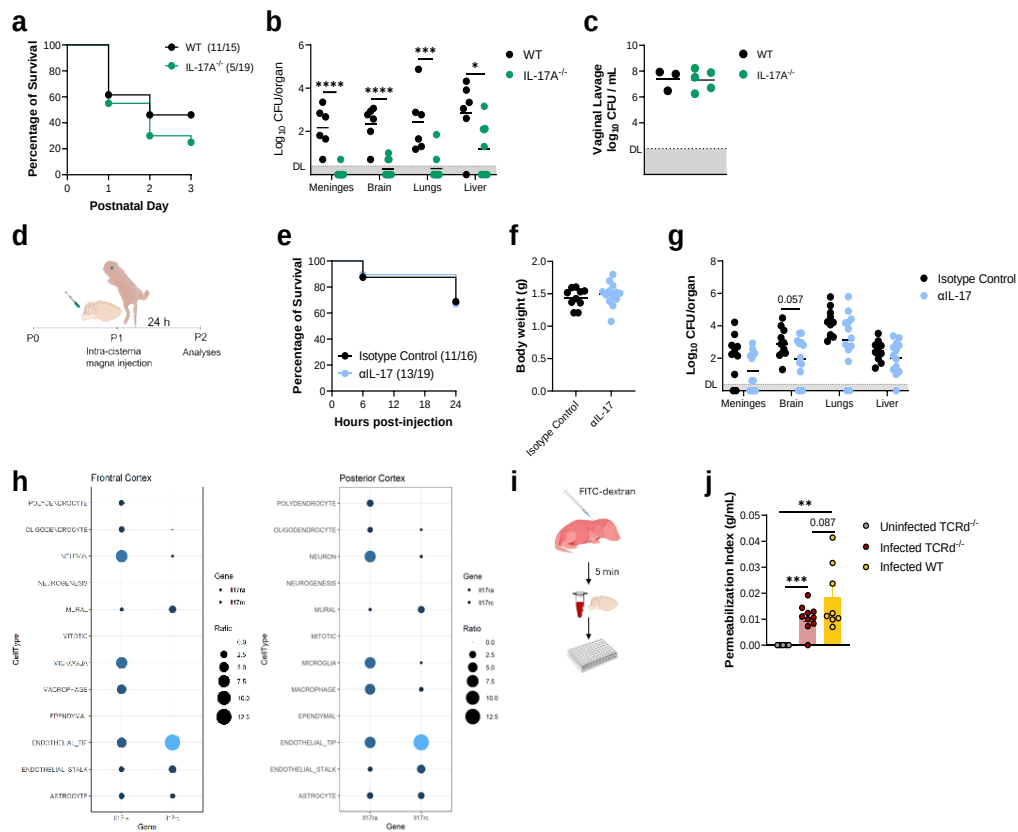


Figure 6. IL-17A increases susceptibility to GBS meningitis through BBB disruption. Pregnant C57BL/6 WT, IL-17A^{-/-} or Tcrd^{-/-} female mice were intravaginally colonised with 3x10⁴ CFU GBS hypervirulent strain BM110, at gestational days 16 and 17. Control animals received PBS. **(a)** Kaplan-Meier survival curve of neonatal mice born from GBS-colonised WT and IL-17A^{-/-} dams, monitored for 3 days. The numbers in parentheses represent the number of animals that survived versus the total number of animals born. **(b)** Bacterial load in meninges, brain, lungs and liver of postnatal (P) 3 WT and IL-17A^{-/-} pups. Each symbol represents one pup (n = 6 WT, n = 9 IL-17A^{-/-}). Students' t-test performed analysis. **(c)** GBS colonisation in vaginal lavage of vaginal colonised WT and IL-17A^{-/-} dams one day after delivery. Each symbol represents one dam (n = 3 WT, n = 4 IL-17A^{-/-}). Comparison by Students' t-test. **(d-g)** Anti-IL-17A or the Isotype IgG control was administrated via intra-cisterna magna to WT P1-infected pups. Animals were analysed 24 hours postinfection (hpi). **(d)** Representative scheme of the αIL-17A administration protocol. **(e)** Kaplan-Meier survival curve of neonatal mice αIL-17A injected and controls, monitored for 24 h. The numbers in parentheses represent the number of animals that survived versus the total number of animals injected. **(f)** Body weight of αIL-17A and Isotype Control injected pups 24 hpi. Each symbol represents one pup (n = 10 Isotype Control, n = 13 αIL-17A). Students' t-test performed analysis. **(g)** Bacterial load in meninges, brain, lungs and liver 24 hpi. Each symbol represents one pup (n = 11 Isotype Control, n = 13 αIL-17A). Students' t-test performed analysis. **(h)** Predicted expression of the IL-17 receptor chains a (Il17ra) and c (Il17rc) in the different cell types, in the frontal (left plot) and posterior (right plot) cortex regions, using previously published single-cell RNA-seq data. The size of each dot represents the average expression of cells expressing each gene. **(i-j)** Postnatal day 2 pups born from were subcutaneously injected with FITC-dextran for BBB permeability evaluation. **(i)** Representative scheme of the blood-brain barrier (BBB) permeability assay. **(j)** Permeability index of the BBB of P1 uninfected Tcrd^{-/-} and infected WT and Tcrd^{-/-} pups. Each symbol represents one pup (n = 5 Uninfected, n = 10 Infected Tcrd^{-/-}, n = 8 Infected WT). Students' t-test performed analysis. Statistical differences (P values) between groups are indicated; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. DL, detection limit.

bacterial meningitis. Since IL-17A^{-/-} mice are born without this cytokine in any tissue, this model is inadequate for determining the functional significance of meningeal IL-17. To circumvent this limitation, pups born from colonised progenitors were injected with anti-IL-17 monoclonal antibodies (α IL-17), or the respective IgG isotype control, directly into the CSF (intra-cisterna magna, i.c.m.) at P1 and analysed 24 h later (Figure 6d). Local blocking of IL-17 did not improve neonatal survival (Figure 6e). Compared to isotype control pups, body weight was no different in α IL-17 treated neonates at the time point tested (Figure 6f). However, while bacterial loads did not differ in the meninges or systemic organs (lungs and liver), α IL-17 injection directly into the pups' CSF resulted in decreased bacterial colonisation in the brain ($P=0.057$) (Figure 6g), indicating localised protection in the absence of this cytokine.

Altogether, our data suggest that $\gamma\delta 17$ T cells play a key role in facilitating bacterial colonisation of the brain during neonatal meningitis. To better understand how $\gamma\delta 17$ T cells were mechanistically mediating this, we next investigated the distribution of IL-17 receptor (IL-17R, both IL-17Ra and IL-17Rc), using an available single-cell sequencing dataset³⁹. IL-17R was expressed on several cell types, across different brain regions. Nevertheless, in regions proximal to the meninges, such as the cortex, endothelial cells were among the populations with a higher percentage of IL-17R expression (Figure 6h). The remaining analysed brain regions can be observed in the supplementary figure 8. Based on these observations, we hypothesised that the blood-brain barrier (BBB) permeability might be altered in infected Tcrd^{-/-} neonates compared to WT pups. Thus, the integrity of the BBB was assessed *in vivo* at P3 using a FITC-dextran assay (Figure 6i). Uninfected Tcrd^{-/-} neonates were used as controls. Following systemic FITC-dextran injection, fluorescence levels in the blood and the brain were quantified. Both infected WT and Tcrd^{-/-} pups showed significant BBB permeability compared to uninfected Tcrd^{-/-} pups (Figure 6j). However, WT infected neonates also revealed increased permeability relative to infected Tcrd^{-/-} ($P=0.087$), indicating that the absence of $\gamma\delta$ T cells helps maintain BBB integrity. In summary, these findings show that meningeal $\gamma\delta$ T cells are important mediators of neonatal bacterial meningitis, facilitating CNS infection through IL-17 secretion and BBB disruption.

Meningeal $\gamma\delta 17$ T cell accumulation in neonatal meningitis leads to ADHD-like behaviour

Given that infants surviving neonatal meningitis experience permanent NDI and that meningeal immunity influences CNS function^{16,49,50}, we investigated whether $\gamma\delta$ T cells contribute to altered behaviour phenotypes in this model. Behavioural assessments were performed throughout the lactation period by evaluating the acquisition of developmental

milestones and later during late adolescence/early adulthood (P42) using a battery of classical behaviour tests. Both male and female animals were included during the developmental phase to provide a comprehensive understanding of early-life effects across sexes and mimicking natural litter compositions. The developing reflex of audition revealed that infected pups, regardless of the presence of $\gamma\delta$ T cells, anticipate this sense by

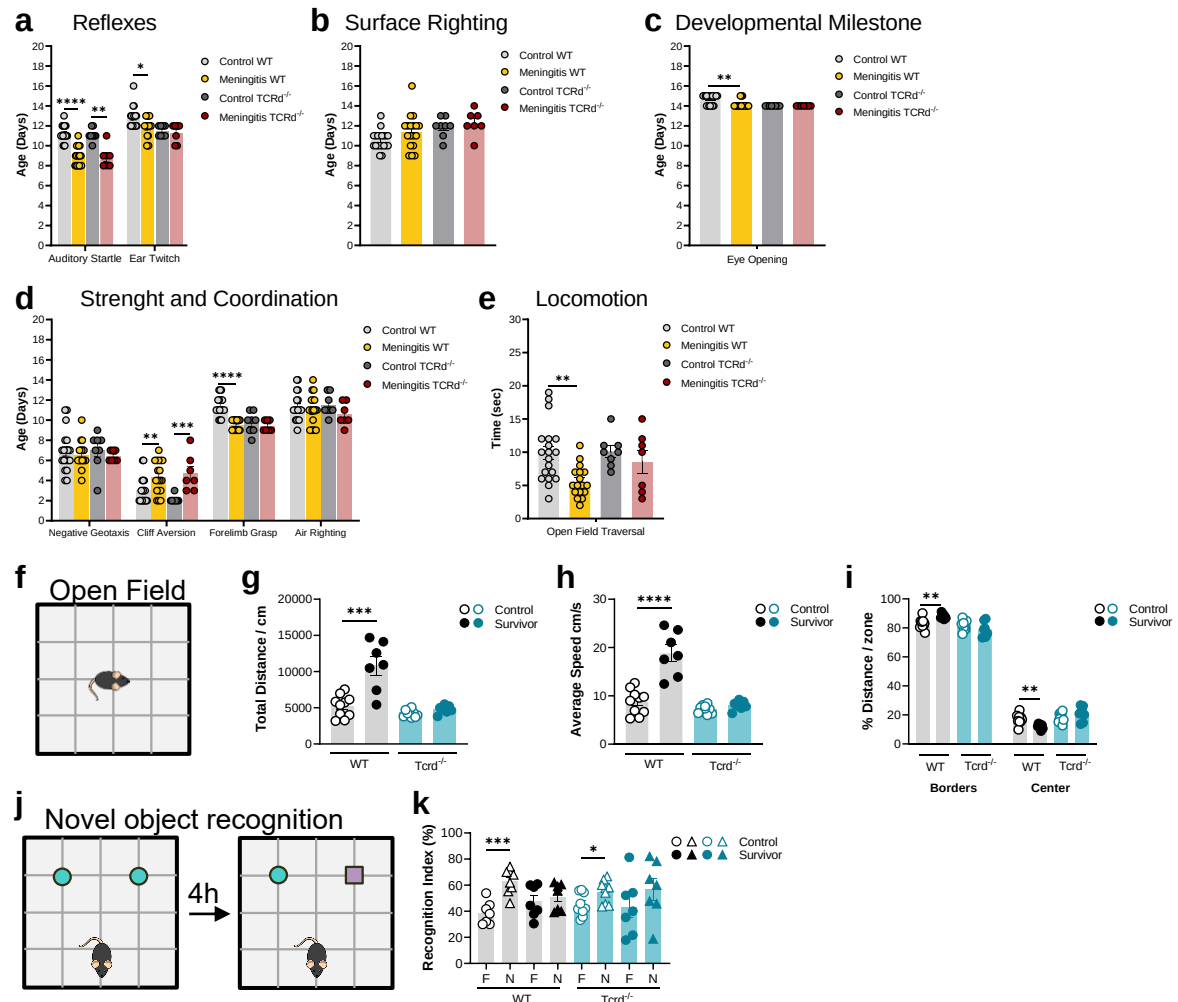


Figure 7. The absence of $\gamma\delta$ T cells restores developmental milestones and behaviour in meningitis survivors. Pregnant C57BL/6 WT or Tcrd^{-/-} female mice were intravaginally colonised with 3×10^4 CFU GBS hypervirulent strain BM110, at gestational days 16 and 17. Control animals received PBS. **(a-e)** Pups born from the indicated groups were tested daily for the first 21 days of life for the indicated reflexes and developmental signs with the tasks determined at specific time points according to milestones. Each symbol indicates data from a single pup ($n = 20$ Control WT, $n = 18$ Infected WT, $n = 8$ Control Tcrd^{-/-}, $n = 7$ Infected Tcrd^{-/-}). Comparisons within genotypes, through Students' t-test. **(f-k)** Male animals born from the indicated groups were allowed to reach adulthood and submitted to behavioural tests. **(f)** Representative scheme of the Open Field test. The total distance covered **(g)**, the average speed **(h)** and the percentage of distance covered by zone **(i)** were used as a measure of hyperactivity and anxious-like behaviour. **(j)** Representative scheme of the novel object recognition test. The recognition index **(k)** was used as a measure of cognitive function. Each symbol represents a single animal ($n = 10$ Control WT, $n = 7$ Survivor WT, $n = 9$ Control Tcrd^{-/-}, $n = 7$ Survivor Tcrd^{-/-}). Error bars indicate mean $\pm$ SEM. Statistical differences (P values) between groups are indicated; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

approximately 2 days compared to their respective controls (Figure 7a). Ear twitch, a reflex involving ear movement in response to touch, showed differences between genotypes (Figure 7a). While infected WT pups presented significantly earlier reflex compared to uninfected control ones, there was no difference between $Tcrd^{-/-}$ groups (Figure 7a). No differences were observed in surface righting among groups (Figure 7b). Eye opening occurred significantly earlier in the WT infected neonates, whereas no differences were found in the $Tcrd^{-/-}$ genotypes, similarly to the ear twitch reflex results (Figure 7c). Regarding strength and coordination, both infected WT and $Tcrd^{-/-}$ neonates presented delayed cliff aversion reflex (Figure 7d). Anterior member strength was assessed through the forelimb grasp test, revealing that infected WT animals completed the test 1.5 days earlier than the control group, similarly to what was observed in the $Tcrd^{-/-}$ groups (Figure 7d). No differences were found in air righting among groups (Figure 7d). Interestingly, locomotion assessment revealed that infected WT pups were able to complete the open field test in 5 seconds, while control pups doubled the time needed (Figure 7e), suggesting hyperactivity in the meningitis group. In the $Tcrd^{-/-}$ genotype, no differences were found between groups (Figure 7e).

Given the known sex differences in adult behaviour and physiology⁵¹, males and females were assessed separately during adulthood (Figure 7, S9 and S10). Surviving WT female mice showed no altered behavioural phenotype, including locomotion, anxious behaviour and cognition (Figure S7). However, surviving WT male mice exhibited significantly increased total distance travelled and higher average speed in the open field (OF) test, indicating hyperactivity, a typical ADHD-like behaviour (Figure 7f-i). In addition, these animals also showed signs of anxiety, as the distance they covered was significantly higher in the borders of the OF arena and, concomitantly, significantly lower at the centre, while showing no differences in the time spent/per zone (Figure 7i and Figure S10e). Notably, no differences were found in the absence of $\gamma\delta$ T cells, with surviving $Tcrd^{-/-}$ behaving as controls (Figure 7f-i). When assessing cognition, through the novel object recognition test, survivors from both genotypes failed to recognise the novel object as being new, unlike their respective control, showing impaired cognition regardless of $\gamma\delta$ T cells' presence (Figure 7j and k, S10f and g). No differences were found between groups and genotypes in the elevated plus maze test and sociability assessment (Figure S10a-d, h and i). As prenatal immune challenges, such as maternal immune activation (MIA), increase the risk for altered behavioural phenotypes in the offspring^{52,53}, we measured the serum levels of several pro- and anti-inflammatory cytokines in GBS-colonised progenitors and healthy controls at E18.5 (Figure S11). Data show a significant decrease in serum levels of IL-1 α in GBS-colonised progenitors (Figure S11). No other differences were observed between groups (Figure S11),

suggesting that behavioural alterations are not driven by MIA. Together, these data indicate a preferential impact of $\gamma\delta$ T cells on ADHD-like behaviours, particularly hyperactivity, in the context of neonatal meningitis.

Neonatal GBS meningitis leads to microglia activation

Our results show that meningeal $\gamma\delta$ T cells facilitate bacterial entry into the CNS during GBS meningitis, contributing to ADHD-like behaviour. We therefore reasoned that an analysis of the meningeal transcriptome during neonatal meningitis in WT pups would provide important information about the mechanism underlying long-term sequelae. Thus, we performed bulk RNA-sequencing (RNA-seq) of the meninges from WT pups with meningitis or healthy controls (Figure 8a). Principal component analysis (PCA) of all expressed genes showed a clear separation between uninfected pups and those with neonatal meningitis (Figure 8b). Differential gene expression analysis identified 569 genes were upregulated in neonatal meningitis compared to healthy pups (Figure 8c). To identify gene sets with similar biological activity with significant changes in transcriptomic levels, we employed a gene set enrichment analysis (GSEA) using mouse hallmark gene sets (Figure 8d and e). Interestingly, all biological processes related to the immune response were found to be significantly downregulated during neonatal meningitis (Figure 8d). In contrast, cellular processes associated with development, differentiation and commitment revealed a significant increase in pathways associated with glia and neurons (Figure 8e). Genes having the highest variation within the top 7 up-regulated pathways are highlighted in Figure 8f. Given the observed altered neuron and glia signature, we quantified their number and assessed their reactivity in several relevant brain regions, namely the prefrontal cortex, somatosensory cortex and hippocampus, in male offspring at P3 by confocal microscopy. In neuronal quantification, using the NeuN marker, statistical difference was observed in the hippocampus, with meningitis pups presenting decreased mean intensity of NeuN, indicating decreased neuronal number (Figure 9 a and b). Microglia quantification showed no differences in their number in all tested brain regions (Figure 9c). We subsequently analysed the morphology of microglia, as this is known to occur in response to stimulation⁵⁴. Imaris semi-automatic 3D reconstructions of microglia revealed no differences in microglia surface area and volume between groups (Figure 9a, d and e). However, microglia phagocytic activity, as determined by CD68 immunostaining, was significantly increased in the hippocampal microglia, with a similar trend observed in the somatosensory cortex ($P=0.052$) (Figure 9f and g). For a deeper characterisation of the microglia status, we next performed flow cytometry analyses in the whole brain at P3 (Figure 9h and i). While the

frequency of microglia was significantly increased in pups with meningitis, their number was statistically decreased, consistent with the decreased brain weight observed in this group at this time point (Figure 9h and 2c). Imaging data were corroborated by a significant increase in SSC-A mean fluorescence intensity (MFI), indicative of higher intracellular

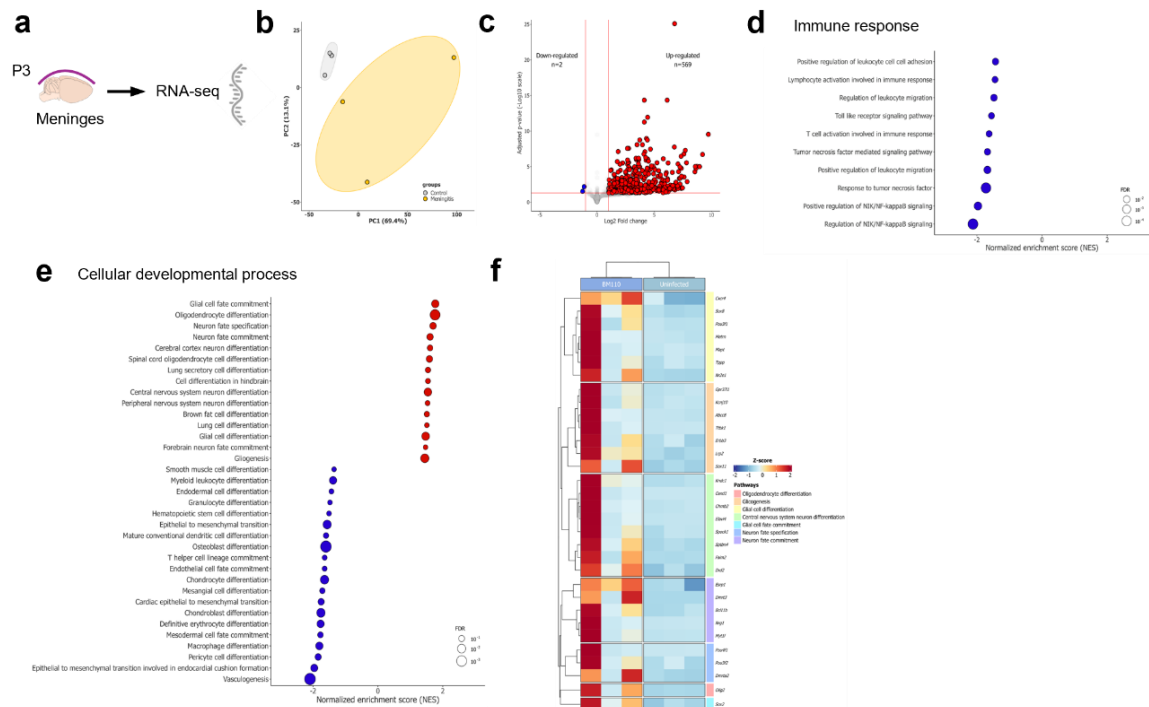


Figure 8. Meningeal RNA-seq analysis reveals altered cellular development in neonatal meningitis.

Pregnant C57BL/6 female mice were intravaginally inoculated with 3×10^4 CFU GBS hypervirulent strain BM110 at gestational days 16 and 17. Control animals received PBS. **(a)** The meninges were isolated from the brain of male pups with meningitis and uninfected controls at postnatal day (P) 3, and total RNA was extracted and sequenced. **(b)** PCA plot showing segregation between uninfected controls (grey) and neonatal meningitis (yellow) meningeal transcriptomes. **(c)** Volcano plot depicting the gene expression differences in the meninges of pups with meningitis compared to uninfected controls. Red dots represent genes expressed at higher levels in neonatal meningitis, while blue dots represent genes with higher expression levels in healthy controls. Grey dots represent genes below the cutoff of significant ($|\log_2 \text{fold change}| \geq 0.5$ and adjusted P values ≤ 0.05). Differential expression was evaluated using the Wald's test, followed by adjustment for multiple testing with the Benjamini-Hochberg correction. Log2-fold change values were shrunk with the apegI method to increase the signal-over-noise ratio of the effect size. **(d-e)** Dot plot of gene set enrichment analysis obtained by functional enrichment of differentially expressed genes in the meninges during neonatal meningitis. The diameter of the dot indicates the degree of significance of the ontology term. Red dots represent terms enriched in pups with meningitis, while blue dots represent terms enriched in healthy mice. **(f)** Heat maps depicting the relative expression values (z-score of each gene across samples) of the top seven gene pathways associated with cellular development. Only differentially expressed genes were represented, excluding genes belonging to more than one pathway. Individual RNA samples result from meninges pooled from 2 pups over 3 independent experiments (n = 3 per group).

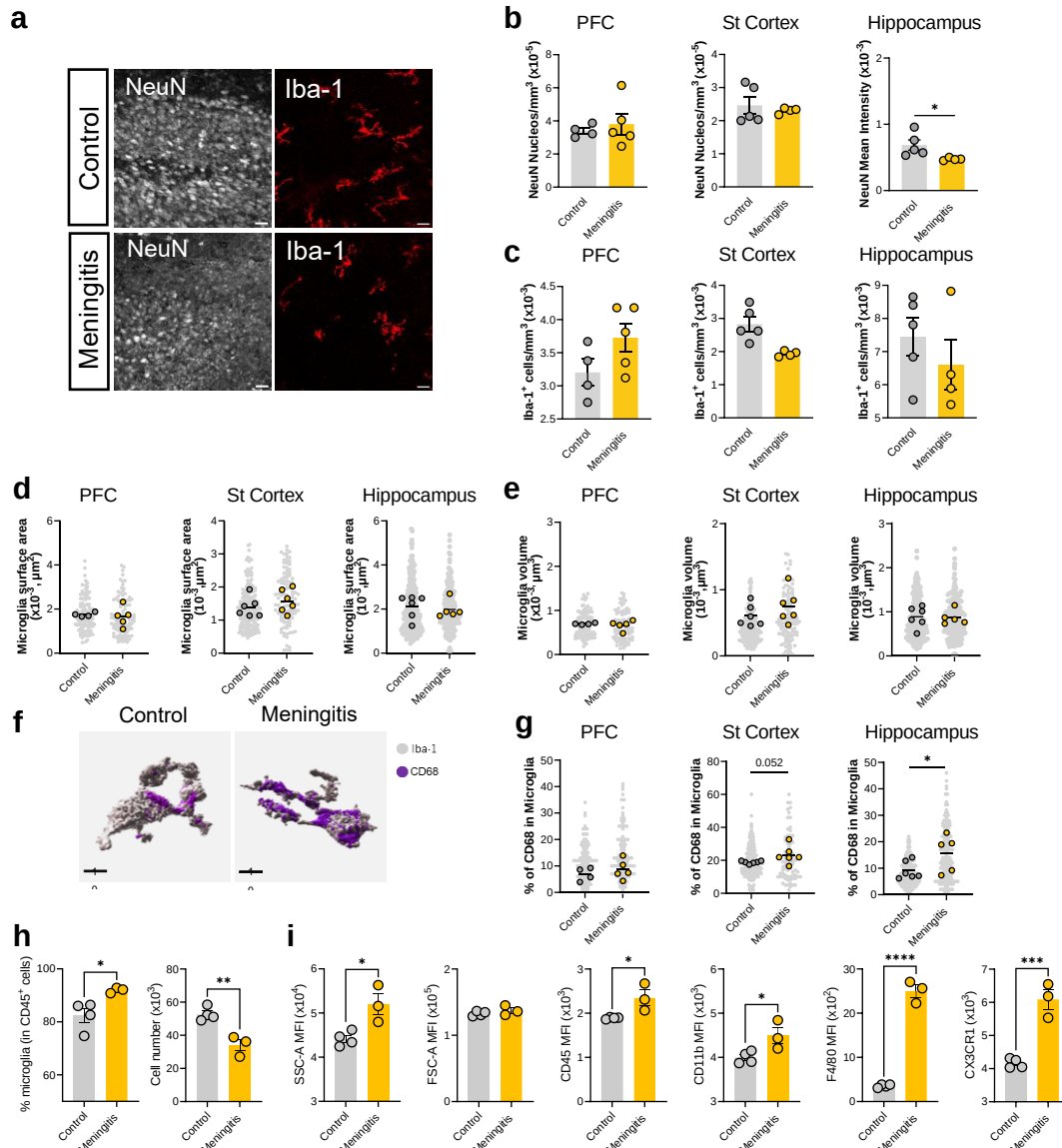


Figure 9. Neonatal GBS infection leads to increased microglial activation. Pregnant C57BL/6 female mice were intravaginally inoculated with 3×10^4 CFU GBS hypervirulent strain BM110 at gestational days 16 and 17. Control animals received PBS. The male progeny was sacrificed at postnatal day (P) 3. **(a)** Representative images of NeuN and Iba1 in the cortex of P3 pups from both groups. Scale bar 20 μm . **(b)** Quantification of NeuN cells in the brain's prefrontal cortex (PFC), somatosensory cortex (St cortex) and hippocampus. Each symbol represents one animal ($n = 4-5$). Error bars indicate mean $\pm$ SEM. **(c)** Quantification of Iba-1 cells in the brain's prefrontal cortex (PFC), somatosensory cortex (St cortex) and hippocampus. Each symbol represents one animal ($n = 4-6$). Error bars indicate mean $\pm$ SEM. Comparisons by Students' t-test. Microglia surface area **(d)** and volume **(e)** at the indicated brain regions. **(f-g)** Quantitative analysis of immunostaining for CD68 inside microglia in the indicated brain regions. **(f)** Representative Imaris 3D reconstruction of microglia labelled with Iba-1, from the cortex of the indicated groups. Lysosomes (CD68, purple) can be visualised inside microglia and quantified. **(g)** Quantification of CD68 in the indicated brain regions. Each coloured symbol represents one animal ($n = 4-6$) and each background grey symbol represents one cell ($n = 115-257$ Control, $n = 106-175$ Meningitis). Comparison by Nested t-test. **(h)** Frequency and number of microglia by flow cytometry. **(i)** Quantification of SSC-A, FSC-A, CD45, CD11b, F4/80 and CX3CR1 on microglia, presented as median fluorescence intensity (MFI). Each symbol represents one animal ($n = 4$ Control, $n = 3$ Meningitis). Error bars indicate mean $\pm$

SEM. Comparisons by Students' t-test. Statistical differences (P values) between groups are indicated. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

granularity and activity (Figure 9i). Moreover, all the other activation markers studied, including CD45, CD11b, F4/80 and CX3CR1, were significantly increased in the microglia of pups suffering from meningitis, confirming their activation status as observed through CD68 confocal microscopy (Figure 9g and i). These results align with the active infection status in the parenchyma of these pups (Figure 1h). Confocal microscopy of adult brains showed no differences in microglia numbers and morphology, as well as no differences in neuronal quantification (Figure S12). Altogether, our data show that neonatal microglia are overall decreased in number during GBS meningitis, but are highly activated, consistent with the infection status of the animals.

Meningeal $\gamma\delta$ 17 T cell accumulation is associated with GBS virulence

Given that the absence of $\gamma\delta$ T cells results in decreased bacterial load in the brain and diminished long-term sequelae, we next wondered whether the microglia status from *Tcrd*^{-/-} pups might be altered. Flow cytometric analyses revealed no evidence of overall microglia activation in infected *Tcrd*^{-/-} pups compared to uninfected controls at P3 (Figure 10a). These findings suggest that $\gamma\delta$ T cell activation and subsequent GBS invasion into the CNS may be linked to microglia reactivity. To test this hypothesis, we next performed infection studies in WT pups using bacterial strains less associated with meningitis, namely the serotype III NEM316 (non-CC17), which does not belong to hypervirulent clonal complex 17, and the attenuated CC17 Δ cylE strain lacking haemolytic/cytolytic activities. Maternal colonisation with either strain resulted in high neonatal survival rates (Figure 10b). In accordance, both attenuated strains exhibited reduced bacterial burden in the brain compared to pups infected with the hypervirulent CC17 strain, reaching significance in the non-CC17 group (Figure 10c). We next assessed meningeal $\gamma\delta$ T cells to determine whether their accumulation was affected by infection with less virulent strains. At P3, no differences were observed between the experimental groups (Figure 10d). We then examined whether pups infected with a less virulent strain exhibited reduced microglial reactivity. Flow cytometric analyses showed no alterations in microglia numbers or the expression of activation markers in pups infected with the non-CC17 strain (Figure 10e). Finally, to further explore whether the presence of bacteria is sufficient to activate microglia, primary microglia cultures were stimulated with hkGBS CC17 for 24 h. The results show that while hkGBS stimulation did not lead to increased expression of the classical microglial activation marker iNOS, it significantly decreased the phagocytic capacity of microglia (Figure 10f). Additionally, hkGBS-stimulated microglia displayed a significant increase in cell area

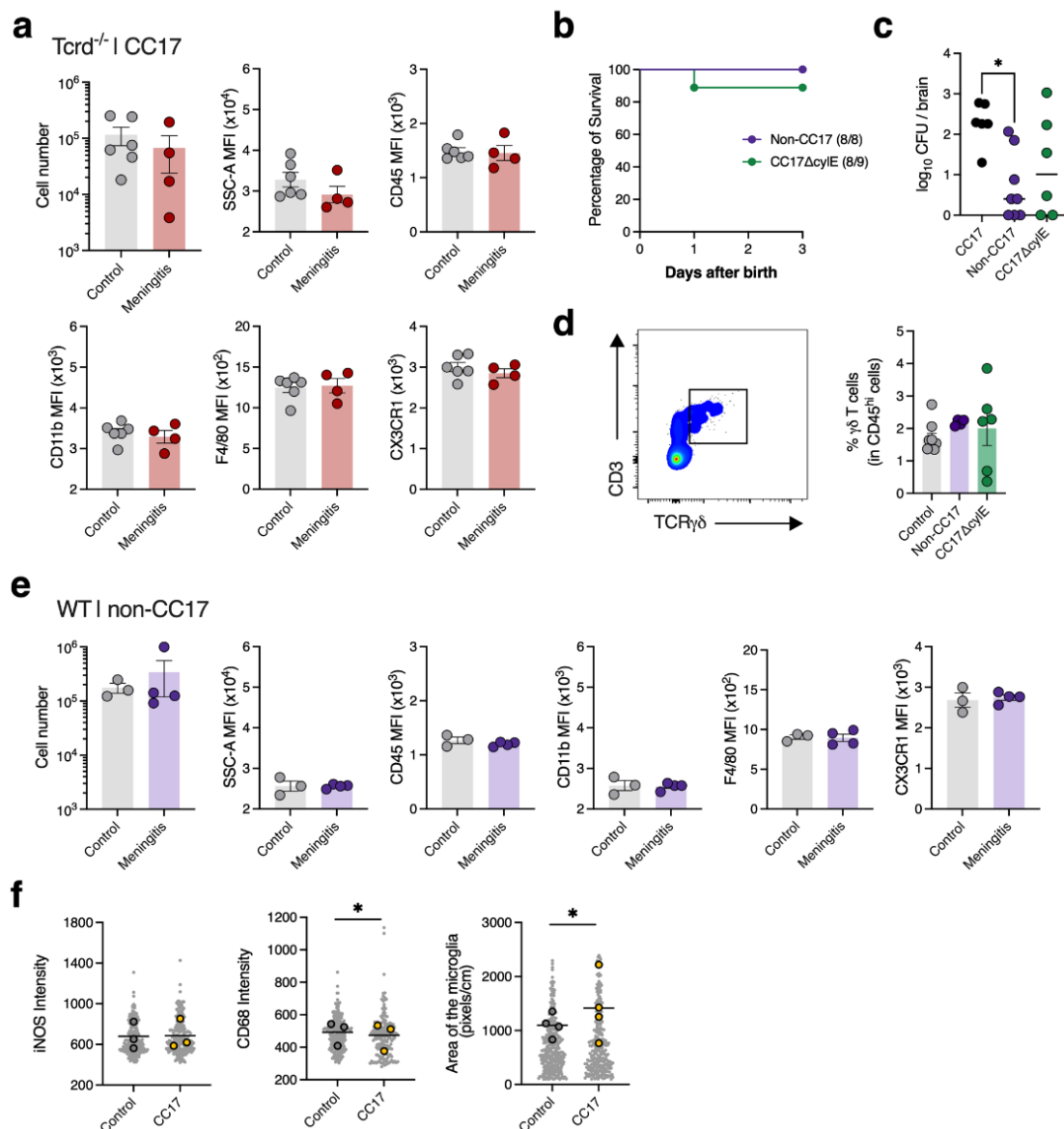


Figure 10. Microglia activation is associated with brain parenchyma invasion. Pregnant C57BL/6 WT or Tcrd^{-/-} female mice were intravaginally inoculated with 3x10⁴ CFU of the indicated GBS strain at gestational days 16 and 17. Control animals received PBS. The male progeny was sacrificed at postnatal day (P) 3. **(a)** Number of microglia by flow cytometry and quantification of SSC-A, CD45, CD11b, F4/80 and CX3CR1 on microglia, presented as median fluorescence intensity (MFI). Each symbol represents one animal (n = 6 Control, n = 4 Meningitis). Comparisons by Students' t-test. **(b)** Kaplan-Meier survival curve of neonatal mice born from Non-CC17 and CC17ΔcylE GBS-colonised WT, monitored for 3 days. The numbers in parentheses represent the number of animals that survived versus the total number of animals born. **(c)** Bacterial load in the brain of P3 Non-CC17 and CC17ΔcylE GBS infected pups. Each symbol represents one pup (n = 6-8). Comparisons by One-way ANOVA. **(d)** Representative flow cytometry scheme showing the gate on γδ T cells (left) and the frequency of this population in Uninfected, Non-CC17 infected and CC17ΔcylE infected at P3. Each symbol represents one pup (n = 9 Uninfected, n = 4 Non-CC17, n = 6 CC17ΔcylE). Error bars indicate mean ± SEM. Comparisons by One-way ANOVA. **(e)** Number of microglia by flow cytometry and quantification of SSC-A, CD45, CD11b, F4/80 and CX3CR1 on microglia, presented as median

fluorescence intensity (MFI). Each symbol represents one animal (n = 3 Control, n = 4 Meningitis). Comparisons by Students' t-test. **(f)** Primary microglial cultures were stimulated with media (Control) or hkGBS (CC17) at a multiplicity of infection of 10 for 24 h. iNOS (left) and CD68 (middle) fluorescence intensity analysed by immunohistochemistry, and microglia area (right). Data presented as mean. Each coloured symbol represents one culture (n = 3) and each background grey symbol represents one cell (n = 216-330 Control, n = 183-280 CC17). Comparisons by nested t-test.

compared to control, indicative of activation (Figure 10f). Altogether, these results suggest that $\gamma\delta$ T cells contribute to GBS virulence and CNS invasion by modulating microglial reactivity, highlighting their potential role in disease pathophysiology.

Discussion

GBS meningitis is the most common neonatal bacterial meningitis, with a significant burden even in the most advanced medical and technological era⁵⁵. However, the mechanisms leading to neurological sequelae are poorly understood. A comprehensive understanding of GBS meningitis pathology is urgently needed, using relevant preclinical models. Here, we used our clinically relevant mouse model that resembles the vertical bacterial transmission, adapted to the C57Bl/6 mice. We uncover a novel mechanism for NDI in GBS meningitis by demonstrating that bacterial stimuli drive lifelong accumulation of $\gamma\delta 17$ T cells in the meninges. This process disrupts BBB integrity and activates microglia leading to ADHD-like behaviour phenotypes in mice.

When evaluating infection kinetics and symptom onset, infected pups presented an expected significantly decreased body weight during the first five days of life. Clinical scoring, used to assess disease severity and predict mortality, revealed nonutility in GBS meningitis. Except in cases of extreme severity, where there are obvious visual signs of disease, predicting mortality was not possible, as animals died even at minimal clinical scoring values. This finding further validates the clinical relevance of our mouse model, given that human symptoms are non-specific or absent in over 30% of cases^{56,57}. Bacterial CFU in the brain revealed a high level of colonisation as early as one day after birth, with 80% of the pups colonised at this age. Moreover, and similar to the unpredictable mortality, the burden of GBS in the brain was not the criterion for clinical scoring level. While brain colonisation is essential for disease, its severity is not directly proportional to bacteria load in this organ.

The meninges contain a dense network of immune cells that intimately interact with the CNS, playing crucial roles in (patho)physiology^{50,58}. Despite this, the role of meningeal immunity in early life has been overlooked. Single-cell RNA-seq revealed for the first time,

to our knowledge, a vast repertoire of immune cells in this tissue already at P3, very similar to the adults' immune profile. Subsequent flow cytometric analysis showed a significant increase in $\gamma\delta$ T cell frequency in the meninges of infected pups at P3. Importantly, the frequency of these cells was independent of brain bacterial load, showing that the presence of bacteria, rather than the quantity, triggers meningeal immunity. Further characterisation confirmed the classical meningeal $\gamma\delta$ T cell phenotype^{26,29}, including TCR V γ 6, tissue-residency phenotype and bias towards IL-17 production. GSEA analyses showed that inflammation-related pathways and response to external stimuli were significantly activated in the pups suffering from meningitis. Interestingly, while overall IL-17 levels were elevated in the meningeal microenvironment, transcriptional regulation of IL-17 in $\gamma\delta$ 17 cells appeared altered during meningitis. This aligns with previously published work showing that commensal-derived signals regulate meningeal $\gamma\delta$ 17 T cells²⁹. Given that GBS colonises the neonatal gut^{16,59}, it is plausible that the gut-meningeal axis could be modulating meningeal $\gamma\delta$ 17 T cells during infection.

Although we were unable to detect IL-17 in the CSF of pups with bacterial meningitis, this is likely due to the limited quantity of CSF volume in neonatal mice. Nevertheless, it cannot be assumed that it is not increased in these animals as cytokines may work at very low levels, undetectable with the current available technology. Our data suggests translational potential for human neonates, as $\gamma\delta$ 17 T cells are among the earliest T cells generated in the foetal human thymus⁶⁰ and have been shown to produce IL-17 in children with bacterial meningitis⁶¹. Importantly, the number of $\gamma\delta$ 17 cells in these children decreases after treatment, suggesting a pathogen-specific response⁶¹, as we also find in our study with $\gamma\delta$ T cells directly responding to GBS. Studies with cord blood cells also show that human neonatal $\gamma\delta$ T cells can produce IL-17 upon polyclonal activation⁶². Additionally, $\gamma\delta$ T cells have already been detected in extremely premature babies and the brain lesions of premature infants with white matter injury^{63,64}.

As every system always works to restore homeostasis, regardless of the situation, we studied older animals to observe when the difference in $\gamma\delta$ T cells disappear. Surprisingly, IL-17-producing $\gamma\delta$ T cells remained significantly elevated, suggesting either increased proliferation or recruitment to the meninges. In steady state, $\gamma\delta$ T cells home to the tissues in the perinatal tissues where they proliferate and self-renew throughout life⁴⁶. Although we did not find differences in the proliferation, the inherently high proliferative capacity of $\gamma\delta$ T cells in steady-state conditions may hide subtle changes. Alternatively, $\gamma\delta$ T cell recruitment to the meninges cannot be discarded. In fact, splenic $\gamma\delta$ T cells were also found to be increased in frequency at P3.

While $\gamma\delta$ T cells are typically associated with pathogen clearance and tissue repair⁶⁵, no differences in the survival of WT and Tcrd^{-/-} pups were observed. Even more striking, although the same colonisation was observed in the lungs and meninges of infected WT and Tcrd^{-/-} pups on the first three days of life, the absence of $\gamma\delta$ T cells massively decreased the number of GBS invading the parenchyma, suggesting a local pathogenic role of this cell population. Meningeal immunity was not affected in the Tcrd^{-/-} pups showing no compensatory mechanisms. IL-17-deficient pups showed decreased bacterial colonisation across all tested organs, demonstrating that IL-17, while protective in other contexts, exacerbates disease in neonatal GBS meningitis. IL-17-mediated responses are normally associated with increased protection against extracellular bacteria, mainly due to the link between IL-17 signalling and neutrophil recruitment⁶⁶. Injection of anti-IL-17 antibodies into the cisterna magna led to decreased bacterial colonisation in the brain, further implicating local release of IL-17 in BBB modulation. These results prove that IL-17, either locally or systemically, despite being literature-established as a critical player against infection, is detrimental in neonatal GBS meningitis. Indeed, we found that Tcrd^{-/-} pups presented decreased BBB permeability. Although a degree of breakdown was also found in these animals, this can be explained by the fact that there are other cells in the meninges producing IL-17, such as type 3 innate lymphoid cells (ILC3), that could add in BBB opening. Moreover, GBS is equipped with several virulence factors likely facilitating brain invasion independently of BBB leakage^{9,67–69}. In agreement with this hypothesis, pups deficient in $\gamma\delta$ T cells presented some degree of bacterial colonisation. $\gamma\delta 17$ T cells have already been implicated in other neurological diseases, including experimental autoimmune encephalomyelitis (EAE) and stroke⁷⁰. Unlike these conditions, where IL-17-driven neutrophil recruitment destroyed the BBB^{71–76}, our findings suggest that meningeal $\gamma\delta 17$ T cells disrupt BBB integrity via direct IL-17 secretion, proving the importance of inter-tissue communication, specifically the role of meningeal immunity in bacterial meningitis. Furthermore, we showed that microglia are directly activated by GBS *in vitro* and that activation is dependent on the GBS tropism to the CNS, as the attenuated GBS strains presenting lower brain colonisation did not have microglia activation. This suggests a reasonable mechanism between the increased meningeal $\gamma\delta 17$ T cells and IL-17, BBB disruption and later microglia activation.

Meningeal $\gamma\delta 17$ T cells have been recently identified as key regulators of neuronal signalling, behaviour, and cognition in both homeostatic and pathological states^{26,29,75,77,78}. In our study, the developmental milestones related to the senses were achieved earlier in WT infected pups. A rat model of maternal separation showed an acceleration of the eye opening⁷⁹, demonstrating that, similarly to our case, early-life stress may trigger sensory

development acceleration. Another study showed that acoustic startle response was exacerbated in adult rats when they were exposed to infection in the neonatal period⁸⁰. Although only testing in adults, they showed that this exacerbated response was only present when adults were first exposed to stress, before the acoustic startle⁸⁰. This is in agreement with our findings, as infected pups presented anticipated auditory startle response. Accelerated sensory development may represent an early ADHD-like trait, as hypersensitivity is commonly associated with this condition⁸¹. Regarding locomotion, WT pups performed the open field test in a significantly reduced time compared to the controls, suggesting hyperactivity. When reaching early adulthood, both WT and *Tcrd*^{-/-} male survivors presented cognitive impairment. The mechanism behind this result is, therefore, unrelated to the $\gamma\delta$ T cells and unrelated with high bacterial burden in the brain. Further studies are needed to address the mechanism behind learning deficits in GBS meningitis. In the open field arena, the WT survivors showed hyperactivity behaviour. Notably, this phenotype was abrogated in the absence of $\gamma\delta$ T cells. In addition to increased travelled distance, WT survivors covered a higher distance in the borders of the arena than the control group. However, they did not spend more time in this area. This means that, on top of hyperactivity, these animals suffer from anxiety. This is in agreement with a previous study showing that $\gamma\delta 17$ T cells seed the meningeal spaces and control anxiety-like behaviour in mice²⁹. Hyperactivity and anxiety are, in fact, two conditions commonly interrelated, being present simultaneously in approximately 25% of children diagnosed with ADHD^{82,83}. Moreover, similar results were observed in models of maternal immune activation, showing brain development alterations consistent with ADHD in their offspring, as well as ADHD-like behaviours^{84–86}. ADHD affects 5% of children worldwide, with 30-60% of these individuals maintaining the impairing symptoms in adulthood^{87,88}. Although human perinatal inflammation was already reported to increase the risk of offspring ADHD^{89–91}, there is few data showing a mechanistic correlation between infection during a critical period of development and the disease development. Our data, support a promising role of the meninges in disease states in infants surviving neonatal bacterial meningitis.

By combining scRNA-seq, flow cytometry, imaging, and behavioural assays with a clinically relevant model of neonatal meningitis, we demonstrate a causal link between dysregulation of meningeal $\gamma\delta 17$ T cells, bacterial invasion into the CNS, and subsequent permanent neurological damage, including ADHD-like behaviour. This study uncovers a previously unrecognised mechanism in GBS meningitis and suggests IL-17 as a potential therapeutic target to alleviate the burden of neuropsychiatric morbidities in infants surviving neonatal meningitis.

References

1. Wilson, C. B., Nizet, V., Maldonado, Y., Remington, J. S. & Klein, J. O. *Remington and Klein's Infectious Diseases of the Fetus and Newborn Infant*. (Elsevier Health Sciences, 2015).
2. Gonçalves, B. P. *et al.* Group B streptococcus infection during pregnancy and infancy: estimates of regional and global burden. *Lancet Glob. Health* 10, e807–e819 (2022).
3. Seale, A. C. *et al.* Estimates of the Burden of Group B Streptococcal Disease Worldwide for Pregnant Women, Stillbirths, and Children. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* 65, S200–S219 (2017).
4. Nanduri, S. A. *et al.* Epidemiology of Invasive Early-Onset and Late-Onset Group B Streptococcal Disease in the United States, 2006 to 2015: Multistate Laboratory and Population-Based Surveillance. *JAMA Pediatr.* 173, 224–233 (2019).
5. O'Sullivan, C. P. *et al.* Group B streptococcal disease in UK and Irish infants younger than 90 days, 2014–15: a prospective surveillance study. *Lancet Infect. Dis.* 19, 83–90 (2019).
6. Bekker, V., Bijlsma, M. W., van de Beek, D., Kuijpers, T. W. & van der Ende, A. Incidence of invasive group B streptococcal disease and pathogen genotype distribution in newborn babies in the Netherlands over 25 years: a nationwide surveillance study. *Lancet Infect. Dis.* 14, 1083–1089 (2014).
7. Plainvert, C. *et al.* Multidrug-Resistant Hypervirulent Group B Streptococcus in Neonatal Invasive Infections, France, 2007–2019. *Emerg. Infect. Dis.* 26, 2721–2724 (2020).
8. Verani, J. R., McGee, L., Schrag, S. J., & Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention (CDC). Prevention of perinatal group B streptococcal disease--revised guidelines from CDC, 2010. *MMWR Recomm. Rep. Morb. Mortal. Wkly. Rep. Recomm. Rep.* 59, 1–36 (2010).
9. Doran, K. S., Liu, G. Y. & Nizet, V. Group B streptococcal beta-hemolysin/cytolysin activates neutrophil signaling pathways in brain endothelium and contributes to development of meningitis. *J. Clin. Invest.* 112, 736–744 (2003).
10. Mu, R. *et al.* Identification of a group B streptococcal fibronectin binding protein, SfbA, that contributes to invasion of brain endothelium and development of meningitis. *Infect. Immun.* 82, 2276–2286 (2014).
11. Lentini, G. *et al.* The plasminogen binding protein PbsP is required for brain invasion by hypervirulent CC17 Group B streptococci. *Sci. Rep.* 8, 14322 (2018).

12. Leib, S. L., Kim, Y. S., Chow, L. L., Sheldon, R. A. & Täuber, M. G. Reactive oxygen intermediates contribute to necrotic and apoptotic neuronal injury in an infant rat model of bacterial meningitis due to group B streptococci. *J. Clin. Invest.* 98, 2632–2639 (1996).
13. Larsen, J. W. *et al.* Experimental group B streptococcal infection in the rhesus monkey. I. Disease production in the neonate. *Am. J. Obstet. Gynecol.* 132, 686–690 (1978).
14. Barichello, T. *et al.* Evaluation of the brain-derived neurotrophic factor, nerve growth factor and memory in adult rats survivors of the neonatal meningitis by *Streptococcus agalactiae*. *Brain Res. Bull.* 92, 56–59 (2013).
15. Reiss, A. *et al.* Bacterial pore-forming cytolysins induce neuronal damage in a rat model of neonatal meningitis. *J. Infect. Dis.* 203, 393–400 (2011).
16. Andrade, E. B. *et al.* A mouse model reproducing the pathophysiology of neonatal group B streptococcal infection. *Nat. Commun.* 9, 3138 (2018).
17. Edwards, M. S. *et al.* Long-term sequelae of group B streptococcal meningitis in infants. *J. Pediatr.* 106, 717–722 (1985).
18. Okike, I. O. *et al.* Trends in bacterial, mycobacterial, and fungal meningitis in England and Wales 2004–11: an observational study. *Lancet Infect. Dis.* 14, 301–307 (2014).
19. Heath, P. T., Okike, I. O. & Oeser, C. Neonatal meningitis: can we do better? *Adv. Exp. Med. Biol.* 719, 11–24 (2011).
20. Kohli-Lynch, M. *et al.* Neurodevelopmental Impairment in Children After Group B Streptococcal Disease Worldwide: Systematic Review and Meta-analyses. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* 65, S190–S199 (2017).
21. Rua, R. & McGavern, D. B. Advances in Meningeal Immunity. *Trends Mol Med* 24, 542–559 (2018).
22. Wolf, S. A. *et al.* CD4-positive T lymphocytes provide a neuroimmunological link in the control of adult hippocampal neurogenesis. *J. Immunol. Baltim. Md 1950* 182, 3979–3984 (2009).
23. Radjavi, A., Smirnov, I. & Kipnis, J. Brain antigen-reactive CD4⁺ T cells are sufficient to support learning behavior in mice with limited T cell repertoire. *Brain. Behav. Immun.* 35, 58–63 (2014).
24. Radjavi, A., Smirnov, I., Derecki, N. & Kipnis, J. Dynamics of the meningeal CD4(+) T-cell repertoire are defined by the cervical lymph nodes and facilitate cognitive task performance in mice. *Mol. Psychiatry* 19, 531–533 (2014).
25. Jeon, S. G. *et al.* Impaired Memory in OT-II Transgenic Mice Is Associated with Decreased Adult Hippocampal Neurogenesis Possibly Induced by Alteration in Th2 Cytokine Levels. *Mol. Cells* 39, 603–610 (2016).

26. Ribeiro, M. *et al.* Meningeal $\gamma\delta$ T cell-derived IL-17 controls synaptic plasticity and short-term memory. *Sci. Immunol.* 4, eaay5199 (2019).
27. Derecki, N. C. *et al.* Regulation of learning and memory by meningeal immunity: a key role for IL-4. *J. Exp. Med.* 207, 1067–1080 (2010).
28. Rattazzi, L. *et al.* CD4⁺ but not CD8⁺ T cells revert the impaired emotional behavior of immunocompromised RAG-1-deficient mice. *Transl. Psychiatry* 3, e280 (2013).
29. Alves de Lima, K. *et al.* Meningeal $\gamma\delta$ T cells regulate anxiety-like behavior via IL-17a signaling in neurons. *Nat. Immunol.* 21, 1421–1429 (2020).
30. Filiano, A. J. *et al.* Unexpected role of interferon- γ in regulating neuronal connectivity and social behaviour. *Nature* 535, 425–429 (2016).
31. Dasgupta, K. & Jeong, J. Developmental biology of the meninges. *genesis* 57, e23288 (2019).
32. Stålhammar-Carlemalm, M., Stenberg, L. & Lindahl, G. Protein rib: a novel group B streptococcal cell surface protein that confers protective immunity and is expressed by most strains causing invasive infections. *J. Exp. Med.* 177, 1593–1603 (1993).
33. Forquin, M.-P. *et al.* The putative glycosyltransferase-encoding gene cylJ and the group B Streptococcus (GBS)-specific gene cylK modulate hemolysin production and virulence of GBS. *Infect. Immun.* 75, 2063–2066 (2007).
34. Fehlhaber, B. *et al.* A sensitive scoring system for the longitudinal clinical evaluation and prediction of lethal disease outcomes in newborn mice. *Sci. Rep.* 9, 5919 (2019).
35. Scheyltjens, I. *et al.* Single-cell RNA and protein profiling of immune cells from the mouse brain and its border tissues. *Nat. Protoc.* 17, 2354–2388 (2022).
36. Van Hove, H. *et al.* A single-cell atlas of mouse brain macrophages reveals unique transcriptional identities shaped by ontogeny and tissue environment. *Nat Neurosci* 22, 1021–1035 (2019).
37. Giladi, A. *et al.* Single-cell characterization of haematopoietic progenitors and their trajectories in homeostasis and perturbed haematopoiesis. *Nat. Cell Biol.* 20, 836–846 (2018).
38. Wu, T. *et al.* clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *The Innovation* 2, 100141 (2021).
39. Saunders, A. *et al.* Molecular Diversity and Specializations among the Cells of the Adult Mouse Brain. *Cell* 174, 1015–1030.e16 (2018).
40. Jin, S. *et al.* Inference and analysis of cell-cell communication using CellChat. *Nat. Commun.* 12, 1088 (2021).
41. Vieira, J. R. *et al.* Endothelial PlexinD1 signaling instructs spinal cord vascularization and motor neuron development. *Neuron* 110, 4074–4089.e6 (2022).

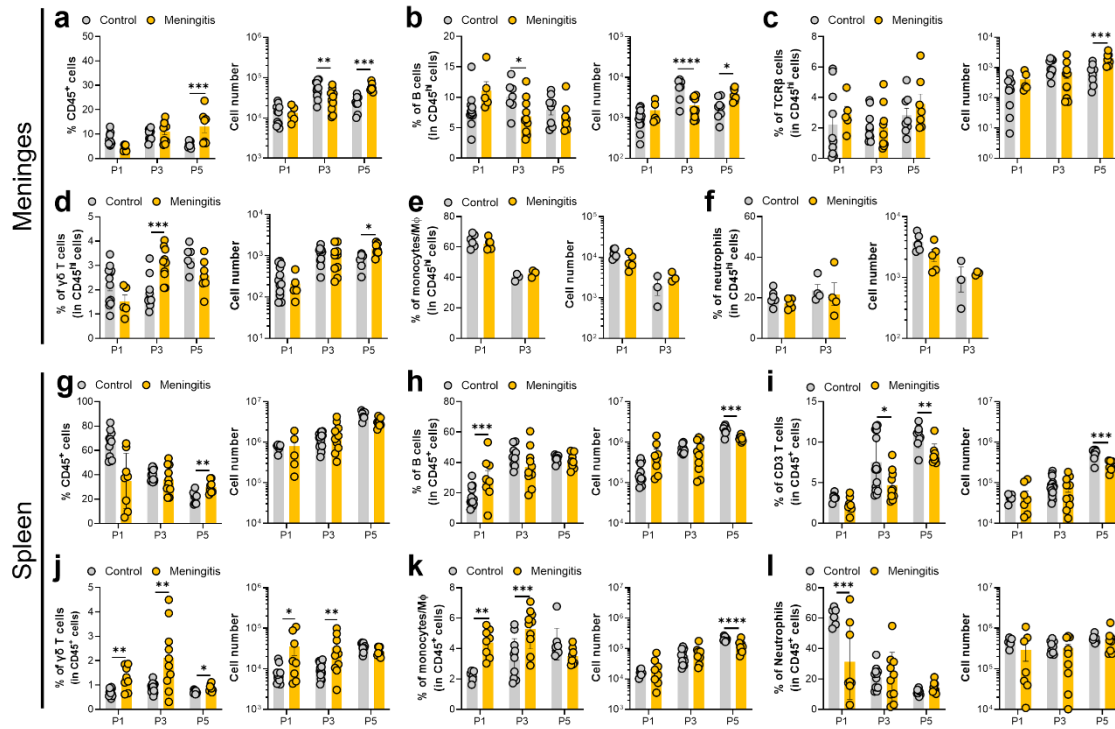
42. Devraj, K., Guérit, S., Macas, J. & Reiss, Y. An In Vivo Blood-brain Barrier Permeability Assay in Mice Using Fluorescently Labeled Tracers. *J. Vis. Exp. JoVE* 57038 (2018) doi:10.3791/57038.
43. Hill, J. M., Lim, M. A. & Stone, M. M. Developmental Milestones in the Newborn Mouse. in *Neuropeptide Techniques* (ed. Gozes, I.) 131–149 (Humana Press, Totowa, NJ, 2008). doi:10.1007/978-1-60327-099-1_10.
44. van Kassel, M. N. *et al.* Sex Differences in Long-term Outcomes After Group B Streptococcal Infections During Infancy in Denmark and the Netherlands: National Cohort Studies of Neurodevelopmental Impairments and Mortality. *Clin. Infect. Dis.* 74, S54–S63 (2022).
45. Turchinovich, G. & Hayday, A. C. Skint-1 identifies a common molecular mechanism for the development of interferon- γ -secreting versus interleukin-17-secreting $\gamma\delta$ T cells. *Immunity* 35, 59–68 (2011).
46. Haas, J. D. *et al.* Development of Interleukin-17-Producing $\gamma\delta$ T Cells Is Restricted to a Functional Embryonic Wave. *Immunity* 37, 48–59 (2012).
47. Papotto, P. H., Yilmaz, B. & Silva-Santos, B. Crosstalk between $\gamma\delta$ T cells and the microbiota. *Nat. Microbiol.* 6, 1110–1117 (2021).
48. Tan, L. *et al.* Single-Cell Transcriptomics Identifies the Adaptation of Scart1+ V γ 6+ T Cells to Skin Residency as Activated Effector Cells. *Cell Rep.* 27, 3657–3671.e4 (2019).
49. Tavares, T., Pinho, L. & Bonifácio Andrade, E. Group B Streptococcal Neonatal Meningitis. *Clin. Microbiol. Rev.* 35, e00079-21 (2022).
50. Rua, R. & McGavern, D. B. Advances in Meningeal Immunity. *Trends Mol. Med.* 24, 542–559 (2018).
51. Szadvári, I., Ostatníková, D. & Babková Durdiaková, J. Sex differences matter: Males and females are equal but not the same. *Physiol. Behav.* 259, 114038 (2023).
52. Pendyala, G. *et al.* Maternal Immune Activation Causes Behavioral Impairments and Altered Cerebellar Cytokine and Synaptic Protein Expression. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* 42, 1435–1446 (2017).
53. You, P. *et al.* Interaction between maternal immune activation and postpartum immune stress in neuropsychiatric phenotypes. *Behav. Brain Res.* 469, 115049 (2024).
54. Nimmerjahn, A., Kirchhoff, F. & Helmchen, F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* 308, 1314–1318 (2005).
55. Thigpen, M. C. *et al.* Bacterial meningitis in the United States, 1998-2007. *N. Engl. J. Med.* 364, 2016–2025 (2011).

56. Wiswell, T. E., Baumgart, S., Gannon, C. M. & Spitzer, A. R. No lumbar puncture in the evaluation for early neonatal sepsis: will meningitis be missed? *Pediatrics* 95, 803–806 (1995).
57. Baud, O. & Aujard, Y. Neonatal bacterial meningitis. *Handb. Clin. Neurol.* 112, 1109–1113 (2013).
58. Su, Y. *et al.* Meningeal immunity and neurological diseases: new approaches, new insights. *J. Neuroinflammation* 20, 125 (2023).
59. Hansen, S. M., Uldbjerg, N., Kilian, M. & Sørensen, U. B. S. Dynamics of *Streptococcus agalactiae* colonization in women during and after pregnancy and in their infants. *J. Clin. Microbiol.* 42, 83–89 (2004).
60. Tan, L. *et al.* A fetal wave of human type 3 effector $\gamma\delta$ cells with restricted TCR diversity persists into adulthood. *Sci. Immunol.* 6, eabf0125 (2021).
61. Caccamo, N. *et al.* Differentiation, phenotype, and function of interleukin-17-producing human V γ 9V δ 2 T cells. *Blood* 118, 129–138 (2011).
62. Ness-Schwickerath, K. J., Jin, C. & Morita, C. T. Cytokine Requirements for the Differentiation and Expansion of IL-17A- and IL-22-producing Human V γ 2V δ 2 T Cells. *J. Immunol. Baltim. Md 1950* 184, 7268–7280 (2010).
63. Albertsson, A.-M. *et al.* $\gamma\delta$ T Cells Contribute to Injury in the Developing Brain. *Am J Pathol* 188, 757–767 (2018).
64. Seki, D. *et al.* Aberrant gut-microbiota-immune-brain axis development in premature neonates with brain damage. *Cell Host Microbe* 29, 1558-1572.e6 (2021).
65. Vantourout, P. & Hayday, A. Six-of-the-best: unique contributions of $\gamma\delta$ T cells to immunology. *Nat. Rev. Immunol.* 13, 88–100 (2013).
66. Mills, K. H. G. IL-17 and IL-17-producing cells in protection versus pathology. *Nat. Rev. Immunol.* 23, 38–54 (2023).
67. Luo, S. *et al.* Quantitative assessment of the blood-brain barrier opening caused by *Streptococcus agalactiae* hyaluronidase in a BALB/c mouse model. *Sci. Rep.* 7, 13529 (2017).
68. Al-Obaidi, M. M. J. & Desa, M. N. M. Mechanisms of Blood Brain Barrier Disruption by Different Types of Bacteria, and Bacterial–Host Interactions Facilitate the Bacterial Pathogen Invading the Brain. *Cell. Mol. Neurobiol.* 38, 1349–1368 (2018).
69. Travier, L. *et al.* Neonatal susceptibility to meningitis results from the immaturity of epithelial barriers and gut microbiota. *Cell Rep.* 35, (2021).
70. Li, Y., Zhang, Y. & Zeng, X. $\gamma\delta$ T Cells Participating in Nervous Systems: A Story of Jekyll and Hyde. *Front. Immunol.* 12, 656097 (2021).
71. Setiadi, A. F. *et al.* IL-17A is associated with the breakdown of the blood-brain barrier in relapsing-remitting multiple sclerosis. *J. Neuroimmunol.* 332, 147–154 (2019).

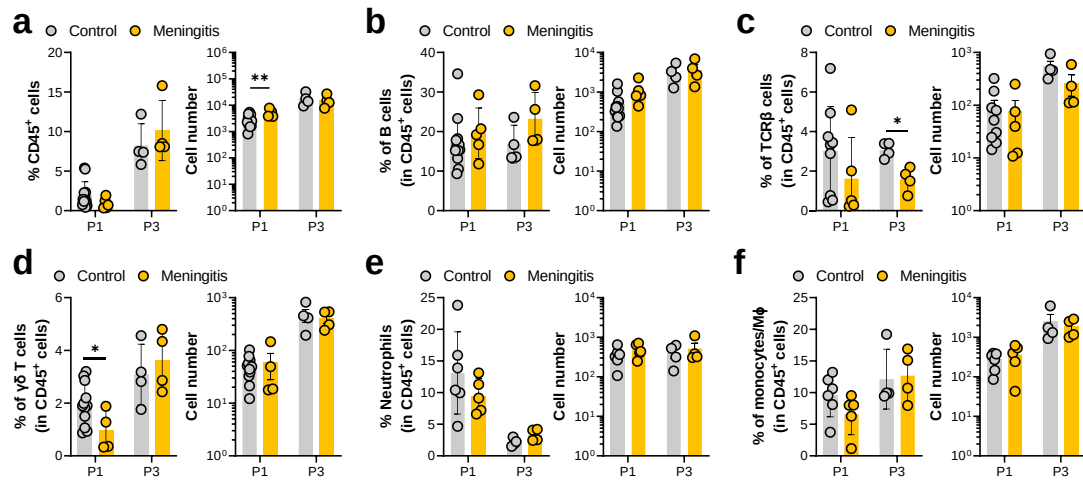
72. Huppert, J. *et al.* Cellular mechanisms of IL-17-induced blood-brain barrier disruption. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* 24, 1023–1034 (2010).
73. Zimmermann, J. *et al.* CNS-targeted production of IL-17A induces glial activation, microvascular pathology and enhances the neuroinflammatory response to systemic endotoxemia. *PLoS One* 8, e57307 (2013).
74. Kroenke, M. A., Carlson, T. J., Andjelkovic, A. V. & Segal, B. M. IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. *J. Exp. Med.* 205, 1535–1541 (2008).
75. Gelderblom, M. *et al.* Neutralization of the IL-17 axis diminishes neutrophil invasion and protects from ischemic stroke. *Blood* 120, 3793–3802 (2012).
76. Arunachalam, P. *et al.* CCR6 (CC Chemokine Receptor 6) Is Essential for the Migration of Detrimental Natural Interleukin-17-Producing $\gamma\delta$ T Cells in Stroke. *Stroke* 48, 1957–1965 (2017).
77. Brigas, H. C. *et al.* IL-17 triggers the onset of cognitive and synaptic deficits in early stages of Alzheimer's disease. *Cell Rep.* 36, 109574 (2021).
78. Benakis, C. *et al.* Commensal microbiota affects ischemic stroke outcome by regulating intestinal $\gamma\delta$ T cells. *Nat. Med.* 22, 516–523 (2016).
79. Mesquita, A. R. *et al.* Neurodevelopment milestone abnormalities in rats exposed to stress in early life. *Neuroscience* 147, 1022–1033 (2007).
80. Walker, F. R., Knott, B. & Hodgson, D. M. Neonatal endotoxin exposure modifies the acoustic startle response and circulating levels of corticosterone in the adult rat but only following acute stress. *J. Psychiatr. Res.* 42, 1094–1103 (2008).
81. Panagiotidi, M., Overton, P. G. & Stafford, T. The relationship between ADHD traits and sensory sensitivity in the general population. *Compr. Psychiatry* 80, 179–185 (2018).
82. Jarrett, M. A. & Ollendick, T. H. A conceptual review of the comorbidity of attention-deficit/hyperactivity disorder and anxiety: Implications for future research and practice. *Clin. Psychol. Rev.* 28, 1266–1280 (2008).
83. Reimherr, F. W., Marchant, B. K., Gift, T. E. & Steans, T. A. ADHD and Anxiety: Clinical Significance and Treatment Implications. *Curr. Psychiatry Rep.* 19, 109 (2017).
84. Penteado, S. H. W. *et al.* Prenatal lipopolysaccharide disrupts maternal behavior, reduces nest odor preference in pups, and induces anxiety: studies of F1 and F2 generations. *Eur. J. Pharmacol.* 738, 342–351 (2014).
85. Makinson, R. *et al.* Intrauterine inflammation induces sex-specific effects on neuroinflammation, white matter, and behavior. *Brain. Behav. Immun.* 66, 277–288 (2017).

86. Arsenault, D., St-Amour, I., Cisbani, G., Rousseau, L.-S. & Cicchetti, F. The different effects of LPS and poly I:C prenatal immune challenges on the behavior, development and inflammatory responses in pregnant mice and their offspring. *Brain. Behav. Immun.* 38, 77–90 (2014).
87. Association, A. P. *Diagnostic and Statistical Manual of Mental Disorders*. (American Psychiatric Publishing, 2000).
88. Ahmed, R., Borst, J. M., Yong, C. W. & Aslani, P. Do parents of children with attention-deficit/hyperactivity disorder (ADHD) receive adequate information about the disorder and its treatments? A qualitative investigation. *Patient Prefer. Adherence* 8, 661–670 (2014).
89. Elhady, M., Elattar, R. S., Elaidy, A. M. A., Abdallah, N. A. & Elmalt, H. A. Role of inflammation in childhood epilepsy and ADHD comorbidity. *Appl. Neuropsychol. Child* 11, 291–296 (2022).
90. Rosenberg, J. B. *et al.* Maternal inflammation during pregnancy is associated with risk of ADHD in children at age 10. *Brain. Behav. Immun.* 115, 450–457 (2024).
91. Takahashi, N. *et al.* Interaction of genetic liability for attention deficit hyperactivity disorder (ADHD) and perinatal inflammation contributes to ADHD symptoms in children. *Brain Behav. Immun. - Health* 30, 100630 (2023).

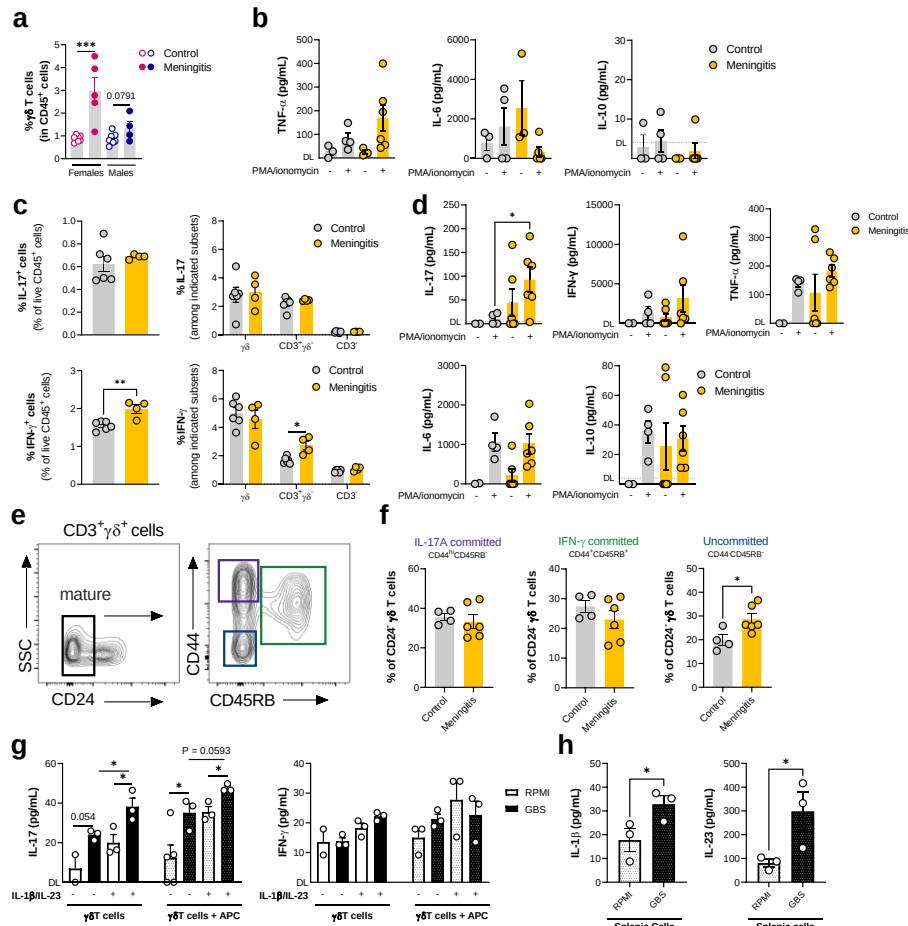
Supplementary Data



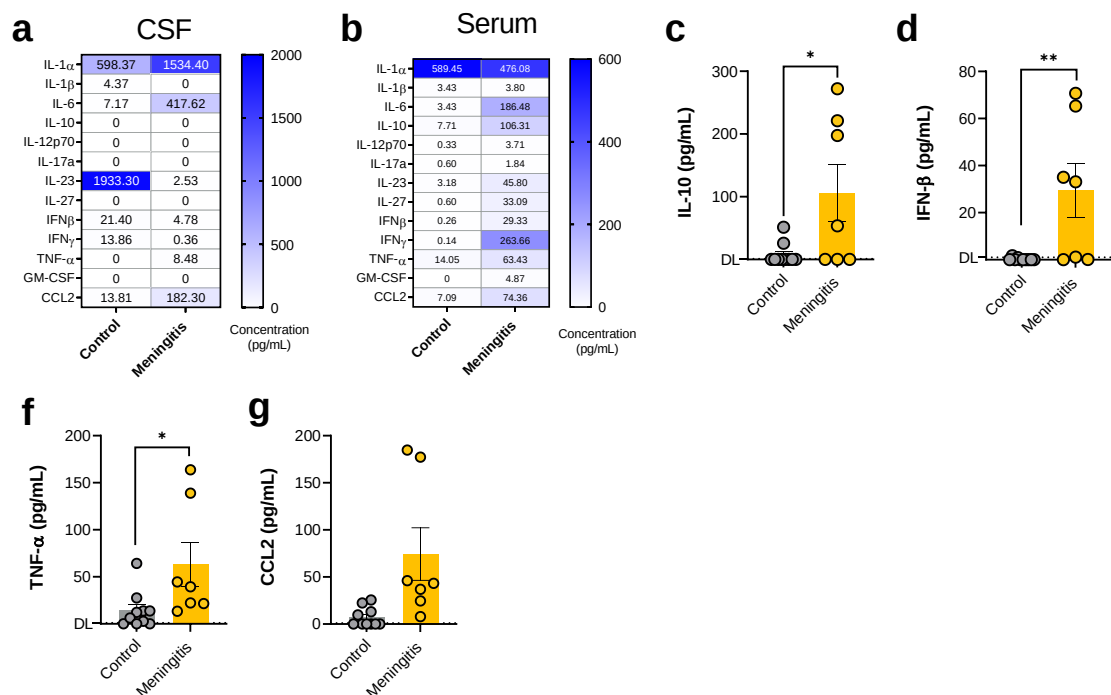
Supplementary Figure 1. Immune kinetic analysis in the meninges and spleen of GBS-infected newborn mice. Pregnant C57BL/6 female mice were intravaginally colonised with 3×10^4 CFU GBS hypervirulent strain BM110, at gestational days 16 and 17. Control animals received PBS. Newborn mice were sacrificed at indicated postnatal days (P). **(a-f)** Frequency and number of indicated populations in the meninges. Each symbol represents a single pup. Error bars indicate the mean $\pm$ SEM ($n = 8-12$ Control, $n = 5-10$ Meningitis). Comparisons by students' t-test within each time point. **(g-l)** Frequency and number of indicated populations in the spleen. Each symbol represents a single pup. Error bars indicate the mean $\pm$ SEM ($n = 8-16$ Control, $n = 7-10$ Meningitis). Comparisons by students' t-test within each time point. Statistical differences (P values) between groups are indicated; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.



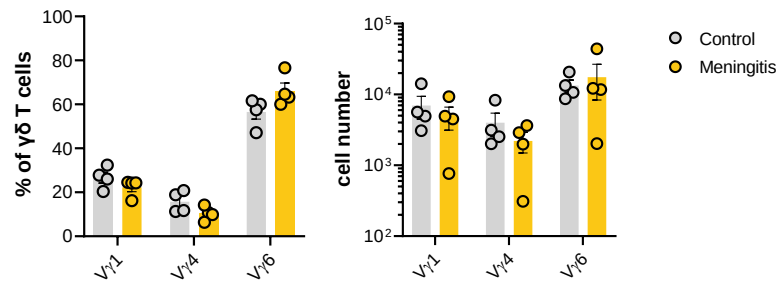
Supplementary Figure 2. Immune kinetic analysis in the brain of GBS-infected newborn mice. Pregnant C57BL/6 female mice were intravaginally colonised with 3×10^4 CFU GBS hypervirulent strain BM110, at gestational days 16 and 17. Control animals received PBS. Newborn mice were sacrificed at indicated postnatal days (P). **(a-f)** Frequency and number of indicated populations. Each symbol represents a single pup. Error bars indicate the mean $\pm$ SEM ($n = 4-12$ Control, $n = 4-5$ Meningitis). Comparisons by students' t-test within each time point. Statistical differences (P values) between groups are indicated; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.



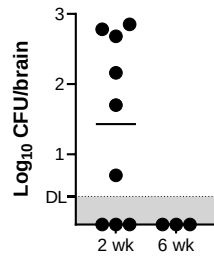
Supplementary Figure 3. Meningeal cytokines and $\gamma\delta$ T cells systemic characterisation in neonatal meningitis. Pregnant C57BL/6 female mice were intravaginally colonised with 3×10^4 CFU GBS hypervirulent strain BM110, at gestational days 16 and 17. Control animals received PBS. Newborn mice were sacrificed at postnatal (P) day 3. **(a-d)** Characterisation of splenic $\gamma\delta$ T cells. **(a)** Frequency of $\gamma\delta$ T cells in the spleen of male and female pups at P3. Each symbol represents one pup ($n = 8$ Control, $n = 4-5$ Meningitis). Error bars indicate mean $\pm$ SEM. Comparisons by Students' t-test. **(b)** Supernatant concentrations of indicated cytokines from ex vivo cultured meningeal cells, isolated from P3 pups, after 24 h of PMA and ionomycin stimulation. Each symbol represents a pool of 2 pups ($n = 6-12$). Data are presented as mean $\pm$ SEM. **(c)** Frequency of IL-17 (top panel) and IFN- γ (bottom panel) produced by splenic cells at P3. Each symbol represents one pup ($n = 6$ Control, $n = 4$ Meningitis). Error bars indicate mean $\pm$ SEM. **(d)** Supernatant concentrations of indicated cytokines from ex vivo cultured splenic cells, isolated from P3 pups, after 24 h of PMA and ionomycin stimulation. Each symbol represents a pool of 2 pups ($n = 6-12$). Data are presented as mean $\pm$ SEM. **(e-f)** Flow cytometric analysis of thymic $\gamma\delta$ T cells' development and commitment. **(e)** Representative flow cytometric gating strategy of committed $\gamma\delta$ T cells' populations. **(f)** Frequency of mature $\gamma\delta$ T cells (CD24 $^{-}$) committed to the production of IL-17A, IFN- γ or uncommitted, respectively. Each symbol represents one pup ($n = 3$ Control, $n = 6$ Meningitis). Error bars indicate mean $\pm$ SEM. Comparisons by Students' t-test. **(g)** Supernatant concentrations of indicated cytokines from ex vivo culture pulmonary $\gamma\delta$ T cells, isolated from P3 pups, after 72 h of stimulation with GBS in the presence or absence of IL-1 β and IL-23 and/or antigen-presenting cells (APC, splenic CD45 $^{+}$ irradiated cells). Each symbol represents a pool of 2 pups ($n = 4-6$). Data are presented as mean $\pm$ SEM. **(h)** IL-1 β and IL-23 concentrations from ex vivo culture of splenic APCs upon 72 h GBS stimulation. Each symbol represents a pool of 2 pups ($n = 6$). Data are presented as mean $\pm$ SEM. Statistical differences (P values) between groups are indicated; *P < 0.05, **P < 0.01, ***P < 0.001. DL, detection limit



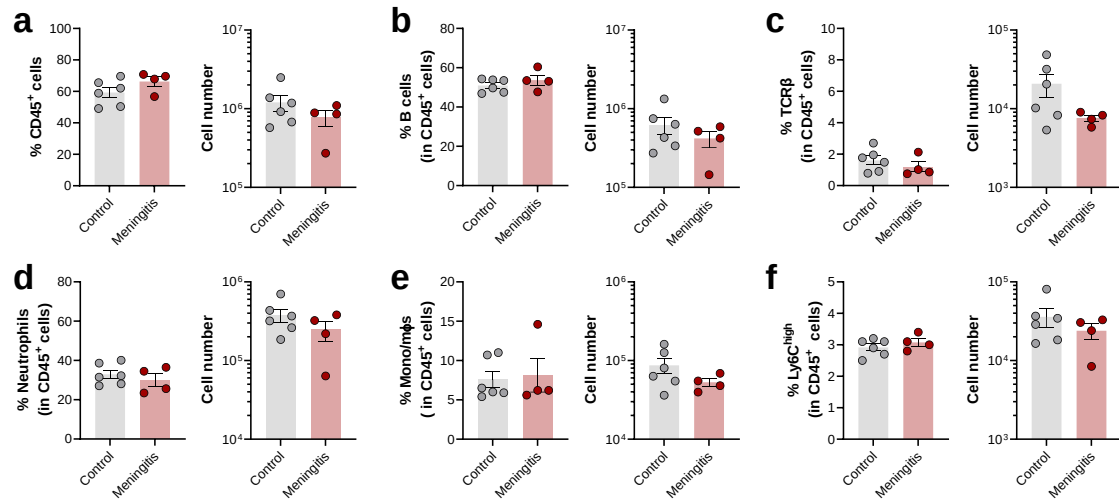
Supplementary Figure 4. Cytokine analysis of the CSF and serum of newborn meningitis mice. Pregnant C57BL/6 female mice were intravaginally colonised with 3×10^4 CFU GBS hypervirulent strain BM110, at gestational days 16 and 17. Control animals received PBS. Cerebral spinal fluid (CSF) and sera were collected at postnatal day (P) 3. **(a-b)** Heatmap of the indicated cytokines' mean concentrations in the CSF **(a)** and serum **(b)** samples. Blue scale indicates the concentration of cytokines in pg/mL. Data presented as mean values ($n = 5$ CSF, $n = 9-11$ Serum). **(c-g)** Concentration of the indicated cytokines in serum samples. Each symbol represents a pool of 2-3 pups ($n = 20$ Control, $n = 14$ Meningitis). Error bars indicate mean $\pm$ SEM. Statistical differences (P values) between groups are indicated; * $P < 0.05$, ** $P < 0.01$.



Supplementary Figure 5. Lung $\gamma\delta$ T cell clones' analysis. Pregnant C57BL/6 female mice were intravaginally colonised with 3×10^4 CFU GBS hypervirulent strain BM110, at gestational days 16 and 17. Control animals received PBS. Frequency (left) and number (right) of lung $V\gamma 1^+$, $V\gamma 4^+$ and $V\gamma 6$ ($V\gamma 1^+V\gamma 4^-$) $\gamma\delta$ T cells, at postnatal day (P) 3. Each symbol represents one pup ($n = 4$). Error bars indicate mean $\pm$ SEM. Comparisons by Students' t-test.

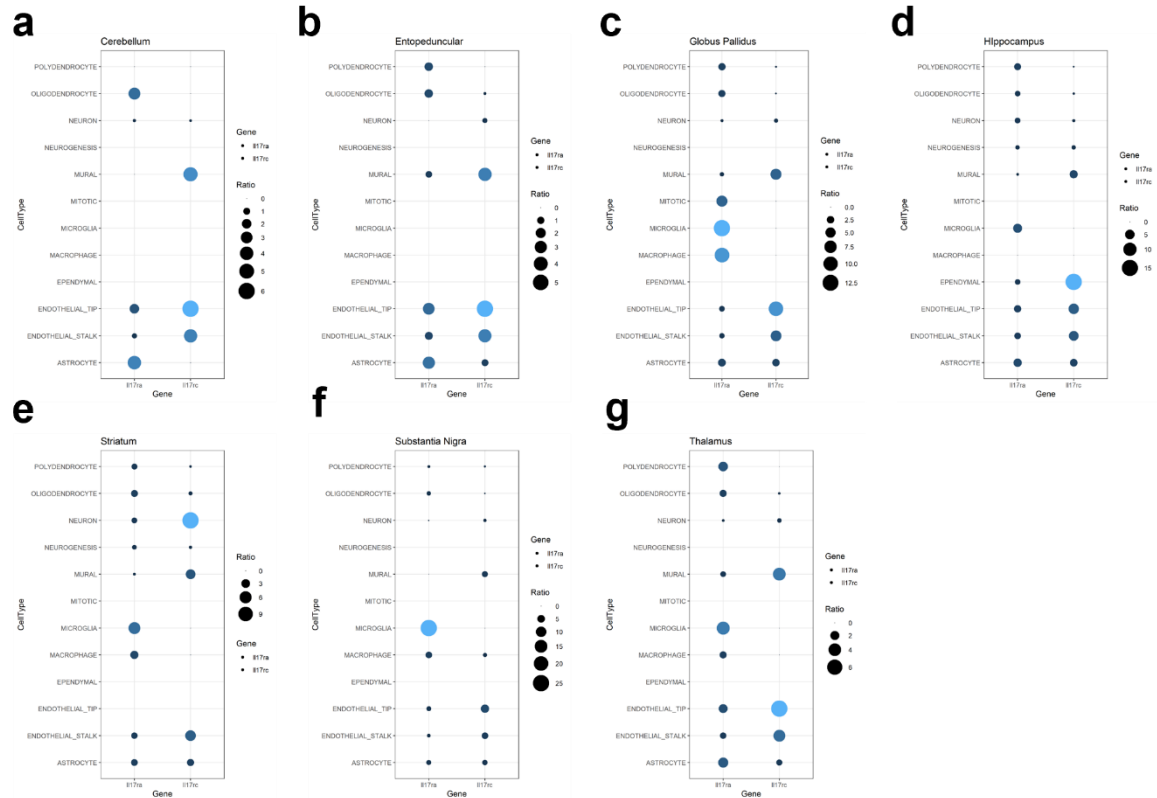


Supplementary Figure 6. GBS BM110 brain load. Pregnant C57BL/6 female mice were intravaginally colonised with 3×10^4 CFU GBS hypervirulent strain BM110, at gestational days 16 and 17. Control animals received PBS.

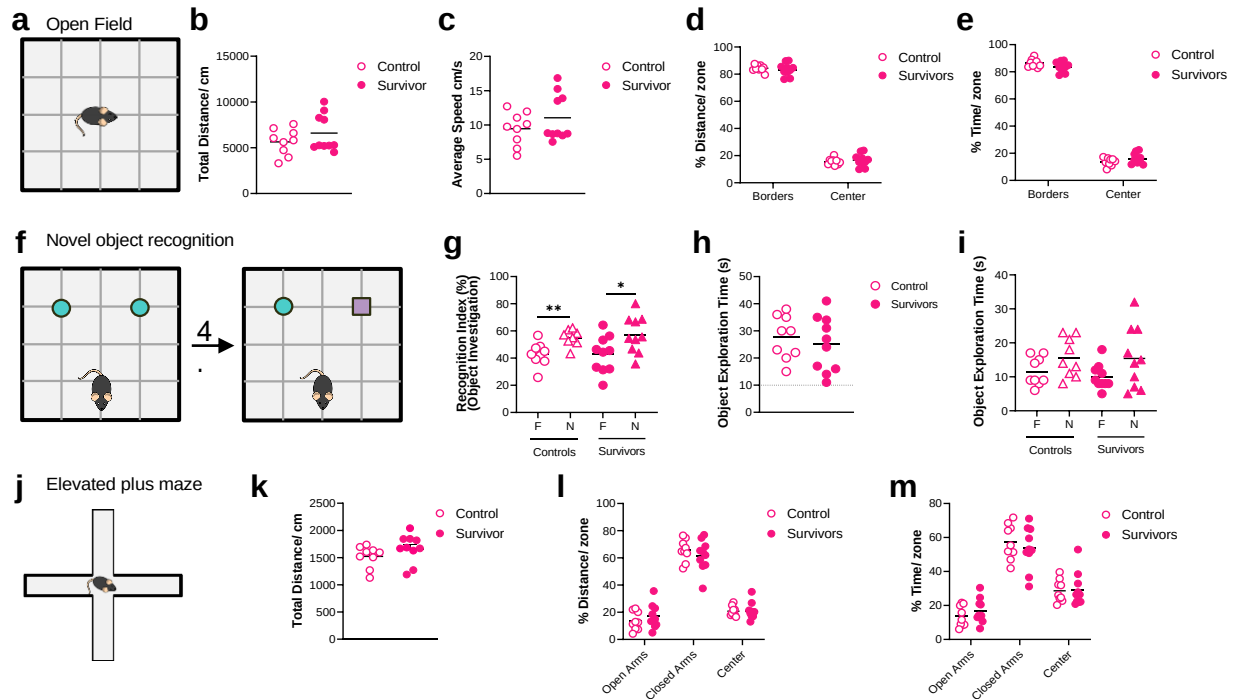


Supplementary Figure 7. Immune kinetic analysis in the spleen of GBS-infected newborn *Tcrd*^{-/-} mice.

Pregnant C57BL/6 female mice were intravaginally colonised with 3×10^4 CFU GBS hypervirulent strain BM110, at gestational days 16 and 17. Control animals received PBS. Newborn mice were sacrificed at postnatal day (P) 3. **(a-f)** Frequency and number of indicated populations. Each symbol represents a single pup. Error bars indicate the mean $\pm$ SEM ($n = 6$ Control, $n = 4$ Meningitis). Comparisons by students' t-test.

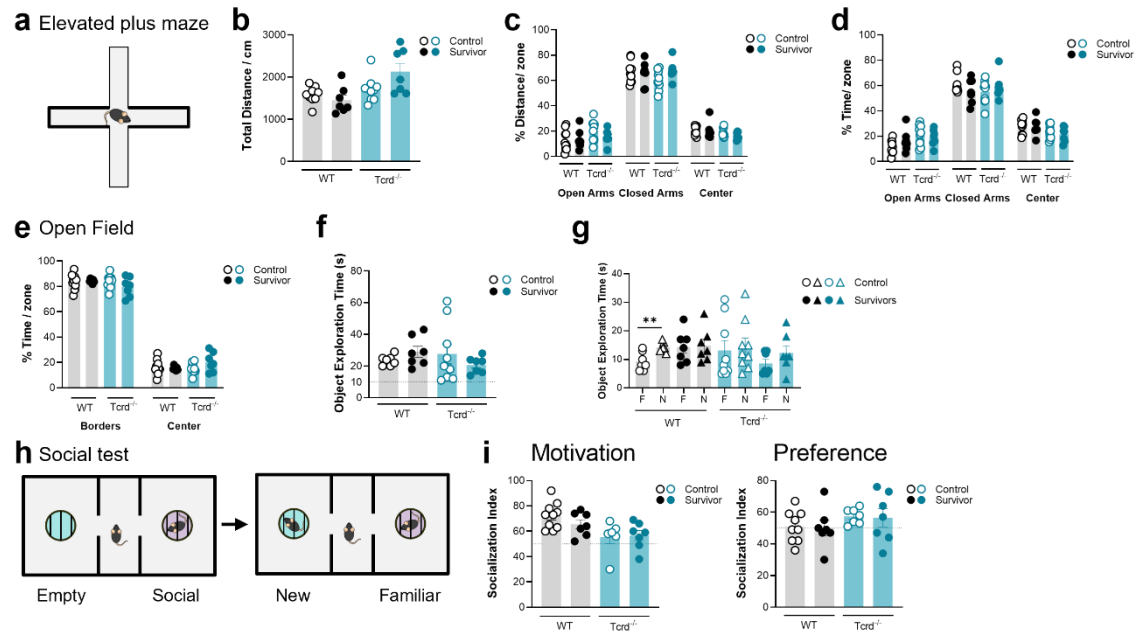


Supplementary Figure 8. Expression of IL-17 receptor in different brain regions. (a-g) Predicted expression of the IL-17 receptor chains a (IL17ra) and c (IL17rc) in the different cell types, in the indicated brain regions, using previously published single-cell RNA-seq data. The size of each dot represent the average expression of cells expressing each gene.



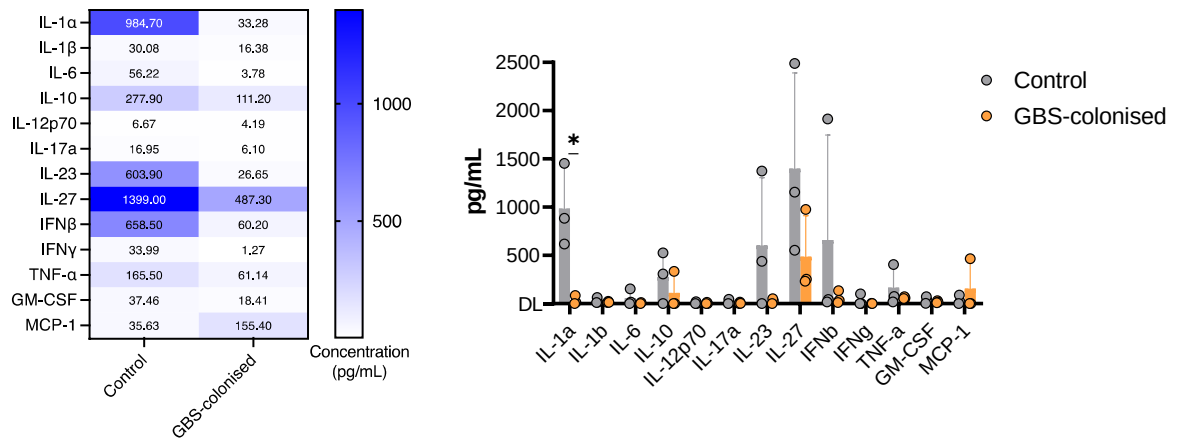
Supplementary Figure 9. Female survivors of neonatal GBS meningitis do not present long-term sequelae.

Pregnant C57BL/6 WT or *Tcrd*^{-/-} female mice were intravaginally colonised with 3×10^4 CFU GBS hypervirulent strain BM110, at gestational days 16 and 17. Control animals received PBS. Females born from the indicated groups were allowed to reach adulthood and submitted to behavioural tests. **(a-e)** Behavioural analysis in the open-field test. **(a)** Representative scheme of the open field test. The total distance covered **(b)**, the average speed **(c)** and the percentage **(d)** and time **(e)** of distance covered by zone were used as a measure of hyperactivity and anxious-like behaviour. **(f-i)** Cognitive test through novel object recognition (NOR). **(f)** Representative scheme of the novel object recognition test. The recognition index **(g)** was used as a measure of cognitive function. The object exploration time **(h)** was used as a control of the test. **(i)** Object exploration time in the familiar (F) and novel (N) object. **(j-m)** Elevated Plus Maze test. **(j)** Representative scheme of the elevated plus maze test. The total distance covered **(k)**, and the percentage of distance **(l)** and time **(m)** covered by zone were used as a measure of anxious-like behaviour. Each symbol represents a single animal ($n = 9$ Control, $n = 10$ Survivor). Data represented as mean. Comparisons by Students' t test.

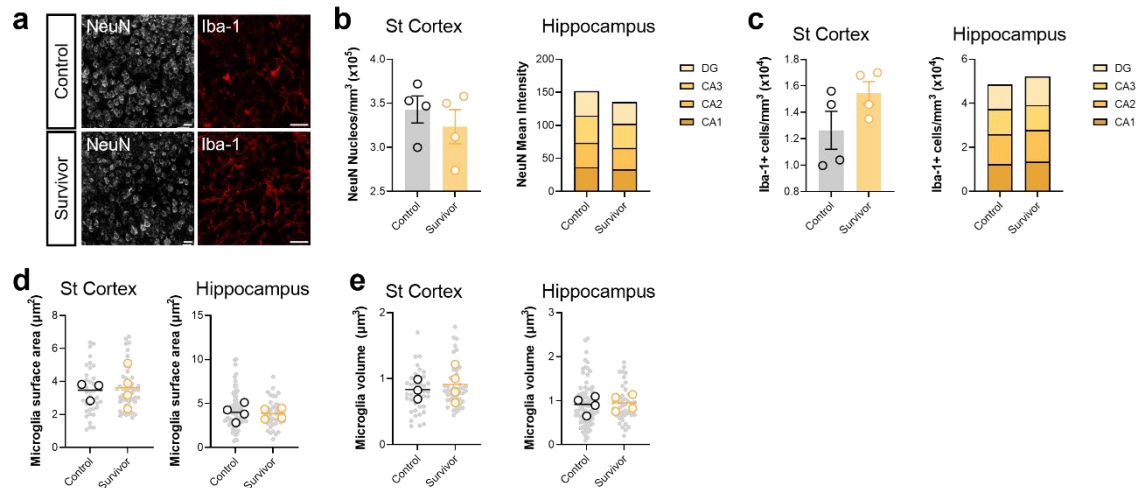


Supplementary Figure 10. Behavioural assessment in male survivors of neonatal GBS meningitis.

Pregnant C57BL/6 WT or Tcrδ^{-/-} female mice were intravaginally colonised with 3x10⁴ CFU GBS hypervirulent strain BM110, at gestational days 16 and 17. Control animals received PBS. Male animals born from the indicated groups were allowed to reach adulthood and submitted to behavioural tests. **(a)** Representative scheme of the Elevated Plus Maze test. The total distance covered **(b)**, and the percentage of distance **(c)** and time **(d)** covered by zone were used as a measure of anxious-like behaviour. **(e)** The percentage of time covered in the indicated regions of the open field test. **(f)** The object exploration time was used as a control of the novel-object recognition test. **(g)** Object exploration time in the familiar (F) and novel (N) object. **(h)** Representative scheme of the social test. The socialisation index in motivation and preference phases **(i)** were used as a measure of social preference. Each symbol represents a single animal (n = 10 Control, n = 7 Survivor). Error bars indicate mean ± SEM. Comparisons by Students' t test within genotypes and social conditions. Statistical differences (P values) between groups are indicated. **P < 0.01.



Supplementary Figure 11. Maternal GBS colonisation does not induce maternal immune activation during pregnancy. Pregnant C57BL/6 WT or Tcrd^{-/-} female mice were intravaginally colonised with 3x10⁴ CFU GBS hypervirulent strain BM110, at gestational days 16 and 17. Control animals received PBS. Sera were collected at embryonic day 18.5. Left, Heatmap of indicated cytokines in the serum. Blue scale indicates the concentration of cytokines in pg/mL. Right, Concentration of the indicated cytokines in serum samples. Each symbol represents a single animal (n = 3 per group). Comparisons by Student's t test. Statistical differences (P values) between groups are indicated. *P < 0.05.



Supplementary Figure 12. Microglia and neuronal quantification in male survivors of neonatal GBS meningitis. Pregnant C57BL/6 female mice were intravaginally inoculated with 3×10^4 CFU GBS hypervirulent strain BM110 at gestational days 16 and 17. Control animals received PBS. The male progeny was sacrificed 6 weeks after birth. **(a)** Representative images of NeuN and Iba1, and Imaris 3D reconstruction of microglia and CD68 in the cortex of 6-week-old animals from both groups. Scale bar 20 μm . **(b)** Quantification of NeuN cells in the brain's somatosensory cortex (St cortex) and hippocampus. **(c)** Quantification of Iba-1 cells in the brain's somatosensory cortex (St cortex) and hippocampus. Each symbol represents one animal ($n = 4$). Error bars indicate mean $\pm$ SEM. Comparisons by Student's t -test. Microglia surface area **(d)** and volume **(e)** at the indicated brain regions. Each coloured symbol represents one animal ($n = 4$) and each background grey symbol represents one cell ($n = 41$ -82 Control, $n = 46$ -48 Survivor). Comparison by Nested t -test.

MANUSCRIPT 3

CGRP SUPPRESSES SIGLEC^{F^{HL}} NEUTROPHIL DEVELOPMENT IN GROUP B *STREPTOCOCCUS* NEONATAL PNEUMONIA

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Abstract

Neonatal pneumonia, a leading cause of morbidity and mortality, is frequently caused by Group B *Streptococcus* (GBS). The immaturity of the neonatal immune system is commonly associated with poor outcomes. However, the protective neonatal immunity in the lungs against this pathogen remains to be completely elucidated. Here, we use a clinically relevant neonatal mouse model of GBS pneumonia to investigate the immune response mechanisms influencing disease severity. We demonstrate that $\gamma\delta$ T cells are significantly increased and neutrophils are effectively recruited to the lungs of infected neonates. However, neutrophils' phenotype varies with disease severity. We show significant infiltration of SiglecF^{hi} neutrophils into the lungs of infected pups with moderate disease, a phenotype associated with enhanced phagocytic capacity and bacterial clearance. In contrast, severe disease is characterised by impaired neutrophil maturation into the SiglecF^{hi} phenotype, resulting in reduced bacterial clearance and disease progression. Moreover, we show that severity is associated with increased expression of calcitonin gene-related peptide (CGRP) in the lungs. CGRP acts on neutrophils to diminish their activation into the SiglecF^{hi} phenotype. Our findings suggest that GBS exploits the neuroimmune axis to evade host immunity through CGRP-mediated suppression of neutrophil activation.

Introduction

Neonatal pneumonia remains a major cause of morbidity and mortality worldwide^{1,2}. Among the pathogens responsible for severe neonatal infections, Group B *Streptococcus* (GBS) is the leading cause of invasive neonatal diseases, including pneumonia and septicaemia³. GBS is a Gram-positive bacterium that is part of the vaginal and gastrointestinal flora of many healthy adults³. However, it can be transmitted vertically from mother to newborn, either *in utero* or *intrapartum*, leading to neonatal infection⁴⁻⁶. The lungs are often the primary organ affected during neonatal GBS infection, given their direct exposure to GBS during delivery as a consequence of aspiration of contaminated fluids. As a result, GBS infection in the lungs triggers inflammation and respiratory complications that can result in significant lung injury^{3,7}. The initial clinical presentation of GBS disease in newborns frequently includes acute respiratory distress, often progressing to pneumonia, which may lead to acute respiratory distress syndrome (ARDS), causing severe lung injury and hypoxemia^{8,9}. It is estimated that between one-third and more than half of affected neonates show symptoms at birth or within 4 to 6 hours after delivery. In fact, GBS is identified as the most common cause of bacterial pneumonia acquired during delivery, with autopsy data from fatal cases showing extensive lobar or multilobar pneumonia in many infants¹⁰.

However, despite progress in neonatal care, the cellular and molecular mechanisms responsible for pathogen clearance in the lungs remain poorly understood.

In neonates, the immune system differs both phenotypically and functionally from that of adults^{11,12}. Neutrophils, the first line of defence in the innate immune system, are key phagocytic cells responsible for pathogen elimination¹³. However, neonatal neutrophils are less efficient than their adult counterparts, exhibiting diminished phagocytic capacity¹⁴, reduced chemotaxis¹⁵, impaired neutrophil extracellular traps (NETs) formation¹⁶, and a generally delayed or weakened response to inflammatory stimuli. These deficiencies contribute to the neonates' increased susceptibility to severe infections^{11,12}. Notably, studies have shown that neutrophils are a highly heterogeneous population. Recently, a distinct subset of neutrophils characterised by high expression of SiglecF (sialic acid-binding immunoglobulin-like lectin F), a lectin typically found on mouse eosinophils, has been identified in lung tumour models and allergic rhinitis^{17,18}.

Recent research has shed light on the role of neuroimmune interactions as important regulators of physiology. The lungs are heavily innervated by nociceptor sensory neurons¹⁹. When activated by noxious inflammatory stimuli, including bacterial products, nociceptors release neuropeptides like calcitonin gene-related peptide (CGRP) and vasointestinal peptide (VIP), which play a crucial role in the regulation of both pain and inflammation²⁰⁻²². Some pathogens have been shown to exploit the CGRP signalling pathway to evade host immunity, thereby enhancing their survival^{6,22,23}. However, the impact of such neuronal signalling on the neonatal lungs during bacterial infection has not been clearly defined.

Here, using a neonatal mouse model of GBS pneumonia, we demonstrate a critical role of the nervous system in modulating the host defence against GBS infection. We show that while neutrophils are effectively recruited to the lungs of infected neonates, their phenotype differs depending on disease severity. Specifically, neutrophils in neonates with severe pneumonia fail to acquire a distinctive profile characterised by high expression of SiglecF. Furthermore, we show that GBS infection triggers the release of CGRP from sensory neurons, which in turn prevents neutrophils from transitioning into the SiglecF^{hi} phenotype. This inability to fully activate impairs the neutrophils' capacity to phagocyte and clear the pathogen, contributing to the severity of the disease.

Methods

Bacterial strains and culture

GBS strain BM110, a capsular serotype III strain and belonging to the clonal complex 17 (CC17) hypervirulent lineage responsible for more than 80% of meningitis cases²⁴, or the GBS strain NEM316 belonging to the CC23²⁵, were cultured in Todd-Hewitt (TH) broth or agar (Difco Laboratories). Overnight cultures of GBS were 1:100 sub-cultured in fresh TH broth and grown at 37 °C until the mid-log phase. Growth was evaluated by monitoring the optical density (OD) at 600 nm (OD₆₀₀ ~0.800) using a spectrophotometer (Biochrom Libra S6+).

Animals

All animal procedures were performed in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and Directive 2010/63/EU and Portuguese rules (DL 113/2013). The animal study protocols were approved by the Ethics Committee of Instituto de Investigação e Inovação em Saúde (i3S), and by the competent national authority Direção Geral de Alimentação e Veterinária (DGAV). C57BL/6 mice were bred and maintained at the i3S under specific pathogen-free conditions, under a 12 h light-dark cycle with *ad libitum* access to food and water. All mice were housed in groups, except for pregnant females, under a controlled environment (22 ± 2 °C, 45 - 55% relative humidity) in ventilated cages. All efforts were made to minimise animal suffering, including the use of cardboard tunnels and sheets as environmental enrichment. All experiments involving neonates were performed in both male and female, and pregnant females were infected at the age of 8-10 weeks. The vertical model of infection is described below.

Neonatal Mouse Model of GBS pneumonia

In the mouse model of infection for human neonatal GBS disease, pregnant C57BL/6 mice were intravaginally (i.vag.) inoculated with 40 µL containing 3-4x10⁴ GBS cells at the gestation (G) days 16 and 17, using a micropipette. Pregnant females delivered spontaneously between G19 and G20, and the pups were maintained with their mothers during the experimental period. The day following birth was considered the postnatal day (P) 1. All experiments were controlled with age-matched mice born from PBS sham progenitors. Animals born from sham and infected pregnant mice were followed until P5 to monitor their survival curves. The pups' weight was registered at indicated time points.

Tissue collection

Neonatal mice (both male and female), selected at different time points as indicated in the text, were deeply anaesthetised by isoflurane inhalation, transcardially perfused with saline solution, and euthanised by decapitation. The lungs and spleen were aseptically removed, weighted, and handled in agreement with its final use. In the case of RNA analysis, a section of lung tissue was immediately snap frozen on dry ice and stored at -80 °C until RNA extraction. When needed, blood was drawn from the mandibular vein using heparin-coated capillaries prior to perfusion. Tissue samples were selected from different litters.

Bacterial load analysis

At specific time points (from P1 to P5) the collected lung was homogenised in 250 µL of PBS with a pellet pestle and a rotor-stator homogeniser. Following homogenisation, serial dilutions were made in sterile saline and plated on TH agar. The plates were incubated overnight at 37 °C and the number of colony-forming units (CFU) was enumerated.

Lung Histopathology

Lungs were perfused and the left lobe was fixed in 10 % buffered formalin. Samples were routinely processed in the TP10120 (Leica®, Wetzlar, Germany) and embedded in paraffin. For staining 3 µm thick sections were cut using the microtome Histocore Autocut (Leica®) and stained with Hematoxylin and Eosin (H&E) or Giemsa, following standard protocols. The slides were digitalised using the Pannoramic MIDI II (3DHISTECH, Budapest, Hungary), with amplification of 40x and visualised using the software CaseViewer (3DHISTECH). Lung inflammation was scored in a blinded fashion by a pathologist. Lung histology was rated semi quantitatively, in a histological score from 0 to 4 based on the percentage of alterations observed: 0 Absence of histological changes; 1, Parenchymal alterations in 1 to 25% of the tissue examined; 2, Parenchymal alterations in 26 to 50% of the tissue examined; 3, Parenchymal alterations in 51 to 75% of the tissue examined; 4, Parenchymal alterations in 76 to 100 % of the tissue examined.

Flow cytometry and cell sorting

Pups (both male and female) were deeply anaesthetised by isoflurane inhalation and euthanised by decapitation. The lungs and spleen were aseptically removed. The lungs were minced into 1-2 mm pieces and digested for 30 minutes (min) at 37°C with 1 mg/mL

collagenase D (Roche) in RPMI 1640 medium (HyClone GE Healthcare Life Sciences). The reaction was stopped with 2 mM EDTA and 0.5% BSA in PBS (PBE). Following the digestion step, the tissue was gently passed through a 20-gauge (G) needle, followed by a 25G, and gently pressed through 100 µm nylon mesh cell strainer. Suspensions were then centrifuged twice at 70g for 1 min, at 4 °C, and the supernatant collected. Finally, cells were centrifuged at 300g, for 4 min at 4 °C, and the supernatant discarded. Red blood cells were lysed in Ammonium-Chloride-Potassium (ACK) solution for 5 min at room temperature and washed in PBE (300g at 4°C for 4 min). The cell pellets were resuspended in ice-cold FACS buffer [2% FBS (heat-inactivated, Biowest), 2 mM EDTA and 0.01% sodium azide in PBS] or sorting buffer (2% FBS, 1 mM EDTA in PBS) and counted.

Spleens were excised, gently dissociated in a 100 µm cell strainer to yield a single cell suspension. Red blood cells were then lysed, and the obtained single-cell suspension was washed and counted.

A total of 1×10^6 cells were plated in 96-well plates for staining. Cells were incubated with FVD eFluor 506 (FACS staining) or FVD eFluor 780 (cell sorting) and then with CD45 FITC (Clone 30-F11), CD69 PE (H1.2F3), CD8 PerCP-Cy5.5 (53-6.7), CD3 Pe-Cy7 (17A2), TCRγδ APC (GL3), TCRβ eFluor 780 (H57-597, eBioscience), Siglec-F PE (E50-2440, BD Biosciences), Ly6C PerCP-Cy5.5 (HK1.4), CD11b Pe-Cy7 (M1/70), CD5 PE (53-7.3, BD Biosciences), TCRβ PE (H57-597, Invitrogen), CD19 APC-Cy7 (6D5), IFNγ PE (XMG1.2), TCRγδ Pe-Cy7 (GL3), CD3 APC-Cy7 (17A2), Ly6G AF647 (1A8), Ki67 Alexa Fluor 488 (16A8), TCRVγ4 PE (UC3-10A6), CD45 PerCP-Cy5.5 (30-F11), TCRVγ1 APC (2.11), TCRβ eFluor 450 (H57-597, Invitrogen), CD44 PerCP-Cy5.5 (IM7), CD4 BV421 (RM4-5), CD62L APC (MEL-14), all from Biolegend unless otherwise mentioned. Cells were analysed in FACSCanto II or FACS Aria flow cytometers (BD Biosciences), using FACS Diva software. Post-acquisition analysis was performed using FlowJo Software v10 (Tree Star).

Intracellular staining

For intracellular staining, single-cell isolates from the lungs were stimulated in complete RPMI medium with PMA (Phorbol 12-Myristate 13-Acetate; 50 ng/ml, Sigma-Aldrich) and Ionomycin (1 µg/mL, Sigma-Aldrich) and Brefeldin A (BFA) (10 µg/mL, Sigma-Aldrich), for 4 h at 37°C, 5% CO₂. Cells were washed with ice-cold DPBS (without Ca²⁺ and Mg²⁺) and stained for extracellular markers as described above. Cells were then permeabilised with a Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and stained for 30 min at 4 °C. Then, cells were washed with 100 µL 1X Permeabilization Buffer and incubated in Permeabilisation Buffer with rat IgG (Sigma-Aldrich) 1:100 and FcBlock 1:500 for 10 min,

at 4°C. Intracellular staining mix, containing anti- IFN- γ -PE (clone XMG1.2, Biolegend) and anti- IL-17-APC (clone TC11-18H10.1, Biolegend) was added and the cells were incubated for 1 h, at room temperature. Cells were washed first in Permeabilisation buffer and then in FACS buffer and resuspended in FACS buffer.

RNA extraction and RT-qPCR

Frozen tissues were retrieved on dry ice and RNA was extracted using the Pure link™ RNA Mini Kit (Ambion). RNA quality was evaluated using Experion™ Eukaryote Total RNA StdSens and RNA concentration was assessed Nanodrop 1000 (Thermo Scientific). RNA was reverse transcribed into cDNA using the SuperScript™ IV First-Strand Synthesis System (Invitrogen), following the manufacturer's guidelines. RT-qPCR reactions were performed on a CFX384 Real-Time PCR Detection System (Bio-Rad) lightcycler using iTaq Universal SYBR Green Supermix (Bio-Rad). cDNA was diluted at 1:5 prior reaction and each sample was analysed in duplicate. Relative gene expression levels were normalised to *Hprt* mRNA levels in each sample using the $2^{-\Delta Ct}$ method. The primer sequences used for the real-time PCR of target gene were (5' to 3'): *Tnfa* Sense (GTGGAAGTGGCAGAAGAG) and anti-sense (ATGAGAAGAGGCTGAGACA), *Il-1b* sense (CAACCAACAAGTGATATTCTCCATG) and anti-sense (GATCCACACTCTCCAGCTGCA), *Il-17* (CTCAGACTACCTCAACCGTTCCA) and anti-sense (TTCCCTCCGCATTGACACA), *Il33* sense (TCCAAGTCCAAGATTTCCTCCG) and anti-sense (CATGCAGTAGACATGGCAGAA), *Il-10* sense (ATTGAATTCCTGGGTGAGAAG) and anti-sense (CACAGGGGAGAAATCGATGACA), *Cgrp* sense (CCTGCAACACTGCCACCTGCG) and anti-sense (GAAGGCTTCAGAGCCACATTG), *Hprt* sense (TCAGTCAACGGGGGACATAAA) and anti-sense (GGGGCTGTACTGCTTAACCAAG).

Bacterial killing assays

For bacterial killing assays, mouse neutrophils were FACS sorted from the lungs of P5 pups, based on the expression of SiglecF (sialic acid-binding immunoglobulin-like lectin F) (CD45⁺Ly6G⁺CD11b⁺SiglecF^{hi} and CD45⁺Ly6G⁺CD11b⁺SiglecF^{neg/low}). Neutrophils were placed in coculture with GBS BM110 at a multiplicity of infection (MOI) of 5, in 200 μ L of RPMI complete medium (without antibiotics). Co-cultures were centrifuged at 300 g for 5 min and incubated for 1 h at 37 °C in 5% CO₂. Upon incubation, cells were washed and further incubated with 200 μ g/mL gentamicin for 30 min, at 37 °C in 5% CO₂. Cells were

washed twice in PBS and lysed by resuspension in 100 μ L of ice-cold ddH₂O. The number of intracellular bacteria was determined by serially dilution and plating in TH agar.

Neutrophil stimulation with CGRP *in vitro*

Following euthanasia, femurs and tibias were dissected from mice. Neutrophils were isolated from the bone marrow as described²⁶. Briefly, bone marrow cells were flushed out using 1 $\times$ HBSS 0.38% sodium citrate buffer in a 5 mL syringe and a 25G needle. Cells were then gently disaggregated by repeatedly aspirating and expelling the solution using an 18G needle and a 5 mL syringe to yield a single-cell solution and centrifuged for 6 min at 230g at room temperature. Cells were washed with 1 $\times$ HBSS 0.38% sodium citrate buffer and the pellet resuspended in 2 mL 1 $\times$ HBSS 0.38% sodium citrate buffer. Percoll working solution (90%) was prepared with 10 $\times$ HBSS buffer (without Ca²⁺ and Mg²⁺ or phenol red), and further diluted with 1 $\times$ HBSS 0.38% sodium citrate to prepare the 72%, 64% and 52% Percoll dilutions. Slowly the cell suspension was added on top of the Percoll (GE Healthcare) gradient and centrifuged at 1545g, 30 min, at room temperature. The enriched population of neutrophils was collected at the 72–64% interphase. After isolation, cells were washed, counted and the purity assessed by flow cytometry. Neutrophils were incubated for 4 h at 37 °C with heat killed GBS BM110 at a MOI of 10 and 50, with or without CGRP (100 nM, Peptide Institute. Inc.). After, cells were washed, surface stained with appropriate markers, fixed with PFA and analysed by flow cytometry.

Data analysis and statistics

Data were plotted and analysed with the GraphPad Prism software (v.10.1.1, GraphPad Software LLC). Means and standard errors of the mean (SEM) were calculated, corresponding to the indicated independent experiments. The log-rank (Mantel–Cox) test was used to analyse the survival curve. The Student's *t* test was used when analysing two different groups. Statistical analysis of changes in two or more groups compared at multiple time points was analysed by One- or Two-way ANOVA. Normality was verified by the Shapiro–Wilk normality test. CFU data were log₁₀ transformed. Differences were considered significant for $P \leq 0.05$. Sample sizes are expressed in each figure legend.

Results

Vertical transmission of GBS leads to lung pathology

The immune system plays a crucial role in protecting against bacterial pneumonia in adults^{27,28}. However, the mechanisms contributing to protective or detrimental immunity and subsequent pathology in neonatal lungs remain poorly understood. To investigate whether vertical transmission of GBS would constitute a suitable model for studying neonatal pneumonia, we first adapted our previously described model of GBS mother-to-progeny transmission²⁹ to the C57BL/6 mice (Figure 1A). Survival curve analysis demonstrated that approximately 60% of pups born from colonised dams succumbed to disease within the first three days of life, with the highest mortality (~40%) occurring between P0 and P1 (Figure 1B), likely due to fulminant septicaemia. This outcome contrasted significantly with the complete absence of mortality in the control group. No more deaths were registered after day 3 (Figure 1B). To assess the progression of infection, bacterial colonisation in the

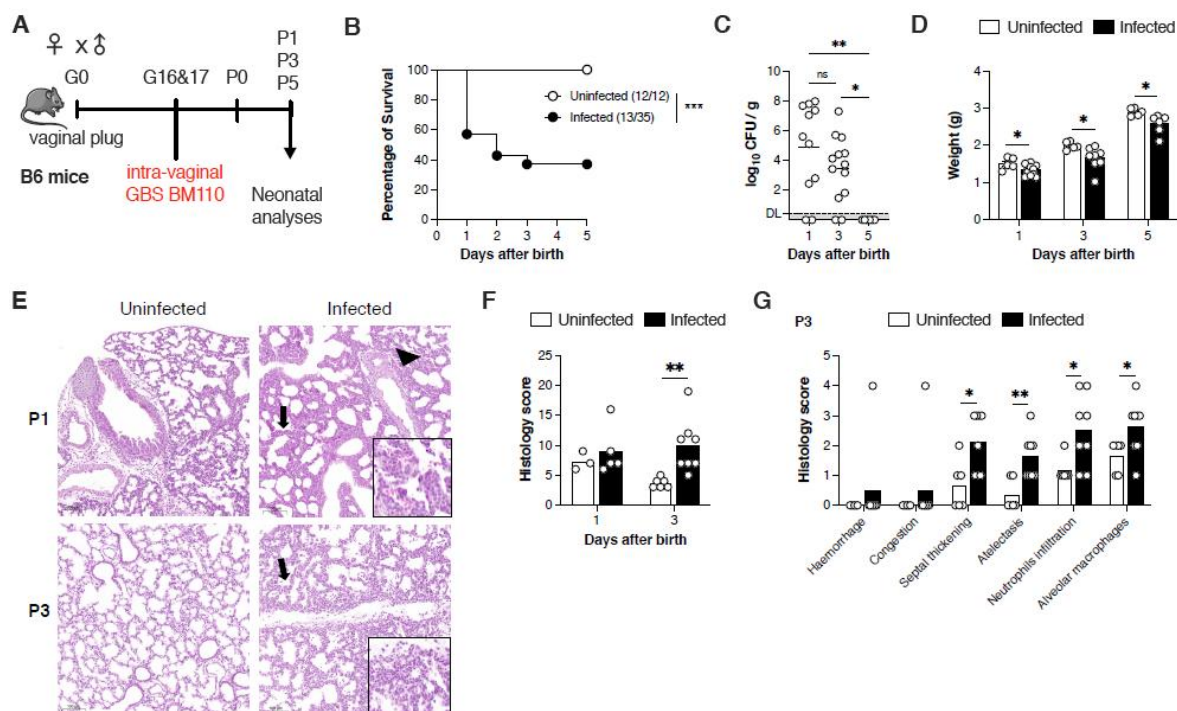


Figure 1. GBS vertical transmission leads to lethal neonatal pneumonia. (A) A schematic illustration of the colonisation model is shown. Pregnant C57BL/6 female mice were intravaginally colonised with 4×10^4 CFU GBS hyper virulent strain BM110, at gestational days 16th and 17th. (B) Kaplan-Meier survival curve of neonatal mice born from GBS-colonized dams, monitored during a 5 days' period. The numbers in parentheses represent the number of animals that survived versus the total number of animals born. (C) Newborn mice were sacrificed at postnatal days 1, 3, and 5 and the lung bacterial loads were determined. Data are presented as mean $\pm$ SEM [n = 11 (P1); n = 13 (P3); n = 6 (P5)]. Each symbol indicates data from single pup. (D) Representative images of tissue slices of lungs of uninfected and infected mice stained with H&E, at indicated timepoints. Scale bar 100 μ m. thickening of the interalveolar septum (septal thickening). Arrowhead indicates haemorrhage. (E) Global histopathology score was attributed to lungs stained with H&E. (F) Summary of histological analysis from the lung of Uninfected (n = 6) and GBS-infected (n = 8) pups at P3. (E and F) Data are combined from two independent experiments and shown as mean $\pm$ SEM. For comparisons between two groups, Student's t test was used. Statistical differences (P values) between groups are indicated. P < 0.05, ** P < 0.01, *** P < 0.001.

neonatal lungs at different time points after delivery was quantified. The maximum burden of bacterial colonisation was observed at P1, with a slight, but not statistically significant, reduction at P3. By P5, bacteria were no longer detectable (Figure 1C). Consistent with bacterial load, infected pups showed significantly reduced body weight gain compared to uninfected controls (Figure 1D).

GBS early-onset disease, occurring in the first week of life, is often accompanied by respiratory complications, including hypoxia and pulmonary hypertension, leading to pneumonia^{5,10,30}, a condition known to cause tissue pathology²⁸. Thus, pulmonary disease severity was assessed by performing H&E and Giemsa staining of lung sections at time points when bacterial burden was detectable (Figure 1E and Supplementary Figure 1). Four anatomic structures were defined when observing the lung sections, namely the interstitium, airways, alveoli, and blood vessels. At P1, the extent of histopathological alterations in infected lungs did not differ significantly from the respective age-matched uninfected controls (Uninfected, 7.33 ± 0.88 ; Infected, 9.00 ± 1.817) (Figure 1F). However, by P3, infected animals exhibited a significant increase in the total histological score compared to their uninfected counterparts (Figure 1F). When analysing each of these findings individually, we found that infected animals were characterised by increased septal thickening, atelectasis, higher neutrophil infiltration, and the presence of alveolar macrophages (Figure 1G). These findings suggest that infection is the factor leading to a worse condition of the neonatal lungs, as reflected by a higher histological score.

Collectively, these data indicate that vertical transmission of GBS results in lung colonisation and pulmonary lesions consistent with pneumonia in neonates.

GBS neonatal pneumonia does not impair neutrophil recruitment to the lungs

The neonatal immune response during neonatal GBS infection is thought to be strongly associated with the progression of infection and disease outcome^{31,32}. It has been shown that during neonatal infection, circulating neutrophils fail to migrate into infected tissues^{31,32}. However, the local immune response at the site of infection during and after disease onset remains poorly characterised. To address this, the main myeloid and lymphoid cell populations in the lungs of infected pups were analysed during the first five days of life and compared to age-matched uninfected controls using flow cytometry (Figure 2A and B, gating strategy). A significant decrease in the frequency of eosinophils was found at P1 and P3 in the lungs of infected pups compared to uninfected controls (Figure 2C). However, the total number of eosinophils did not differ between groups (Figure 2C). By P5, although their relative percentage was similar between groups, the total numbers were significantly

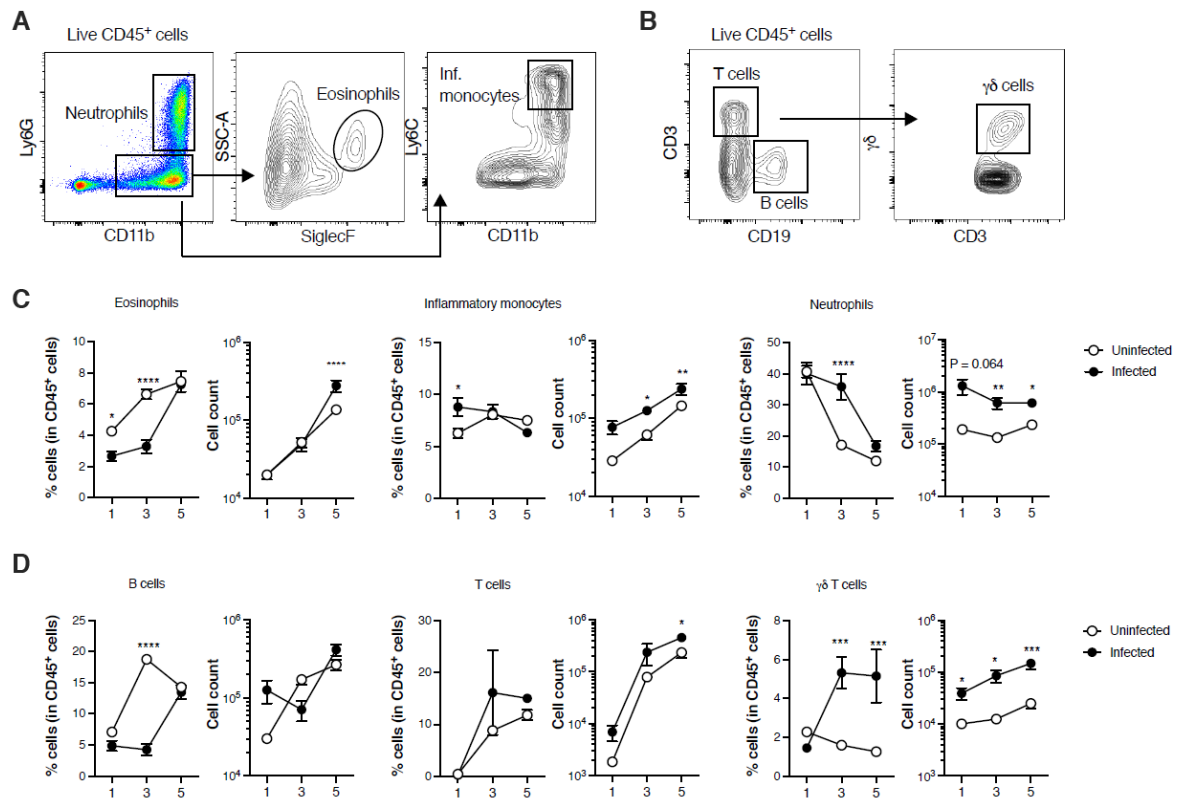


Figure 2. Immune cellular dynamics during GBS neonatal pneumonia. Pregnant C57BL/6 female mice were intravaginally colonised with 4×10^4 CFU GBS hyper virulent strain BM110, at gestational days 16th and 17th. Animals from uninfected and infected groups were sacrificed at indicated time points and the lung was collected for flow cytometry analysis **(A)** Representative gating strategy used to define the myeloid cells. Cells were gated in live CD45⁺ cells. Neutrophils were defined as CD11b⁺Ly6G⁺, eosinophils were defined as CD11b⁺Ly6G⁺SiglecF⁺SSC-A^{int/hi}, inflammatory monocytes were defined as CD11b⁺Ly6G⁺Ly6C^{hi}. **(B)** Representative gating strategy used to define lymphoid cells. Cells were gated in live CD45⁺ cells. T cells were defined as CD3⁺CD19⁻, B cells were defined as CD3⁻CD19⁺, γδ T cells were defined as CD3⁺CD19⁺γδ⁺. **(C)** Frequency and number of indicated myeloid cells. Data are presented as mean $\pm$ SEM, each symbol represents data from single pup [n = 6 (P1, uninfected); n = 11 (P1, infected); n = 11 (P3, uninfected); n = 10 (P3, infected); n = 7-8 (P5, uninfected); n = 8 (P5, infected)]. **(D)** Frequency and number of indicated lymphoid cells. Data is presented as mean $\pm$ SEM, each symbol represents data from single pup [n = 6 (P1, uninfected); n = 11 (P1, infected); n = 5-8 (P3, uninfected); n = 8-10 (P3, infected); n = 8 (P5, uninfected); n = 8 (P5, infected)]. Comparisons by two-way ANOVA. Statistical differences (P values) between groups are indicated. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

increased in infected animals (Figure 2C). The total numbers of inflammatory monocytes were also increased in the lungs of infected pups compared to uninfected controls, reaching significance at P3 and P5 (Figure 2C). Strikingly, neutrophils exhibited the most pronounced increase among the myeloid populations, with a significant increase in both frequency and cell number in infected pups, at P3 (Figure 2C).

In the lymphoid compartment, γδ T cells were markedly altered in infected neonates. Their numbers were significantly increased at P1, and both their frequency and numbers were

elevated at P3 and P5, suggesting that they could be recruited or expanding in response to infection (Figure 2D). As for the total CD3⁺ T cells, this population only showed a significant increase in cell counts at P5, with higher numbers in infected pups compared to controls (Figure 2D). In control pups, the frequency of B cells quickly increased from P1 to P3. However, this was not observed in infected animals, where the percentage of B cells was significantly reduced at P3. By P5, no differences were observed in the frequency of B cells between groups (Figure 2D).

Neutrophil recruitment to infected tissues is a hallmark of inflammation and bacterial infections and is partially driven by IL-17A (referred hereon as IL-17). Thus, we sought to determine whether this cytokine was increased during neonatal pneumonia and its cellular source. $\gamma\delta$ T cells are an important source of IL-17, available to respond in neonates, and we have found that these cells were increased during neonatal GBS pneumonia. When analysing their phenotype, our data revealed that $\gamma\delta$ T cells were predominantly IL-17 producers in infected neonatal lungs, after *ex vivo* stimulation with PMA and ionomycin, although IFN- γ -producing $\gamma\delta$ T cells were also detected at P3 (Figure 3A-C). While infection led to increase frequencies of both IL-17- and IFN- γ -producing $\gamma\delta$ T cells, this increase was more pronounced in the IL-17-producing subset (Figure 3A-C). Upon gating on IL-17-positive cells, we found that the lungs of GBS-infected pups presented high frequencies of $\gamma\delta$ T cells among total IL-17-producing cells, accounting for approximately 70% of total IL-17-producing cells in this tissue (Figure 3B). Given that $\gamma\delta$ T cells are associated with lung homeostasis, tissue repair and maintenance of barrier integrity (e.g., by promoting epithelial cell survival)^{33,34}, we investigated whether $\gamma\delta$ T cells remained altered at later time points post-infection. Since two weeks after birth entails the peak of pulmonary immune system development during the alveolarisation period and the beginning of microvascular maturation^{35,36}, we ascertained whether lung tissue presented with pathological alterations. Infected animals presented a larger area of tissue with histopathological abnormalities, with at least three distinct pathological findings present simultaneously, suggesting that surviving infants might develop respiratory sequelae (Figure 3D and 3E). At this time point, no differences were observed in the number of $\gamma\delta$ T cells or neutrophils between infected and uninfected animals (Figure 3F and 3G). Additionally, evaluation of the number of other myeloid cells (eosinophils and inflammatory monocytes) revealed no differences between groups, either in the lungs or systemically in the spleen (Supplementary Figure 2). To further explore the phenotype of $\gamma\delta$ T cells, their residency profile was analysed using the residency markers CD69, CD44 and CD62L (Figure 3H, gating strategy). Our data revealed that $\gamma\delta$ T cells in the lungs of animals surviving neonatal pneumonia exhibited an activated

CD69⁺CD44⁺CD62L⁻ profile, consistent with tissue-resident effector T cells (Figure 3H-J),

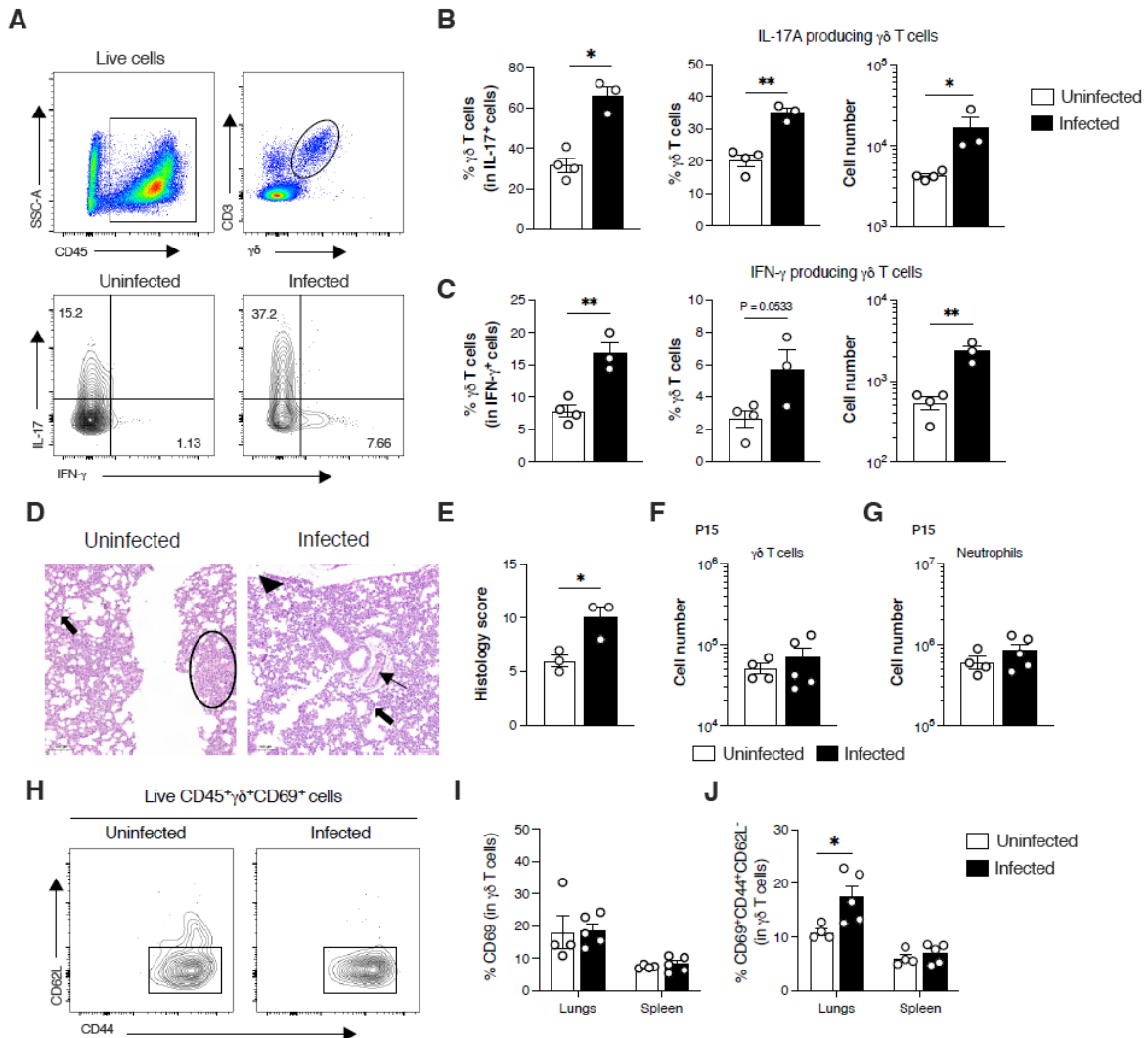


Figure 3. $\gamma\delta$ T cells are a major source of IL-17A during GBS neonatal pneumonia. Pregnant C57BL/6 female mice were intravaginally colonised with 4×10^4 CFU GBS hyper virulent strain BM110, at gestational days 16th and 17th. Animals from uninfected and infected groups were sacrificed at P3 or P15. **(A)** Representative gating strategy used to define $\gamma\delta$ T cells and IL-17A and IFN- γ positive cells. Live cells were gated in SSC-A vs CD45⁺ cells. The following cells were gated within CD45⁺ cells. $\gamma\delta$ T cells were defined as CD3⁺ $\gamma\delta$ ⁺. The intracellular markers IL-17A or IFN- γ were analysed withing $\gamma\delta$ T. **(B-C)** Frequency and number of IL-17 **(B)** and IFN- γ **(C)** produced by $\gamma\delta$ T cells in the lungs at P3. Data is presented as mean $\pm$ SEM, each symbol indicates data from a single pup [n = 4 (uninfected); n = 3 (infected)]. **(D)** Representative images of tissue slices of lungs of uninfected and infected mice stained with H&E, at P15. Scale bar 100 μ m. Black arrows indicate thickening of the interalveolar septum (septal thickening). Arrowhead indicates hemorrhage. Thin black arrow indicates vascular congestion. Ellipse indicates atelectasis. **(E)** Global histopathology score was attributed to lungs stained with H&E. Data are combined from two independent experiments and shown as mean $\pm$ SEM, each symbol represents data from single pup (n = 3). **(F)** Number of $\gamma\delta$ T cells in the lungs at P15. Data is presented as mean $\pm$ SEM [n = 4 (uninfected); n = 5 (infected)]. **(G)** Number of neutrophils in the lungs at P15. Data is presented as mean $\pm$ SEM [n = 4 (uninfected); n = 5 (infected)]. **(H)** Representative contour plots of tissue resident $\gamma\delta$ T cell. The profile of tissue resident of $\gamma\delta$ T cells was define by CD45⁺ $\gamma\delta$ ⁺CD69⁺CD44⁺CD62L⁻. **(I-J)** Frequency of indicated cell populations among $\gamma\delta$ T cells. Data is presented as mean $\pm$ SEM [n = 4 (uninfected); n = 5 (infected)]. Comparisons by Student's t-test. Statistical differences (P values) between groups are indicated. * P < 0.05; ** P < 0.01.

suggesting that these cells might have the potential to rapidly expand in response to a secondary challenge.

Altogether, these findings show that infected pups are able to efficiently recruit neutrophils into the lungs early in the course of infection. Furthermore, $\gamma\delta$ T cells mount a rapid response to GBS infection and are a major source of IL-17, indicating their potential role in neutrophil recruitment.

SiglecF^{hi} neutrophils accumulate in the neonatal lungs during moderate disease

Given that infected pups efficiently recruited neutrophils to infected tissue, we sought to understand the underlying factors contributing to the observed mortality. To this end, our analysis was stratified by disease severity at P3, a time point at which physical distress are clearly manifested in severely affected animals. When bacterial colonisation in the lungs was stratified by severity (moderate or severe), we found that animals with severe disease had a significantly higher bacterial load compared to those with moderate disease, indicating a diminished capacity to control the infection (Figure 4A). To determine whether disease severity was associated with increased local inflammation, the expression of genes encoding key inflammatory cytokines, such as TNF- α , IL-1 β , IL-33, IL-17, and the anti-inflammatory cytokine IL-10 was determined in the lungs of pups presenting moderate or severe pathology by real-time qPCR (RT-qPCR). The results revealed a relatively weak inflammatory profile, with a significant increase only in the pro-inflammatory cytokine *Il1b* in animals with severe disease, compared to uninfected controls (Figure 4B). Despite differences in bacterial load, the gene expression of *Tnfa*, *Il33*, *Il17* and *Il10* did not significantly differ between moderate and severe groups (Figure 4B). However, a tendency towards higher gene expression of *Tnfa* was observed in pups with severe disease ($P = 0.0567$) (Figure 4B).

Based on previous findings showing efficient neutrophil recruitment to the lungs at early stages of infection (Figure 2C, right), it was next assessed whether the neutrophil population was altered in pups with severe disease (Figure 4C, gating strategy). The obtained results showed that both pups with moderate and severe disease had significantly higher frequency and numbers of neutrophils compared to uninfected controls (Figure 4D). Interestingly, no differences were observed in this population between pups with moderate and severe GBS disease (Figure 4D). Given that neutrophils are a heterogeneous population, we hypothesised that the neutrophils present in the lungs during neonatal pneumonia could exhibit distinct phenotypes depending on lung pathology. To address this, we performed a deeper analysis of lungs neutrophils and identified a subset co-expressing the lectin

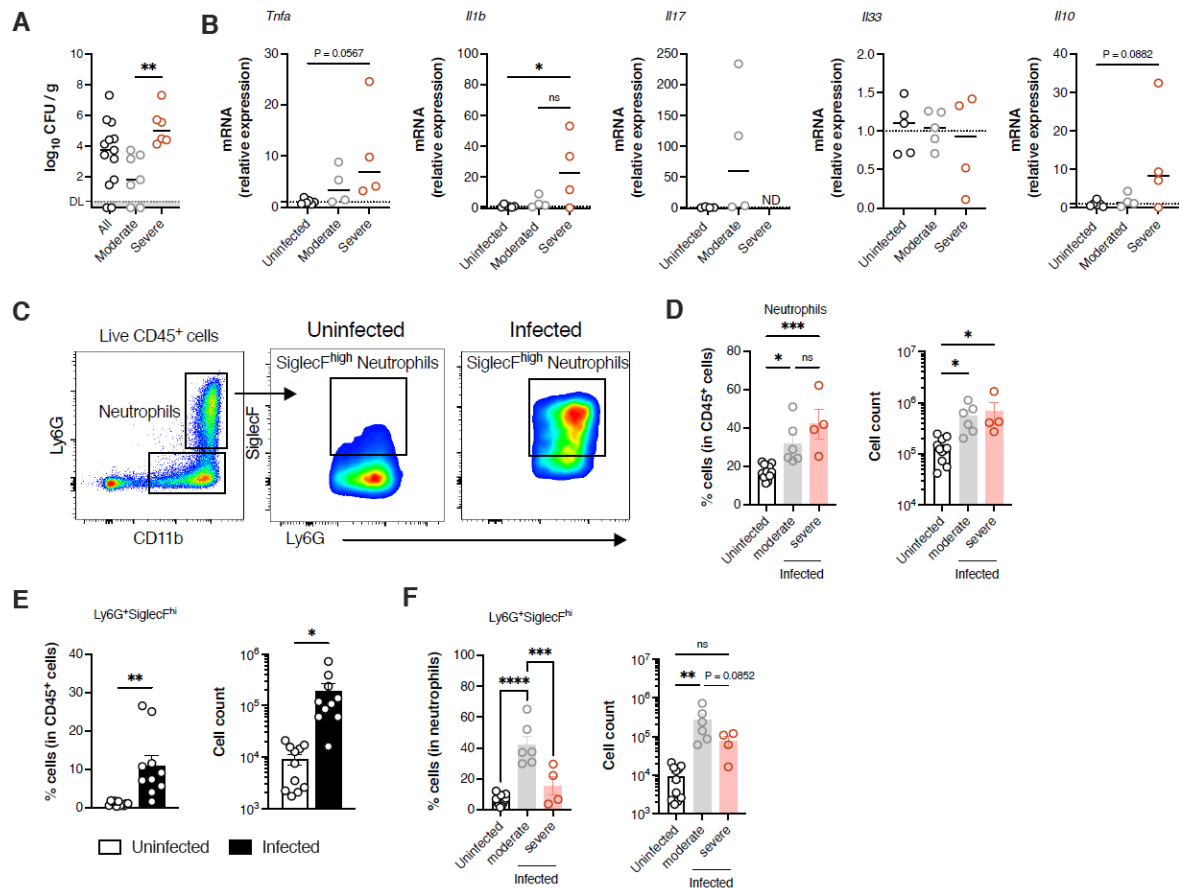


Figure 4. SiglecF^{hi} neutrophils are recruited to the lungs during moderate neonatal pneumonia. Pregnant C57BL/6 female mice were intravaginally colonised with 4×10^4 CFU GBS hyper virulent strain BM110, at gestational days 16th and 17th. Animals from uninfected and infected groups were sacrificed at P3. Animals stratification was made by dividing data as All (all infected animals), Moderate (infected animals with moderate signs of disease) and Severe (infected animals with severe signs of disease). **(A)** Bacterial loads in the lung determined by disease severity. Data are presented as mean [n = 13 (all); n = 7 (moderate); n = 6 (severe)]. **(B)** Relative gene expression of the indicated pro- and anti-inflammatory cytokines evaluated by RT-qPCR, normalized for the reference gene Hprt. Samples are stratified by disease severity. Data are presented as mean $\pm$ SEM [n = 5 (uninfected); n = 4-5 (moderate); n = 4 (severe)]. **(C)** Representative gating strategy used to define neutrophils profile. Cells were gated in live CD45⁺ cells. Neutrophils were defined CD11b⁺Ly6G⁺. Profile of neutrophils was defined as CD11b⁺Ly6G⁺SiglecF^{hi} or CD11b⁺Ly6G⁺SiglecF^{neg/low}. **(D)** Frequency and number of neutrophils in uninfected, moderate and severe groups. Data is presented as mean $\pm$ SEM [n = 11 (uninfected); n = 6 (moderate); n = 4 (severe)]. **(E)** Frequency and number of SiglecF^{hi} neutrophils in uninfected and infected animals. Data are presented as mean $\pm$ SEM [n = 11 (uninfected); n = 10 (infected)]. **(F)** Frequency and number of SiglecF^{hi} neutrophils in the lung of infected pups according to disease severity. Data are presented as mean $\pm$ SEM [n = 11 (uninfected); n = 6 (moderate); n = 4-5 (severe)]. Each symbol indicates data from single pup. For comparisons between two groups, Student's t test was used. Comparisons between three groups, one-way ANOVA with multiple comparisons was used. Statistical differences (P values) between groups are indicated. * P < 0.05; ** P < 0.01; *** P < 0.001, and **** P < 0.0001. ND, not detected. ns, not significant.

SiglecF, designated as SiglecF^{hi} neutrophils (figure 4C). The lungs of infected animals exhibited significantly higher numbers of the SiglecF^{hi} neutrophils compared to uninfected controls, where this population was rare (Figure 4E). Furthermore, pups with moderate

disease presented a significant increase in the frequency of SiglecF^{hi} neutrophils within the total neutrophil population. In contrast, this increase was not pronounced in animals with severe disease, suggesting that the presence of SiglecF^{hi} neutrophils could be important in bacterial clearance and contribute to neonatal survival (Figure 4F).

SiglecF^{hi} neutrophils exhibit enhanced phagocytic capacity and contribute to bacterial clearance

Having found that neutrophils are efficiently recruited into the lungs of neonatal mice during GBS pneumonia and that their phenotype varies with disease severity, we sought to investigate whether SiglecF^{hi} neutrophils play a role in local bacterial clearance. To assess their potential contribution, the phagocytic capacity of SiglecF^{hi} neutrophils was evaluated by performing an *ex vivo* bacterial killing assay. Neutrophils were sorted from the neonatal lungs based on their expression of SiglecF (CD45⁺Ly6G⁺CD11b⁺SiglecF^{hi} and CD45⁺Ly6G⁺CD11b⁺SiglecF^{neg/low}) and incubated with GBS (MOI = 5). The percentage of phagocytosis was then obtained. Our results demonstrated a significant increase in phagocytic capacity of the SiglecF^{hi} subset compared to the SiglecF^{neg/low} neutrophils (Figure 5A). To further elucidate the origin of the SiglecF^{hi} neutrophils during GBS pneumonia, the presence of this population was assessed in the peripheral blood and spleen of infected pups at P3, comparing the results with uninfected controls. The total number of circulating neutrophils, as well as the frequency of SiglecF^{hi} subset among neutrophils, did not differ between the groups (Figure 5B and 5C). Additionally, analysis of mean fluorescence intensity (MFI) revealed no significant differences in the levels of SiglecF in the cell surface (Figure 5D). Similarly, no differences in the frequency and number of neutrophils were observed in the spleen (Figure 5E), nor in the SiglecF^{hi} subset (Figure 5F), suggesting that SiglecF^{neg/low} neutrophils may acquire the SiglecF^{hi} phenotype in response to stimuli given within the lungs environment.

To determine whether the differences in neutrophil phenotypes were exclusive to the hypervirulent CC17 GBS strain used, and to model the disease in a less severe scenario, we conducted infection studies using a non-CC17 GBS strain (NEM316), which belongs to capsular serotype III and the CC23 clone³⁷. Compared with pups born from CC17-colonised mothers, pups born to non-CC17 colonised progenitors showed significantly increased survival (Figure 5G). Moreover, we found no significant difference in bacterial burden between non-CC17 and CC17 infected pups presenting moderate disease (Figure 5H). Concomitantly, the expression of *Il1b* in non-CC17-infected pups did not differ significantly from uninfected controls (Figure 5I). We further investigated whether non-CC17-infected

animals would also present increased neutrophils in the lungs, as previously observed with

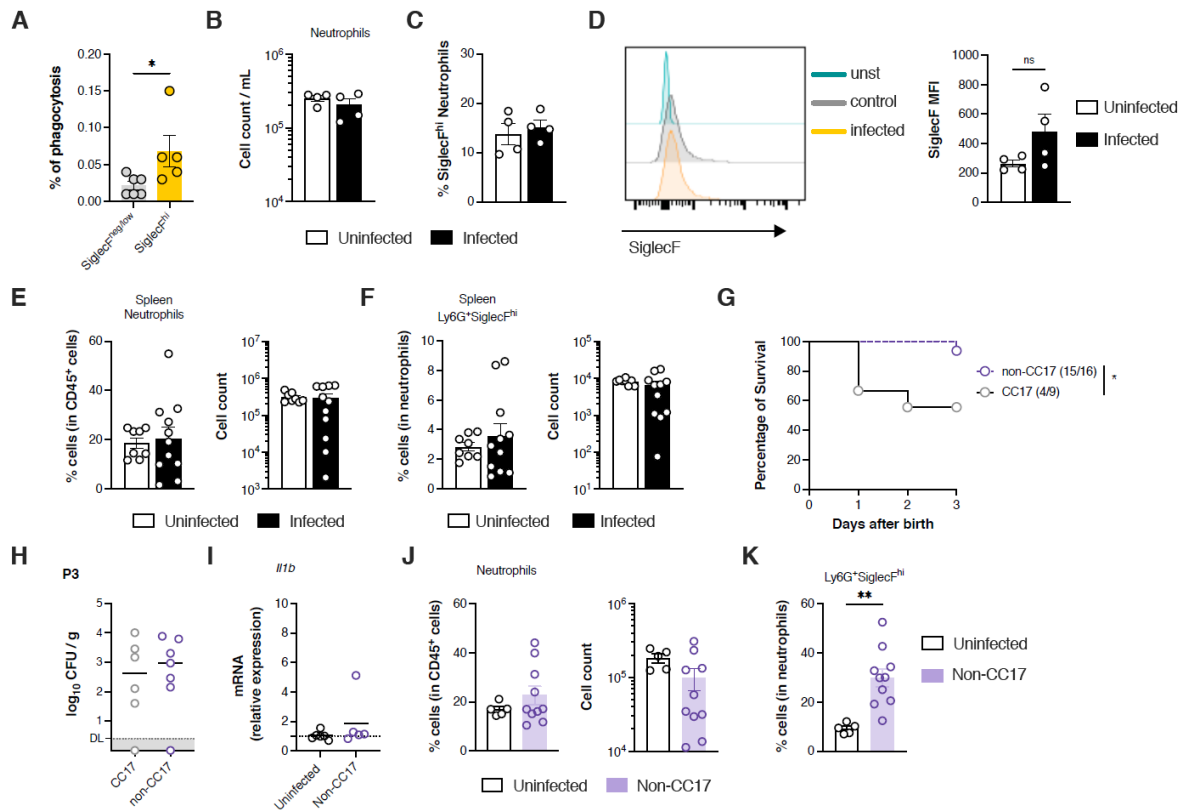


Figure 5. SiglecF^{hi} neutrophils have higher phagocytic capacity and contribute to host defence in GBS neonatal pneumonia. (A) Gentamycin protection assay for phagocytosis analysis of SiglecF^{hi} and SiglecF^{neg/low} sorted from the neonatal mouse lung after 1-h incubation with GBS at a MOI of 5 and enumeration of CFUs determined by serial dilution plating. Pooled data from two independent experiments (n = 6, SiglecF^{neg/low}; n = 5, SiglecF^{hi}). Each symbol indicates a replica. Statistical differences (P values) between groups are indicated. Comparisons by Student's t-test. * P < 0.05. (B-F) Pregnant C57BL/6 female mice were intravaginally colonised with 4x10⁴ CFU GBS hyper virulent strain BM110, at gestational days 16th and 17th. Animals from uninfected and infected groups were sacrificed at P3. (B) Cell number of circulating neutrophils per mL of blood. (C) Percentage of SiglecF^{hi} neutrophils in circulation. (D) Quantification of SiglecF on neutrophils, presented as mean fluorescence intensity (MFI). Representative histograms are shown. Yellow line, infected pups with moderate disease; Grey line, uninfected pups; Green line, unstained. Data are presented as mean ± SEM (n = 4 per group). (E) Frequency and number of neutrophils in the spleen. Data is presented as mean ± SEM [n = 8 (uninfected); n = 11 (infected)]. (F) Frequency and number of SiglecF^{hi} neutrophils in the spleen. Data is presented as mean ± SEM [n = 8 (uninfected); n = 11 (infected)]. (G-K) Pregnant C57BL/6 female mice were intravaginally colonised with 4x10⁴ CFU of GBS hyper virulent strain BM110 (CC17) or the NEM316 strain (non-CC17), at gestational days 16th and 17th. (G) Kaplan-Meier survival curve of neonatal mice born from GBS-colonised dams, monitored during a 3 days' period. The numbers in parentheses represent the number of animals that survived versus the total number of animals born. (H) Newborn mice were sacrificed at postnatal day 3 and the lung bacterial loads were determined. Data are presented as mean ± SEM [n = 6 (CC17); n = 7 (non-CC17)]. (I) Relative expression of Il1b analysed by RT-qPCR and normalized for the reference gene Hprt. Data are presented as mean ± SEM [n = 6 (uninfected); n = 5 (non-CC17)]. (J) Frequency and number of neutrophils in the lung of pups born from non-CC17 colonised dams. Data are presented as mean ± SEM [n = 5 (uninfected); n = 10 (non-CC17)]. (K) Frequency of Ly6G⁺SiglecF^{hi} neutrophils in the lung. Data are presented as mean ± SEM [n = 5 (uninfected); n = 10 (non-CC17)]. Each symbol indicates data from single pup. Comparisons by Student's t-test. Statistical differences (P values) between groups are indicated. ** P < 0.01. ns, not significant.

the CC17 strain. Notably, no significant differences in either the percentage or number of neutrophils were detected between uninfected and non-CC17-infected pups (Figure 5J). However, approximately 30% of neutrophils in the lungs of non-CC17-infected pups exhibited the SiglecF^{hi} phenotype, similarly to the results obtained from animals infected with the virulent strain and exhibiting moderate disease (Figure 5K).

In summary, the accumulation of SiglecF^{hi} neutrophils in the lungs of the infected animals, combined with their increased phagocytic capacity, sustains the hypothesis that this immune population plays a critical role in bacterial clearance during neonatal GBS pneumonia.

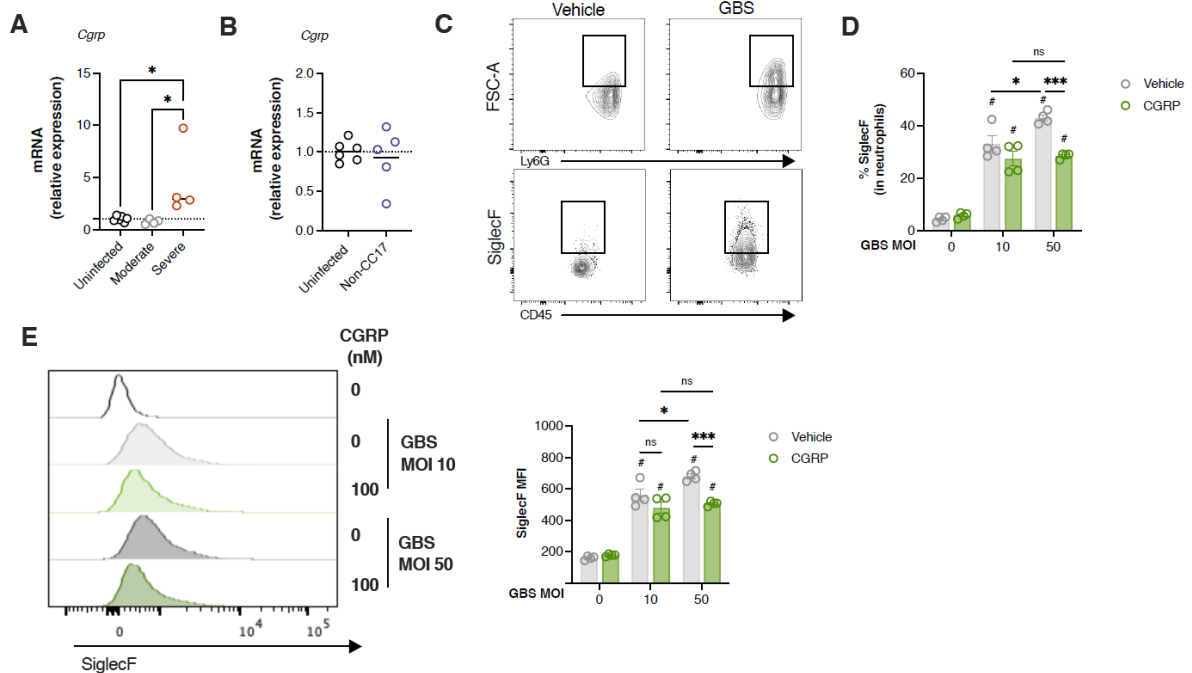


Figure 6. CGRP suppress neutrophil activation during GBS infection. (A-B) Pregnant C57BL/6 female mice were intravaginally colonised with 4×10^4 CFU of GBS hyper virulent strain BM110 (CC17) or the NEM316 strain (non-CC17), at gestational days 16th and 17th. Animals from uninfected and infected groups were sacrificed at P3. For pups born from CC17 progenitors, samples are stratified by disease severity as Moderate (infected animals with moderate signs of disease) and Severe (infected animals with severe signs of disease). Relative gene expression of *Cgrp* evaluated by RT-qPCR, normalized for the reference gene *Hprt*. Data are presented as mean $\pm$ SEM [$n = 6$ (uninfected); $n = 4$ (moderate); $n = 4$ (severe); $n = 5$ (Non-CC17)]. **(C-E)** Mouse neutrophils were incubated with heat-killed GBS at the indicated MOI in the presence of CGRP or vehicle for 4 h. The frequency of SiglecF^{hi} neutrophils was determined by flow cytometry analyses. **(C)** Representative gating strategy used to analyse neutrophils. **(D)** Frequency of SiglecF^{hi} among neutrophils in the indicated groups. Data is presented as mean $\pm$ SEM. **(E)** Left, Histogram overlays of representative examples of SiglecF channel fluorescence of neutrophils incubated with the indicated conditions. Right, quantification of SiglecF on neutrophils, presented as mean fluorescence intensity (MFI). Data are presented as mean $\pm$ SEM ($n = 4$ samples/group). Statistical differences (P values) between groups are indicated. Statistic on the top of each condition represents a comparison with controls. #, * $P < 0.05$, *** $P < 0.001$. ns, not significant.

Severe GBS pneumonia induces CGRP expression in the lungs dampening SiglecF^{hi} neutrophil development

Nociceptor activation triggers the release of calcitonin gene-related peptide (CGRP) from nerve terminals²² and has been implicated in bacteria-induced neuroimmune evasion by inhibiting neutrophil recruitment and killing^{6,23}. Thus, we next sought to investigate whether this neuropeptide is upregulated in our vertical transmission model of GBS pneumonia, by analysing the relative expression of *Cgrp* in the lungs of infected neonates. A significant increase in *Cgrp* expression was observed in animals with severe disease compared to both uninfected controls and animals with moderate disease (Figure 6A). In contrast, pups infected with the non-CC17 strain did not present any change in *Cgrp* gene expression (Figure 6B).

Given that at this time point, animals with severe disease were still capable of efficiently recruiting neutrophils while also upregulating the *Cgrp* gene, we next examined whether CGRP release by sensory neurons could suppress the conversion of neutrophils into the SiglecF^{hi} phenotype. To this end, neutrophils isolated from the bone-marrow were incubated with different MOI of heat-killed GBS in the presence of CGRP or vehicle control. Bacterial stimulation resulted in an increase in neutrophil size, as indicated by enhanced neutrophil forward scatter (FSC), suggesting activation of these cells (Figure 6C). Among FSC^{hi} neutrophils, GBS stimulation significantly increased the proportion of the SiglecF^{hi} phenotype in a dose-dependent manner (Figure 6D). However, when neutrophils were incubated with CGRP, this increase in the SiglecF^{hi} population was significantly inhibited, particularly at the higher dose (MOI = 50) (Figure 6D). In line with this finding, CGRP also led to a reduced increase in SiglecF MFI (Figure 6E).

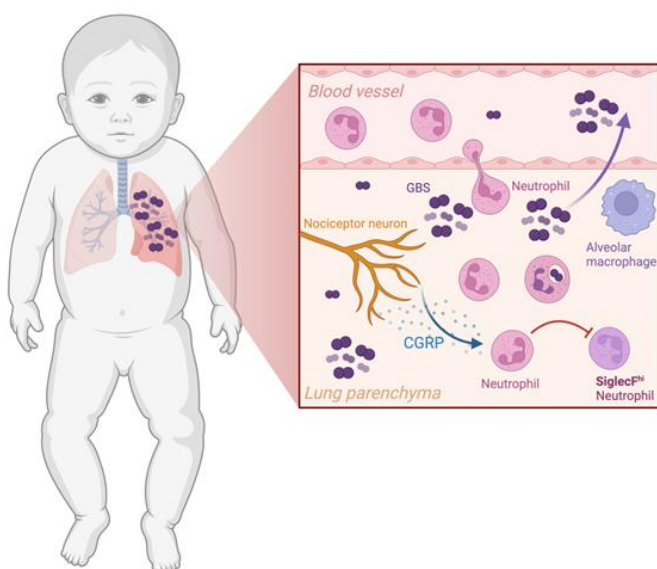


Figure 7. Schematic representation of a proposed mechanism by which GBS hijack the neuro-immune axis to evade the host innate immune system. Group B Streptococcus (GBS) interact and activate nociceptor neurons in the lung, which releases the neuropeptide CGRP. CGRP appears to hinder neutrophil activation into SiglecF^{hi} neutrophil, a profile associated with increased bacterial clearance. This illustration is for explanatory purposes and is not drawn to scale. Created with Biorender.com.

In conclusion, our findings suggest that CGRP upregulation during severe GBS pneumonia impairs the development of SiglecF^{hi} neutrophils, potentially hindering effective bacterial clearance and contributing to worsened disease outcomes (Figure 7).

Discussion

The greatest risk of death from pneumonia in childhood is in the neonatal period. GBS disease is typically marked by respiratory symptoms within the first few hours of life, reflecting an initial pulmonary focus of infection³⁸. It is estimated that more than 80% of infected newborns present with respiratory pathology, which may predispose them to more severe pulmonary complications, such as pneumonia^{3,7}. Differences in immune system development likely contribute to the increased vulnerability of newborns to pneumonia compared to adults³⁹. However, a lack of in-depth understanding of neonatal immunity in response to bacterial pneumonia limits our ability to effectively reduce neonatal infections and mortality.

Traditionally, neutrophils have been viewed as the main culprits behind inefficient bacterial clearance in neonates. In this work, we used a vertical transmission model of neonatal GBS pneumonia and demonstrated that, while neutrophils are effectively recruited into the lungs of neonates, their phenotype varies with disease severity. We identified a subset of neutrophils, characterised by high SiglecF expression (SiglecF^{hi}), present in neonates with moderate disease but absent in those with severe disease. Our findings further suggest that GBS infection triggers the release of CGRP from sensory neurons, inhibiting neutrophil maturation into the SiglecF^{hi} phenotype in cases of severe disease.

The neonatal immune system is widely considered immature, which is commonly linked to an increased risk of neonatal pneumonia. Numerous studies have demonstrated impaired neutrophil amplification, mobilisation, and function, especially during severe sepsis, a condition that can follow or co-occur with neonatal pneumonia. This impaired response often leads to inadequate bacterial clearance, allowing the pathogen to spread and exacerbating disease severity⁴⁰⁻⁴². To date, therapies aimed at enhancing granulopoiesis during sepsis, including the use of myeloid-specific growth factors, such as granulocyte-colony stimulating factor (G-CSF) and granulocyte/macrophage colony-stimulating factor (GM-CSF), have not proven effective in improving survival in septic infants⁴³. Elevated endogenous G-CSF levels in septic neonates suggest that end-organ unresponsiveness may contribute to this lack of therapeutic efficacy⁴⁴. In our study, we found that neutrophils were efficiently recruited into the lungs of pups with neonatal pneumonia. This contrasts with our previous studies, which found that inhibiting TLR2 or IL-10 signalling improved

neonatal survival by promoting effective neutrophil migration into infected tissues and enhancing bacterial clearance^{31,32}. A possible explanation for these discrepancies might rely on the infection route used. In the present study, we used a vertical transmission model of GBS, which more closely mimics human pathophysiology, whereas previous studies used hematogenous dissemination. This reinforces the importance of using appropriate animal models when studying human diseases. We similarly showed that neutrophils are a relevant population for bacterial clearance, however, while neutrophils are essential, their phenotype - rather than just their presence - affects bacterial clearance and disease severity.

Further analysis of lungs neutrophil phenotypes during neonatal pneumonia unexpectedly revealed a neutrophil population co-expressing the eosinophil-specific surface marker SiglecF, a rare population in uninfected animals. Notably, we report here for the first time that neutrophils in animals with severe disease fail to transition into the SiglecF^{hi} phenotype. Recently discovered, SiglecF-expressing neutrophils have been implicated in various diseases, including lung tumours^{17,45}, infarcted heart^{46,47}, airway inflammation and allergy⁴⁸, chronic kidney disease⁴⁹, and bacterial infection⁵⁰. These cells have been shown to have a greater capacity for producing reactive oxygen species (ROS) and forming NETs^{18,45,50}, which could enhance bacterial control in our model. Indeed, we found that SiglecF^{hi} neutrophils isolated from neonatal lungs exhibited higher phagocytic capacity, supporting their crucial role in bacterial clearance and neonatal survival. Furthermore, in a less severe model of neonatal disease, where pathology was reduced and survival increased, neutrophils expressed SiglecF, supporting our hypothesis that increased severity in GBS pneumonia may be linked to bacterial immune evasion through neutrophil inhibition.

There is growing recognition that the nervous system regulation of immunity is essential for tissue homeostasis and defence. In recent years, the role of neuropeptides in immune modulation has gained considerable attention, highlighting a unique crosstalk between neurons and immune cells. Peripheral neurons, including sensory neurons, can release neuropeptides locally, which in turn regulate immune responses through signalling via receptors expressed on immune cells^{51,52}. CGRP, a potent vasodilator and neuropeptide primarily found in the peripheral and central nervous systems (CNS), exerts diverse effects on immune cells and inflammatory processes, influencing pain regulation, inflammation, and host defence mechanisms^{20-22,53,54}. Emerging studies have identified the CGRP pathway as a key factor in immune evasion strategies used by various pathogens, including GBS^{6,23,55}.

Recently, in an adult mouse model of GBS meningitis, it was shown that genetic ablation of Nav1.8 sensory neurons, a voltage-gated sodium channel expressed by nociceptors, decreased meningeal and brain colonisation by GBS⁵⁵. This study found that GBS activated

nociceptors to release CGRP in the meninges, contributing to meningitis pathogenesis, with systemic CGRP injection increasing CNS invasion⁵⁵. During *Streptococcus pyogenes* infection, activation of nociceptor neurons led to CGRP release, which in turn inhibited neutrophil recruitment to infected skin, causing dermonecrosis and weight loss²³. Similarly, the selective genetic ablation of TRPV1+ sensory neurons inhibited CGRP release, restoring neutrophil recruitment to infection sites²³. Activation of lungs sensory neurons has also been shown to inhibit neutrophil recruitment in a model of lethal *Staphylococcus aureus* pneumonia⁶. Notably, although we observed efficient neutrophil recruitment in the acute phase of neonatal pneumonia, they failed to activate into a more phagocytic phenotype, likely due to CGRP. However, it is possible that CGRP upregulation also acts as a regulatory mechanism to prevent excessive inflammation. CGRP has been shown to mediate immune suppression in pulmonary tissue, mainly by inhibiting neutrophil recruitment and $\gamma\delta$ T cell-mediated defence in bacterial lungs infections⁶. Additionally, CGRP can induce IL-10⁵⁶, and systemic IL-10 levels increase during neonatal GBS infection^{31,32}. Thus, immunosuppression could diminish effective and protective antibacterial immunity, thereby increasing susceptibility, as previously described in a mouse model of neonatal GBS sepsis³¹.

Our study also demonstrates that neonatal tissue-resident $\gamma\delta$ T cells respond rapidly to infection and serve as a primary source of IL-17 in the lungs, which promotes neutrophil recruitment and likely enhances anti-pathogen immunity. $\gamma\delta$ T cells have been shown to secrete IL-17 during infections with *Mycobacterium tuberculosis*, *Escherichia coli*, and *Bordetella pertussis*, facilitating neutrophil recruitment^{50,57,58}. Our study suggests that this mechanism may play an important role in GBS clearance, as $\gamma\delta$ T cells produce IL-17, leading to neutrophil recruitment into the lungs and acting as innate first responders. In mice infected with *B. pertussis*, lung-resident $\gamma\delta$ T cells are an early source of IL-17, which is essential for protection⁵⁹. IL-17 production upon nasal infection with *B. pertussis* is associated with the recruitment of SiglecF⁺ neutrophils, which are also crucial for protection⁵⁰. However, the presence of $\gamma\delta$ T cells may also exacerbate local inflammation in certain circumstances. In breast cancer metastasis, IL-17-producing $\gamma\delta$ T cells drive systemic expansion and polarisation of neutrophils, which then suppress antigen-specific CD8⁺ T cells⁶⁰. In infections and inflammatory disorders, the $\gamma\delta$ T cell/IL-17/neutrophil axis can drive disease pathogenesis^{60,61}. Thus, it is plausible that $\gamma\delta$ T cells could contribute to disease severity rather than bacterial control. It has been shown that nociceptor neurons regulate lung-resident $\gamma\delta$ T cells, as mice either genetically modified or with chemically ablated nociceptor neurons have higher numbers of these cells⁶. In our model, CGRP released by nociceptor sensory neurons may also regulate lung-resident $\gamma\delta$ T cells

independently of neutrophils. Alternatively, CGRP could suppress $\gamma\delta$ T cell activity in animals with severe disease, reducing IL-17 production and consequently impairing neutrophil recruitment and bacterial clearance.

Collectively, our study uncovers that higher severity in GBS pneumonia might be linked to the bacteria's ability to evade the host immune system by inhibiting neutrophil activation, thereby impairing infection control. Based on our findings, we hypothesise that GBS hijacks the neuroimmune axis by inducing high levels of CGRP, which inhibits neutrophil activation. CGRP inhibition or modulation of neuroimmune interactions could restore neutrophil function and enhance bacterial clearance, providing a potential therapeutic target for severe neonatal infectious diseases.

References

1. Collaborators, G. B. D. U.-M. Global, regional, and national progress towards Sustainable Development Goal 3.2 for neonatal and child health: all-cause and cause-specific mortality findings from the Global Burden of Disease Study 2019. *Lancet* 398, 870-905, doi:10.1016/S0140-6736(21)01207-1 (2021).
2. Hooven, T. A. & Polin, R. A. Pneumonia. *Semin Fetal Neonatal Med* 22, 206-213, doi:10.1016/j.siny.2017.03.002 (2017).
3. Maldonado, Y., Nizet, V., Barnett, E. D., Edwards, K. M. & Malley, R. *Remington and Klein's Infectious Diseases of the Fetus and Newborn Infant*. (Elsevier Health Sciences, 2024).
4. Tavares, T., Pinho, L. & Bonifacio Andrade, E. Group B Streptococcal Neonatal Meningitis. *Clin Microbiol Rev*, e0007921, doi:10.1128/cmr.00079-21 (2022).
5. Randis, T. M., Baker, J. A. & Ratner, A. J. Group B streptococcal infections. *Pediatrics in Review* 38, 254-262 (2017).
6. Baral, P. et al. Nociceptor sensory neurons suppress neutrophil and $\gamma\delta$ T cell responses in bacterial lung infections and lethal pneumonia. *Nat Med* 24, 417-426, doi:10.1038/nm.4501 (2018).
7. Gynecology, O. Prevention of group B streptococcal earlyonset disease in newborns: ACOG committee opinion, number 797. *Obstet Gynecol* 135, e51-72 (2020).
8. Ranieri, V. M. et al. Acute respiratory distress syndrome: the Berlin Definition. *JAMA: Journal of the American Medical Association* 307 (2012).

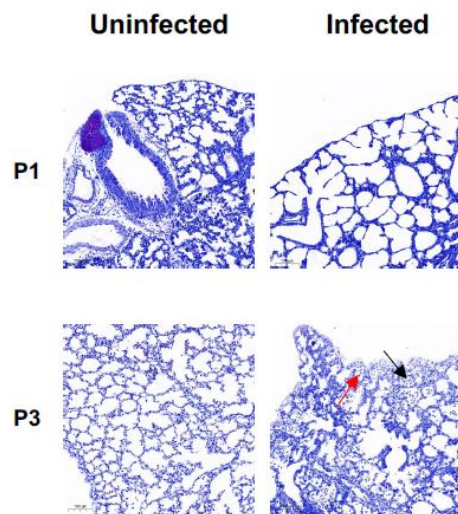
9. Group, P. A. L. I. C. C. Pediatric acute respiratory distress syndrome: consensus recommendations from the Pediatric Acute Lung Injury Consensus Conference. *Pediatric critical care medicine* 16, 428-439 (2015).
10. Hemming, V. G., McCloskey, D. W. & Hill, H. R. Pneumonia in the neonate associated with group B streptococcal septicemia. *American Journal of Diseases of Children* 130, 1231-1233 (1976).
11. Tsafaras, G. P., Ntontsi, P. & Xanthou, G. Advantages and Limitations of the Neonatal Immune System. *Front Pediatr* 8, 5, doi:10.3389/fped.2020.00005 (2020).
12. Kollmann, T. R., Kampmann, B., Mazmanian, S. K., Marchant, A. & Levy, O. Protecting the Newborn and Young Infant from Infectious Diseases: Lessons from Immune Ontogeny. *Immunity* 46, 350-363, doi:10.1016/j.immuni.2017.03.009 (2017).
13. Burn, G. L., Foti, A., Marsman, G., Patel, D. F. & Zychlinsky, A. The Neutrophil. *Immunity* 54, 1377-1391, doi:10.1016/j.immuni.2021.06.006 (2021).
14. Levy, O. *et al.* Impaired innate immunity in the newborn: newborn neutrophils are deficient in bactericidal/permeability-increasing protein. *Pediatrics* 104, 1327-1333, doi:10.1542/peds.104.6.1327 (1999).
15. Turkmen, M., Satar, M. & Atici, A. Neutrophil chemotaxis and random migration in preterm and term infants with sepsis. *Am J Perinatol* 17, 107-112, doi:10.1055/s-2000-9507 (2000).
16. Yost, C. C. *et al.* Impaired neutrophil extracellular trap (NET) formation: a novel innate immune deficiency of human neonates. *Blood* 113, 6419-6427, doi:10.1182/blood-2008-07-171629 (2009).
17. Pfirschke, C. *et al.* Tumor-Promoting Ly-6G(+) SiglecF(high) Cells Are Mature and Long-Lived Neutrophils. *Cell Rep* 32, 108164, doi:10.1016/j.celrep.2020.108164 (2020).
18. Matsui, M., Nagakubo, D., Satooka, H. & Hirata, T. A novel Siglec-F(+) neutrophil subset in the mouse nasal mucosa exhibits an activated phenotype and is increased in an allergic rhinitis model. *Biochem Biophys Res Commun* 526, 599-606, doi:10.1016/j.bbrc.2020.03.122 (2020).
19. Blake, K. J., Jiang, X. R. & Chiu, I. M. Neuronal Regulation of Immunity in the Skin and Lungs. *Trends Neurosci* 42, 537-551, doi:10.1016/j.tins.2019.05.005 (2019).
20. Chiu, I. M., Pinho-Ribeiro, F. A. & Woolf, C. J. Pain and infection: pathogen detection by nociceptors. *Pain* 157, 1192-1193 (2016).
21. Trankner, D., Hahne, N., Sugino, K., Hoon, M. A. & Zuker, C. Population of sensory neurons essential for asthmatic hyperreactivity of inflamed airways. *Proc Natl Acad Sci U S A* 111, 11515-11520, doi:10.1073/pnas.1411032111 (2014).

22. Baral, P., Udit, S. & Chiu, I. M. Pain and immunity: implications for host defence. *Nat Rev Immunol* 19, 433-447, doi:10.1038/s41577-019-0147-2 (2019).
23. Pinho-Ribeiro, F. A. *et al.* Blocking neuronal signaling to immune cells treats streptococcal invasive infection. *Cell* 173, 1083-1097. e1022 (2018).
24. Lin, F.-Y. C. *et al.* Phylogenetic lineages of invasive and colonizing strains of serotype III group B streptococci from neonates: a multicenter prospective study. *Journal of clinical microbiology* 44, 1257-1261 (2006).
25. Jones, N. *et al.* Multilocus sequence typing system for group B streptococcus. *Journal of clinical microbiology* 41, 2530-2536 (2003).
26. Ubags, N. D. & Suratt, B. T. Isolation and characterization of mouse neutrophils. *Lung Innate Immunity and Inflammation: Methods and Protocols*, 45-57 (2018).
27. Korkmaz, F. T. & Traber, K. E. Innate immune responses in pneumonia. *Pneumonia (Nathan)* 15, 4, doi:10.1186/s41479-023-00106-8 (2023).
28. Torres, A. *et al.* Pneumonia. *Nat Rev Dis Primers* 7, 25, doi:10.1038/s41572-021-00259-0 (2021).
29. Andrade, E. B. *et al.* A mouse model reproducing the pathophysiology of neonatal group B streptococcal infection. *Nat Commun* 9, 3138, doi:10.1038/s41467-018-05492-y (2018).
30. Le Doare, K. & Heath, P. T. An overview of global GBS epidemiology. *Vaccine* 31, D7-D12 (2013).
31. Andrade, E. B. *et al.* TLR2-induced IL-10 production impairs neutrophil recruitment to infected tissues during neonatal bacterial sepsis. *J Immunol* 191, 4759-4768, doi:10.4049/jimmunol.1301752 (2013).
32. Madureira, P. *et al.* Inhibition of IL-10 production by maternal antibodies against Group B Streptococcus GAPDH confers immunity to offspring by favoring neutrophil recruitment. *PLoS Pathog* 7, e1002363, doi:10.1371/journal.ppat.1002363 (2011).
33. Xi-zhi, J. G. *et al.* Lung $\gamma\delta$ T cells mediate protective responses during neonatal influenza infection that are associated with type 2 immunity. *Immunity* 49, 531-544. e536 (2018).
34. Khairallah, C., Chu, T. H. & Sheridan, B. S. Tissue adaptations of memory and tissue-resident gamma delta T cells. *Frontiers in immunology* 9, 2636 (2018).
35. Schittny, J. C., Mund, S. I. & Stampanoni, M. Evidence and structural mechanism for late lung alveolarization. *American Journal of Physiology-Lung Cellular and Molecular Physiology* 294, L246-L254 (2008).
36. de Kleer, I. M. *et al.* Perinatal Activation of the Interleukin-33 Pathway Promotes Type 2 Immunity in the Developing Lung. *Immunity* 45, 1285-1298, doi:10.1016/j.immuni.2016.10.031 (2016).

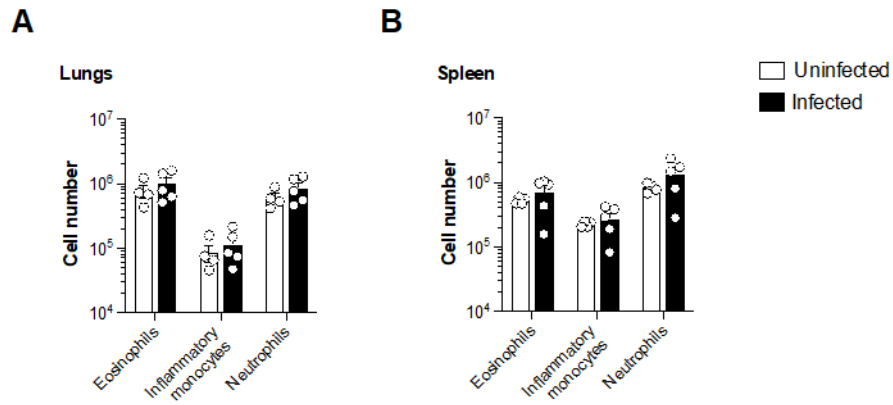
37. Glaser, P. *et al.* Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. *Mol Microbiol* 45, 1499-1513, doi:10.1046/j.1365-2958.2002.03126.x (2002).
38. Vollman, J. H., Smith, W. L., Ballard, E. T. & Light, I. J. Early onset group B streptococcal disease: clinical, roentgenographic, and pathologic features. *J Pediatr* 89, 199-203, doi:10.1016/s0022-3476(76)80447-7 (1976).
39. Dowling, D. J. & Levy, O. Ontogeny of early life immunity. *Trends Immunol* 35, 299-310, doi:10.1016/j.it.2014.04.007 (2014).
40. Benjamim, C. F., Ferreira, S. H. & Cunha, F. Q. Role of nitric oxide in the failure of neutrophil migration in sepsis. *J Infect Dis* 182, 214-223, doi:10.1086/315682 (2000).
41. Alves-Filho, J. C., Spiller, F. & Cunha, F. Q. Neutrophil paralysis in sepsis. *Shock* 34 Suppl 1, 15-21, doi:10.1097/SHK.0b013e3181e7e61b (2010).
42. Crosara-Alberto, D. P. *et al.* Involvement of NO in the failure of neutrophil migration in sepsis induced by *Staphylococcus aureus*. *Br J Pharmacol* 136, 645-658, doi:10.1038/sj.bjp.0704734 (2002).
43. Bernstein, H. M., Calhoun, D. A. & Christensen, R. D. Use of myeloid colony-stimulating factors in neonates with septicemia. *Curr Opin Pediatr* 14, 91-94, doi:10.1097/00008480-200202000-00016 (2002).
44. Gessler, P. *et al.* Serum concentrations of granulocyte colony-stimulating factor in healthy term and preterm neonates and in those with various diseases including bacterial infections. *Blood* 82, 3177-3182 (1993).
45. Engblom, C. *et al.* Osteoblasts remotely supply lung tumors with cancer-promoting SiglecF(high) neutrophils. *Science* 358, doi:10.1126/science.aal5081 (2017).
46. Calcagno, D. M. *et al.* SiglecF(HI) Marks Late-Stage Neutrophils of the Infarcted Heart: A Single-Cell Transcriptomic Analysis of Neutrophil Diversification. *J Am Heart Assoc* 10, e019019, doi:10.1161/JAHA.120.019019 (2021).
47. Vafadarnejad, E. *et al.* Dynamics of Cardiac Neutrophil Diversity in Murine Myocardial Infarction. *Circ Res* 127, e232-e249, doi:10.1161/CIRCRESAHA.120.317200 (2020).
48. Shin, J. W. *et al.* A unique population of neutrophils generated by air pollutant-induced lung damage exacerbates airway inflammation. *J Allergy Clin Immunol* 149, 1253-1269 e1258, doi:10.1016/j.jaci.2021.09.031 (2022).
49. Ryu, S. *et al.* Siglec-F-expressing neutrophils are essential for creating a profibrotic microenvironment in renal fibrosis. *J Clin Invest* 132, doi:10.1172/JCI156876 (2022).
50. Borkner, L., Curham, L. M., Wilk, M. M., Moran, B. & Mills, K. H. G. IL-17 mediates protective immunity against nasal infection with *Bordetella pertussis* by mobilizing

- neutrophils, especially Siglec-F(+) neutrophils. *Mucosal Immunol* 14, 1183-1202, doi:10.1038/s41385-021-00407-5 (2021).
51. Schiller, M., Ben-Shaanan, T. L. & Rolls, A. Neuronal regulation of immunity: why, how and where? *Nat Rev Immunol* 21, 20-36, doi:10.1038/s41577-020-0387-1 (2021).
 52. Huh, J. R. & Veiga-Fernandes, H. Neuroimmune circuits in inter-organ communication. *Nat Rev Immunol* 20, 217-228, doi:10.1038/s41577-019-0247-z (2020).
 53. Arkless, K., Argunhan, F. & Brain, S. D. CGRP Discovery and Timeline. *Handb Exp Pharmacol* 255, 1-12, doi:10.1007/164_2018_129 (2019).
 54. Assas, B. M., Pennock, J. I. & Miyan, J. A. Calcitonin gene-related peptide is a key neurotransmitter in the neuro-immune axis. *Front Neurosci* 8, 23, doi:10.3389/fnins.2014.00023 (2014).
 55. Pinho-Ribeiro, F. A. *et al.* Bacteria hijack a meningeal neuroimmune axis to facilitate brain invasion. *Nature* 615, 472-481, doi:10.1038/s41586-023-05753-x (2023).
 56. Jusek, G., Reim, D., Tsujikawa, K. & Holzmann, B. Deficiency of the CGRP receptor component RAMP1 attenuates immunosuppression during the early phase of septic peritonitis. *Immunobiology* 217, 761-767, doi:10.1016/j.imbio.2012.04.009 (2012).
 57. Lockhart, E., Green, A. M. & Flynn, J. L. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. *J Immunol* 177, 4662-4669, doi:10.4049/jimmunol.177.7.4662 (2006).
 58. Shibata, K., Yamada, H., Hara, H., Kishihara, K. & Yoshikai, Y. Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production. *J Immunol* 178, 4466-4472, doi:10.4049/jimmunol.178.7.4466 (2007).
 59. Misiak, A., Wilk, M. M., Raverdeau, M. & Mills, K. H. IL-17-Producing Innate and Pathogen-Specific Tissue Resident Memory gammadelta T Cells Expand in the Lungs of Bordetella pertussis-Infected Mice. *J Immunol* 198, 363-374, doi:10.4049/jimmunol.1601024 (2017).
 60. Coffelt, S. B. *et al.* IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. *Nature* 522, 345-348, doi:10.1038/nature14282 (2015).
 61. Sutton, C. E. *et al.* Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* 31, 331-341, doi:10.1016/j.immuni.2009.08.001 (2009).

Supplementary Data



Supplementary Figure 1. Histopathology of the neonatal lung upon GBS pneumonia. Pregnant C57BL/6 female mice were intravaginally colonised with 4×10^4 CFU GBS hyper virulent strain BM110, at gestational days 16th and 17th. Representative images of lung stained with Giemsa Hare showed for each time point. Scale bar, 100 μ m. Black arrow indicates presence of alveolar macrophages. Red arrow indicate neutrophils in the alveoli.



Supplementary Figure 2. Myeloid cell profile after recovery from neonatal pneumonia. Pregnant C57BL/6 female mice were intravaginally colonised with 4×10^4 CFU GBS hyper virulent strain BM110, at gestational days 16th and 17th. Animals from uninfected and infected groups were sacrificed at P15. Flow cytometry analysis of indicated myeloid cells in the lungs (**A**) and spleen (**B**). Data is presented as mean $\pm$ SEM [$n = 4$ (uninfected); $n = 5$ (infected)].

CHAPTER III

THESIS DISCUSSION

Neonatal meningitis remains a major health burden worldwide. Group B *Streptococcus* (GBS), the primary pathogen causing meningitis, is responsible for more than 80% of cases⁹⁰. Incredibly, despite revolutionary improvements in technology and intensive care units over the past decades, the incidence of GBS neonatal meningitis has remained stable since the 1970s, with no significant reduction in morbidity. In fact, the incidence has increased in developed countries in the last few years^{324–326}. The only approved treatment against GBS is based on infection prevention through *intrapartum* antibiotic administration to colonised pregnant women, which is not efficient neither for preventing nor reducing meningitis¹¹⁴. The widespread use of antibiotics is contributing to increasing bacterial resistance, turning the treatment insufficient. We remain completely impotent in not only fighting the pathogen but also treating towards reducing the high morbidity associated with this infection. New approaches are a must to defeat neonatal meningitis. Antibiotic and symptomatic treatment are not enough anymore. They were never enough. However, if we cannot find a treatment, we must sit and think why. In my opinion, before finding a treatment, we need to understand the disease further. Our current knowledge is still scarce. What is the course of bacterial spreading upon infection? How does the disease evolve in terms of symptomatology and progression? What is the local immune response in each affected organ? What is happening in the brain and its borders that leads to neurological damage? Which cells and molecules are responsible for this damage? What are the mechanisms of brain injury? Why do the severity and morbidity vary so widely among individuals, often

unrelated to symptomatology severity? Should we treat asymptomatic and severely sick babies the same way? These are some of the million questions we still need to answer. Understanding the infection's pathophysiology is required to develop new and efficient treatments. In my thesis project, we aimed at unravelling the CNS pathophysiology, subdivided into the following aims:

- To study the kinetics of bacterial dissemination during neonatal GBS infection, alongside the onset of symptomatology and disease progression;
- To characterise the dynamics of disease-specific immune cells and their transcriptional profiles in the meningeal spaces during neonatal meningitis;
- To unravel the mechanisms responsible for GBS-induced NDI;
- To characterise the neuroimmune interactions during neonatal GBS infection.

Diagnosing meningitis in the neonatal period is particularly difficult. Approximately 30% of newborns do not present symptoms³²⁷. Additionally, these include fever, lethargy, vomiting, and respiratory failure, which are often subtle and non-specific⁷¹. Therefore, distinguishing between flu, sepsis, gastroenteritis or meningitis is challenging. Neonates are incapable of speaking and expressing the region, type, and level of pain, as well as the symptoms they feel, further complicating the initial diagnostic process. Diagnostic tests to detect bacteria include blood and CSF cultures, the latter being the standard gold analysis^{75,328}. However, while blood cultures are frequently unprecise and negative in up to 50% of meningitis cases, CSF collection involves the invasive and skilled lumbar puncture (LP) procedure^{75,328,329}. LP is not performed in all cases, such as in asymptomatic newborns or when the doctors do not suspect meningitis^{75,328}. Nevertheless, neither case warrants the absence of meningitis. Moreover, the LP procedure is not performed in half of the infants in intensive care nurseries being treated for sepsis, and even if meningitis is suspected, LP is a technically difficult procedure and, therefore, often deferred due to concerns about the risk of complications^{75,330}. In addition to these challenges, in 75% of cases, LP is performed in infants already undergoing antibiotic treatment, biasing CSF culture results⁷⁵. In developing countries, the scenario is even worse and likely underestimated, as access to healthcare is limited, with scarce human and material resources. Therefore, we are not currently equipped with adequate understanding and resources to predict death or morbidity in neonates experiencing meningitis³²⁸.

To address these problems, we adapted our mouse model of GBS infection to the C57BL/6 mice³³¹. The model relies on the colonisation of the pregnant females' vaginal canal, closely mimicking the human vertical transmission and its outcomes³³¹. We demonstrated that the symptomatology appearance in the newborn mice is unrelated to the bacterial colonisation

level in the brain or mortality prediction (Manuscript 2), which aligns with the challenges mentioned above in diagnosing human neonatal meningitis. These findings validate the accuracy of our mouse model of vertical transmission in replicating the establishment of neonatal meningitis and its clinical outcomes, including disease progression and death (Manuscript 2). Using an appropriate animal model is critical for studying the respective human diseases. The better we mimic the infection route and its pathophysiology, the more reliable and translatable the study outcomes are. Nonetheless, our project faced limitations in conducting progressive, longitudinal studies of the disease. We were unable to follow the same pup over time to truly observe the kinetics of GBS organ colonisation, its multiplication and the associated symptomatology appearance. Instead, our bacterial colonisation studies relied on the euthanasia of animals for organ plating and CFU counting. To overcome this limitation, we engineered a bioluminescent strain of GBS (Manuscript 1). We initially transformed a hypervirulent GBS strain with the plasmid pSL101P32, containing the *luxABCDE* operon encoding a bacterial luciferase and its substrate. However, this strain proved unsuitable for *in vivo* studies, as no bioluminescent signal was detected, regardless of the bacterial infection dose. We then transformed the bacteria with the plasmid pLZ12Km2-P23R:TA:ffluc, encoding for the red-shifted firefly luciferase. This system requires the administration of luciferin, the luciferase substrate, but provides improved tissue penetration. In fact, the bacterial infection dose was proportional to the average radiance level measured in subcutaneously infected pups. However, in our vaginal model of infection, GBS was able to dispose of the plasmid, rendering it unsuitable for long-term *in vivo* studies. Lastly, we incorporated the red-shifted firefly luciferase in the GBS genome in collaboration with Arnaud Firon from the Pasteur Institute (Paris, France). This genomic integration overcame the plasmid loss observed in longer longitudinal studies. However, the disadvantage of this system is that each bacterial cell carries only one copy of the luciferase gene, limiting its bioluminescent signal. As we showed in Manuscript 1, the signal in the brain was insufficient to associate with differences in brain colonisation, not being suitable for our studies. Few bacteria are enough to cause meningitis, a number below the detection limit of the genomic luciferase system. This system is, however, suitable for moderate and severe systemic infections, representing an advancement for accurate longitudinal infection studies. Additionally, it contributes to the implementation of the 3R's principles in ethical animal experiments, by reducing the number of animals required and refining experimental procedures. These bioluminescent systems can also be tested for other suitable pathogens worldwide and their respective animal models, providing a valuable resource for research worldwide. In this thesis project, we maintained the traditional CFU counting method throughout the experiments.

Regarding GBS transmission, neonates usually acquire the bacteria during birth when crossing the vaginal canal, through the inspiration or aspiration of the mother's contaminated fluids¹¹⁴. Therefore, the first infected organs are the lungs and the gut. In accordance, respiratory symptoms are the most common signs of infection, present in more than 80% of cases^{71,332}. In Manuscript 3, we showed that our mouse model effectively recreates human lung infection and pathology from day one of life. These findings highlight the importance of developing robust and adequate infection models, so that the outcomes are translational to humans, the ultimate goal of biomedical research.

Several models have been widely used to study GBS neonatal infection, ranging from local infections, including intra-cisterna magna or intra-nasal routes, to haematogenous ones, such as subcutaneous infections. These models led to the hypothesis that brain damage is caused by a massive infiltration of immune cells into the parenchyma^{333–335}. However, our clinically relevant mouse model challenges this assumption. We found no parenchymal immune cell infiltration, showing no differences in myeloid cell populations at postnatal day (P) 3, and only a slight decrease in the frequency of TCR β cells (Manuscript 2). This suggests that the mechanism underlying brain damage in neonatal meningitis remains unclear. These results once again highlight the need for disease models that comprehensively mimic human pathophysiology. The previously established mouse models focused on one particular aspect of GBS infection, disregarding it as a complex and inter-organ disease. I believe our animal mouse model is the answer as we can still study specific aspects of pathophysiology while maintaining organ interactions as they occur in humans. Therefore, if organs like the brain or lungs contribute to disease outcomes, their roles are inherently accounted for in our model.

Our hypothesis to explain the neurodevelopmental impairment in GBS meningitis survivors was that the brain borders, specifically the meninges, are contributing to the brain damage. In the last two decades, the meninges have emerged as an immunological tissue niche important in the homeostasis of the CNS and far more than just a physical barrier (Review in Annex 1). Their involvement in disease processes is an even more recent topic of research, and our knowledge remains scarce. In our project, we found that pups with meningitis have significantly increased frequency of $\gamma\delta$ T cells at P3. Moreover, there is an enrichment of IL-17 in the meningeal environment, produced by $\gamma\delta$ T cells (Manuscript 2). $\gamma\delta$ T cells are well known for their protective role in pathogenic infections, including fungal, bacterial and viral infections^{336,337}. Unlike $\alpha\beta$ T cells, which require APCs to present antigens in the context of MHC, $\gamma\delta$ T cells can directly respond to pathogens (Manuscript 2). They have unique T cell receptors (TCRs) and can express almost all Toll-like receptors (TLRs) to recognise pathogenic antigens and rapidly initiate their immune response^{338,339}.

Furthermore, $\gamma\delta$ T cells recognise and respond to signals from the host itself without the need of TCR engagement, including the cytokines IL-1 β and IL-23³⁴⁰. These responses include the release of cytotoxic molecules, such as granzymes and perforins, which kill infected cells, and the production of potent cytokines to activate and/or recruit other immune cells (e.g., neutrophils, DCs, macrophages, T cells, etc.) for pathogen clearance and tissue repair^{338,341–343}. For instance, $\gamma\delta$ T cells are the primary cells recruiting neutrophils upon *Escherichia coli* intraperitoneal infection, via IL-17 production³⁴⁴. The production of this cytokine by $\gamma\delta$ T cells was also observed as critical in *Staphylococcus aureus* cutaneous infection, being dependent on IL-1 β and IL-23³⁴⁵. Both Tcrd^{-/-} and IL-17R^{-/-} mice presented increasing bacterial burden and skin lesions, implying $\gamma\delta$ T cells as protectors in dermal infection³⁴⁵. Moreover, $\gamma\delta$ T cells are important for the recruitment of T lymphocytes to the skin. While controlling pathogen clearance, these cells also work towards tissue repair, by responding to stressed or damaged keratinocytes with keratinocyte growth factors, inducing their proliferation³⁴⁶. In pulmonary infection by *Streptococcus pneumonia*, $\gamma\delta$ T cells were found to be critical to protect the lung from injury³⁴⁷. These cells directly kill alveolar macrophages and DCs, controlling their number during infection. Tcrd^{-/-} infected mice presented twice the number of alveolar macrophages and thrice the DCs, resulting in granuloma formation³⁴⁷. Therefore, $\gamma\delta$ T cells are the orchestra masters, being excellent cell communicators linking innate and adaptive immunity, and coordinating the immune network to optimise the host response to infection. Similarly, IL-17 is a well-established pro-inflammatory cytokine critical for pathogen clearance. In candidiasis, IL-17 receptor (IL-17R) deficient mice presented severe infection due to impaired neutrophil recruitment³⁴⁸. Additionally, IL-17 has an important role in prolonging neutrophil survival, through the activation of pericytes to produce granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), highlighting the importance of this cytokine in steady-state haematopoiesis³⁴⁹. In addition, pericytes activated by IL-17 are also involved in the enhanced neutrophil phagocytosis ability and production of pro-inflammatory cytokines, like IL-1 β and TNF- α ³⁴⁹. IL-17 is crucial for *S. aureus* clearance from the nasal cavity, as it induces the production of antimicrobial peptides³⁵⁰. In fact, healthy humans colonised with *S. aureus* presented elevated levels of human antimicrobial peptides in their nasal secretions compared to non-colonised healthy humans³⁵⁰. Thus, the enriched $\gamma\delta$ 17 T cells and IL-17 environment in the meninges in our project align with their important roles in protecting the host. To confirm the importance of these cells in GBS clearance, we studied mice lacking $\gamma\delta$ T cells or IL-17. Surprisingly, Tcrd^{-/-} pups born from GBS-colonised progenitors showed significantly reduced GBS colonisation in the brain, while presenting similar bacterial loads in the lungs and meninges compared to wild-type (WT) controls (Manuscript 2). This means that the absence of $\gamma\delta$ T cells protects the brain

against GBS parenchymal invasion. Moreover, contrary to what we were expecting, *Tcrd*^{-/-} infected pups did not present altered mortality. Similarly, infected *IL-17*^{-/-} pups were also protected against parenchymal GBS invasion and showed no differences in survival compared to WT infected pups. Furthermore, local blockade of IL-17, through intra-cisterna magna administration of anti-IL17, has led to brain protection against GBS colonisation. These findings led us to conclude that IL-17 produced by $\gamma\delta$ T cells is detrimental to the brain during GBS meningitis. Considering the critical role of systemic IL-17 in host protection against extracellular bacteria, these results defy the literature-established concept of $\gamma\delta$ T cells as protective during infection by these pathogens.

We wondered if IL-17 produced by $\gamma\delta$ T cells contributed to blood-brain barrier (BBB) disruption. Our hypothesis was confirmed *ex vivo* as *Tcrd*^{-/-} infected pups presented a higher permeabilisation index than uninfected controls but lower than the WT infected counterparts (Manuscript 2). Therefore, a meningeal enrichment in $\gamma\delta$ T cells, particularly in IL-17, during neonatal meningitis contributes to the BBB disruption potentiating GBS parenchymal invasion. The detailed mechanism of IL-17-mediated BBB disruption remains to be elucidated in future studies. IL-17 has been associated with several neurological disorders such as autism spectrum disorder, epilepsy, chronic migraine or multiple sclerosis (MS)^{351,352}. In a mouse model of MS, administration of anti-IL-17 antibodies attenuated the clinical scoring of experimental autoimmune encephalomyelitis (EAE) development³⁵³. Furthermore, the CSF from MS patients revealed increased levels of IL-17 and correlated with albumin quotient (Qalb), a measure of patients' BBB integrity impairment³⁵³. Studies using the human brain endothelial cell line hCMEC/D3 showed that treatment with recombinant IL-17 reduced the gene expression of tight junction protein ZO-1, as well as *Cdh5* and *Pecam1*, encoding an adherens junction protein and adhesion molecule, respectively³⁵³. Kebir *et al.* showed that blood-brain barrier endothelial cells (BBB-ECs) in MS lesions express IL-17R, which are absent in control white matter³⁵⁴. Using an *in vitro* monolayer model of human BBB-ECs and treating with IL-17 led to increased BBB permeability with concomitant reduced expression of ZO-1 and occludin, proving the direct impact of the cytokine in BBB disruption³⁵⁴. The BBB breakdown allowed the transverse of CD4⁺CD45RO⁺ IL-17-producing lymphocytes, 60% of them co-expressing granzyme B and production of IL-17 and IL-22, ultimately killing neurons through granzyme B-mediated mechanisms³⁵⁴. An *in vitro* study by Huppert *et al.* demonstrated that the murine BBB-ECs monolayer behaved equally, showing decreased levels of occludin and alterations in the distribution of ZO-1 at the cell surface upon IL-17 treatment³⁵⁵. In the presence of IL-17, BBB-ECs increased the intracellular production of ROS through NADPH and xanthine oxidases, which further activated the endothelial contractile machinery and reduced

occludin levels³⁵⁵. These results were confirmed *in vivo* in an EAE mouse model using an inhibitor of the endothelial contractile machinery³⁵⁵, paving the way for understanding the mechanisms of IL-17-mediated BBB disruption. In addition, and in agreement with Kebir et al. human cell studies, IL-17 treatment enhanced monocytic adhesion and traversal of the BBB-ECs³⁵⁵. In our project, we can speculate that an identical mechanism leads to the BBB disruption via meningeal IL-17. Further quantification of tight junction proteins will likely confirm the BBB increased permeabilisation index observed in WT pups with meningitis (Manuscript 2). To explore the role of IL-17 in BBB disruption during neonatal meningitis in greater detail, we will collaborate with Helena Azevedo, Group Leader of the i3S Molecular Biomaterials group, who is currently developing a 3D human BBB *in vitro* model that we will use to explore in detail the role of IL-17 and its mechanism in BBB disruption in neonatal GBS meningitis. Using this system will provide insight into the translational mechanisms, further validating our mouse model outcomes. Moreover, the evaluation of the BBB disruption extent may allow us to decipher why it does not result in immune cell infiltration into the parenchyma. It might be the case that BBB is not sufficiently disrupted to allow cell transversal. As ROS production by the BBB-ECs contributes to BBB integrity impairment, we can wonder if microglia, which are significantly activated in the parenchyma of meningitis pups (Manuscript 2), present increased levels of ROS production that would facilitate the breakdown of tight junction proteins. Additionally, besides expressing IL-17R at their surface, microglia are also capable of producing IL-17^{356,357}. Therefore, IL-17 quantification in the brain of pups with meningitis compared to uninfected counterparts should be addressed. Although we do not show parenchyma immune cell infiltration, we cannot discard that parenchymal cells are increasing cytokine production, either IL-17 or others, and contributing to BBB permeability together with the meningeal $\gamma\delta$ 17 T cells.

In addition to contributing to BBB breakdown, an overproduction of the proinflammatory IL-17 may turn antibacterial properties into tissue-persistent inflammation. IL-17 is pathogenic in numerous neurological disorders, such as chronic neuroinflammatory conditions, MS and EAE, Alzheimer's disease, and ischemic brain injury³⁵⁸. MS is an IL-17-mediated autoimmune disease, and before the discovery of Th17 cells, autopsies already revealed increased IL-17 expression in MS plaques and that its gene is the most expressed in the CNS of MS patients³⁵⁹. Furthermore, brain tissues from these patients presented significantly increased CD4⁺ and CD8⁺ IL-17-producing T cells in the lesions, with IL-17 detected in these cells, astrocytes and oligodendrocytes³⁶⁰. Besides producing IL-17, all glial cells (microglia, astrocytes and oligodendrocytes) express IL-17R³⁶¹. Moreover, CD161^{high} CCR6⁺ $\gamma\delta$ T cells, which are IL-17 producers, were significantly increased in the CSF of relapsed MS patients, but not in patients with infectious diseases³⁶². These results

are in line with ours. Due to technical limitations, we have not examined $\gamma\delta$ T cells in the CSF, as we can only collect a maximum of 3 μ L of CSF per pup, making it impossible to analyse. Even pooling samples from up to three pups yielded cytokine levels below detection limits in multiplex analyses, demonstrating the difficulty of studying neonatal CSF. To evaluate cells, we would have to pool samples from a large number of pups. Moreover, as our bioluminescent *gluc*GBS strain has a main disadvantage of limited brain detection in our mouse model of infection (Manuscript 1) and clinical scoring is not predictable of meningitis (Manuscript 2), we would not be able to pool animals according to the disease manifestation and level of colonisation, which could compromise the results. Nonetheless, while patients with infectious diseases did not present differences in $\gamma\delta$ T cell in the CSF, one study, to our knowledge, reported increased levels of IL-17 in the CSF of children with bacterial meningitis³⁶³. Our data show increased $\gamma\delta$ T cells in the meninges and increased levels of IL-17 throughout life (Manuscript 2), suggesting that IL-17 may signal to glial cells and contribute to neurologic damage.

High IL-17 expression is also observed in ischemic injured brain tissue in animal models and postmortem human tissues^{364–366}. Upon cerebral artery occlusion, levels of IL-17 increase in the brain³⁶⁶. Moreover, using an *in vitro* model of oxygen-glucose deprivation, the authors showed that IL-17 contribute to neuronal death in a dose-dependent manner³⁶⁶. Moreover, $\gamma\delta$ T cells were found as major contributors to the late phase of ischemic reperfusion (tissue damage caused when blood supply returns to that tissue)³⁶⁷. The IL-17 produced by these cells accounted for over 94% of total IL-17-producing T cells³⁶⁷. Moreover, adoptive transfer of IL-17^{-/-} T lymphocytes, but not IFN- γ ^{-/-} cells, resulted in significantly less brain damage³⁶⁷. Tcrd^{-/-} or anti- $\gamma\delta$ -antibodies treated mice showed decreased infarct volume translating into neuroprotection³⁶⁷. Therefore, $\gamma\delta$ 17 T cells exacerbate neuronal damage. Neurons possess IL-17 receptors, and it has already been demonstrated that they can directly respond to IL-17 in neonatal and adult mice^{368,211}. We will soon study if this communication is occurring during GBS neonatal meningitis and its outcomes. *In vitro* culture of neurons with IL-17, or $\gamma\delta$ 17 T cells and/or glial cells from infected animals and further neural mortality assessment will elucidate the mechanisms of neurological impairment in this disease.

In conclusion, increased IL-17 levels due to meningeal $\gamma\delta$ T cells contribute to BBB disruption and may lead to neuronal damage, either directly or indirectly via glial cells. The detailed signalling cascades upon IL-17 recognition remain to be elucidated.

In this project, one of the most puzzling results is the absence of myeloid cell recruitment into the parenchyma (Manuscript 2). We show in Manuscript 3 an increased number of $\gamma\delta$

T cells in the lungs during neonatal pneumonia. Concomitantly, there is a massive neutrophil recruitment to the tissue to help clear the bacteria. However, the same does not occur in the CNS, as there is no recruitment of neutrophils to the brain or meninges (Manuscript 2). It is even more perplexing given that the BBB is more permeable due to the meningeal $\gamma\delta$ T cells, which could facilitate cell infiltration. Basic immunological principles dictate that neutrophils are typically recruited to the site of infection and are the first responders. In the neonatal period, neutrophils have even increased importance as newborns mostly rely on their innate immune system for protection. We can speculate that the CNS may prioritise maintain tissue homeostasis and prevent tissue damage. But which cells are responsible for this regulation? Meningeal $\gamma\delta$ T cells are still producing IL-17, a cytokine known to induce neutrophil recruitment, so they might not be responsible. One hypothesis is related to the neuroimmune crosstalk between neurons and neutrophils. The neuropeptide CGRP was proven to act on neutrophils, monocytes and macrophages in models of skin and muscle injury, preventing the recruitment of these cells and promoting tissue healing³⁶⁹. Additionally, CGRP was shown to be released by meningeal nociceptor nerves in response to GBS²⁴³. Systemic injection of CGRP increased GBS parenchymal invasion in an adult mouse model of infection and its absence, due to ablation of nociceptors, decreased meningeal and brain GBS load²⁴³. Therefore, GBS may trigger meningeal nociceptors to evade the immune response. This way, immune cells are not recruited, potentiating GBS survival and multiplication. In this context, the lack of neutrophil recruitment may reflect an immune evasion strategy rather than a protective mechanism by the CNS. In the lungs, we showed that CGRP reduces SiglecF expression in neutrophils, reducing their phagocytic capacity in severely sick animals, unlike moderately sick counterparts (Manuscript 3). It would be interesting to evaluate if meningeal neutrophils also present differences in the SiglecF expression that impairs GBS clearance, and if it correlates to disease severity as observed in the lungs. It is reasonable that the basic immune concepts of neutrophil recruitment are also the basic evading mechanisms of pathogens. Nevertheless, neutrophil recruitment is not impaired in the lungs. This discrepancy might be due to the higher level of GBS colonisation in the lungs, even in the moderately sick pups, where CGRP is unable of dampening neutrophil recruitment at 100%.

The mechanisms of neutrophil recruitment differ between organs, which could also explain why neutrophils are recruited to the lungs of GBS-infected pups but not to the CNS. Neutrophil recruitment into the lungs is mainly chemokine-dependent through the CXC motif chemokine receptor (CXCR) 2, the main chemokine receptor in these cells³⁷⁰. Inhibition of this receptor translated into a 50% reduction of neutrophil recruitment to the lungs, as well as irradiation of WT mice followed by *Cxcr2*^{-/-} bone marrow transplantation^{371,372}. Mice with

both $Cxcr2^{-/-}$ genotype and with $Cxcr2^{-/-}$ bone marrow transplantation lacked neutrophil recruitment³⁷². Nonetheless, previous studies from our group using a neonatal mouse model of sepsis, showed that circulatory neutrophils had reduced levels of CXCR2. However, the impaired neutrophil recruitment to the lungs was attributed to IL-10 overproduction rather than being CXCR2-mediated³⁷³, highlighting that CXCR2 is not exclusively responsible for neutrophil recruitment to the lungs. In our vaginal mouse model, however, we observed neutrophil recruitment to the lungs (Manuscript 3), despite showing increased levels of serum IL-10 in Manuscript 2. These findings suggest that mechanism of neutrophil recruitment may vary depending on the infection route. In the CNS, CXCR2 also play a role in BBB breakdown and neutrophil infiltration in a model of EAE³⁷⁴. Blocking or genetically depleting this receptor prevented EAE, including BBB disruption and leukocyte infiltration³⁷⁴. Of note, this study was performed on the spinal cords, not the brain, our target organ. An Alzheimer's disease model showed increased expression of intercellular adhesion molecule 1 (ICAM-1) and selectins in the brain compared to controls³⁷⁵. Depletion or inhibition of the neutrophil ICAM-1 ligand leukocyte function-associated antigen 1 (LFA-1) reduced Alzheimer's neuropathology and improved memory in mice already suffering from Alzheimer³⁷⁵, showing that preventing neutrophil CNS invasion protects against neuronal damage. The brains of EAE mice also demonstrated increased expression of ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1), and their blockade inhibited endothelial lymphocyte adhesion³⁷⁶. In conclusion, the lungs and the brain present tissue-specific mechanisms of neutrophil recruitment signalling cascades, and we can speculate this as a reason for meningitis pups lacking neutrophil recruitment into the CNS in our project. It does not explain, however, which cells and molecules could be inhibiting these mechanisms, and further studies should be addressed. The meninges, CSF and blood cytokine analysis showed no significant differences in IFN- γ , TNF- α and IL-6 (Manuscript 2), cytokines known to activate ICAM-1 expression³⁷⁷. Furthermore, IL-10 is significantly increased in the serum of pups with meningitis, which could contribute to reduced CNS neutrophil recruitment, despite not being important for recruitment in the lungs in this model (Manuscript 3). Previous studies from our group showed that IL-10, in an haematogeneous mouse model of neonatal sepsis, is responsible for impaired neutrophil recruitment to the sites of infection³⁷³. Mice lacking IL-10 were resistant to GBS infection due to significantly increased neutrophil migration to the infected organs³⁷⁸. Furthermore, a specific maternal vaccination against GBS decreased the production of IL-10, resulting in increased neutrophil recruitment, showcasing a GBS virulence mechanism³⁷⁸. No differences in the meninges nor CSF regarding IL-10 were found, but almost 100% of the values were below detection levels, preventing us from concluding the role of IL-10 in CNS neutrophil recruitment. The

3D BBB model will allow us to explore all these mechanisms and clarify the neutrophil non-recruitment mystery.

In conclusion, we need to consider the two previously stated levels in neutrophil recruitment: level 1- the blood vessel- endothelium and associated perivascular cells, and level 2- organ-specific resident cell activation and neutrophil recruitment³⁷⁹. In our case, a third level should be considered: GBS in the CNS. GBS neonatal meningitis may impact these levels or act itself to prevent neutrophil recruitment. Whether it is a CNS protection or GBS evasion mechanism remains to be elucidated in future studies.

The next step in our project was to evaluate when homeostasis was achieved upon neonatal meningitis. Animals with two weeks old no longer showed differences in the lung's immune cells, namely in neutrophils and $\gamma\delta$ T cells (Manuscript 3). However, lung pathology was present, with at least three different histopathologic pulmonary abnormalities present at the same time. This result suggests that meningitis survivors may suffer from respiratory sequelae, which are very common in surviving humans. Pneumonia in early life has been linked to lung dysfunction, although studies remain scarce. A mouse study of neonatal *Chlamydia trachomatis* pneumonia showed long-term pulmonary deficits. Juveniles (5-week-old) and adults (8-week-old) suffered from airway hyperreactivity and lung compliance (calculated as lung volume/pressure) upon neonatal meningitis³⁸⁰. An empiric-based human study stated that children whose mothers recall having pneumonia during the first year of life were more susceptible to respiratory illness than children whose mothers do not recall lung diseases³⁸¹. A cohort with 1392 British adults born in 1958 who had pneumonia until 7 years of age presented reduced ventilatory function³⁸². A systematic review and meta-analysis, including studies from 1970 to 2011, of patients with pneumonia during childhood and without pre-existing risk factors for pulmonary diseases showed an incidence of at least one major lung sequelae (restrictive lung disease, obstructive lung disease, bronchiectasis) of over 10% of these individuals³⁸³. Minor sequelae (chronic bronchitis, asthma, other abnormal pulmonary function, other respiratory disease) presented a ~7% incidence. It is important to note that 61% of this meta-analysis individuals are patients diagnosed with pneumonia before 2 years of age. Therefore, despite non-existing studies, to our knowledge, in GBS neonatal pneumonia vs long-term sequelae, our results of lung pathology during mouse childhood suggest that survivors may suffer from lung dysfunction later in life. Animal studies should be performed to validate this hypothesis.

Regarding meningeal homeostasis achievement upon neonatal GBS meningitis, our results show that $\gamma\delta 17$ T cells remained increased in the meninges of neonatal infection survivors throughout life (Manuscript 2). As $\gamma\delta$ T cells are self-renewing within the organs after thymic

migration, this chronic increase might suggest an early imprint on these cells. The STAT transcription factor 5 (STAT5) was recently shown to be required for post-thymic $\gamma\delta$ T17 development and tissue-specific imprinting in the neonatal microenvironment³⁸⁴. In STAT5-deficient mice, $\gamma\delta$ T17 cells were absent from all adult organs, showcasing the importance of STAT5 signalling cascade³⁸⁴. It is, therefore, plausible to wonder if there is any alteration in this cascade in the opposite direction, or other signalling pathway imprint, leading to constant increased $\gamma\delta$ T17 cell number in the meninges.

Chronic IL-17 also leads to the question of the long-term consequences of a meningeal chronic inflammatory environment. In a second pathogenic challenge, will IL-17 potentiate a rapid bacterial clearance, as it is already increased before the infection, or will it contribute to tissue pathology? The answer may not be easy as it is most likely a multifactorial question. It may depend, for example, on the pathogen, the tissues it colonises and the infection severity. Nonetheless, it is worthy of future studies. Moreover, could chronic meningeal IL-17 enrichment contribute to immune-mediated neurologic disorders later in life or exacerbate negative neuronal outcomes? Further studies with survivors of our mouse models vs naïve controls can address these potential long-term sequelae.

As already mentioned in this thesis, up to 50% of neonatal GBS meningitis survivors suffer from permanent neurologic impairment. Thus, we performed a battery of tests in the neonatal period and early adulthood to evaluate the short- and long-term consequences caused by $\gamma\delta$ T cells in neonatal GBS meningitis. Regarding sex differences, although they were not observed in the developmental period, adult females did not present sequelae, in opposition to males (Manuscript 2). Nonetheless, it is known that in humans males are more likely to suffer from neurodevelopmental impairment upon GBS meningitis³⁸⁵. During development, WT-infected pups anticipated the maturation of sensory reflexes, such as auditory startle, ear twitch and eye-opening (Manuscript 2). A rat model of maternal separation showed anticipation of the eye opening reflex³⁸⁶, showing that, similar to our case, early-life stress may trigger sensory development acceleration. Another study showed that acoustic startle was exacerbated in adult rats exposed to neonatal infection³⁸⁷. This intensified response was only present when adults were exposed to stress before the acoustic startle³⁸⁷, validating the need for acute stress prior to sensory hypersensitivity. Although we have not tested the olfactory sense in our study, early stress was shown to affect the pup's odour attachment to the mother³⁸⁸. Neonatal pups instinctively learn the maternal odour and show typical social behaviour and attachment with the mother^{389–391}. As pups prepare for independence, corticosterone production by the amygdala increases its plasticity, resulting in maternal odour avoidance³⁹². This maturation mechanism was proved to be accelerated in stressed pups due to fragmented maternal care during impoverished

nesting material³⁸⁸. The alteration of this attachment/fear circuit has been linked to long-term behavioural and neural deficits, including depression and anxiety in humans^{393–397}. In accordance, human studies demonstrated that children who experienced maternal deprivation in early life or who live with depressed mothers present larger amygdalas than the respective controls^{398,399}. This translated into an acceleration of maturation, proved by adult-like interactions of the medial prefrontal cortex with the amygdala in children suffering from parental deprivation⁴⁰⁰. These results might relate to our findings. As in our model, the animals are stressed due to infection, their senses develop earlier in an attempt to increase their independence and, therefore, their survival chances. Interestingly, *Tcrd*^{-/-} groups presented no differences regardless of infection (Manuscript 2). Moreover, they were always developing equally to the infected WT pups and, consequently, different from WT baseline animals. The only exception was the auditory startle test, where infected *Tcrd*^{-/-} pups responded significantly earlier than the genotype controls. These results imply an important role for $\gamma\delta$ T cells during homeostatic brain development. Although there is a lack of studies in this field, meningeal $\gamma\delta$ T cells were recently implicated in adults for their homeostatic functions in the brain, including anxiety and memory regulation and synaptic plasticity^{211,212,401}. It is therefore reasonable to hypothesise that $\gamma\delta$ T cells play equal importance during brain development.

Still in the developing period, WT infected pups also performed the open field traversal test in a significantly reduced time compared to the controls, suggesting hyperactivity (Manuscript 2). This disorder was also observed in the male adults who survived GBS meningitis in the perinatal period. In the open field test, WT survivors ran a significantly larger distance compared to WT controls and *Tcrd*^{-/-} groups (controls and survivors). Concomitantly, their average speed was also significantly higher. These results indicate hyperactivity. In addition to increased running distance, they covered a significantly increased distance in the borders compared to the control group. However, they did not spend additional time in this area. This means that, on top of hyperactivity, these animals display anxiety-like behaviour, as the increased locomotion was only achieved in the borders but not in the centre of the arena. These two conditions, hyperactivity and anxiety, are commonly inter-related, being present simultaneously in approximately 25% of children diagnosed with ADHD^{402,403}. Moreover, similar results were observed in models of maternal immune activation^{404,405}. Adult animals born from mothers with intrauterine inflammation presented a hyperlocomotion in the open field arena, specifically at the borders but not in the centre in agreement with hyperactivity and anxiety⁴⁰⁵. Moreover, animals presented increased rearing behaviour, associated with risk assessment, suggesting increased preoccupation and, thus, anxiety⁴⁰⁵. In humans, perinatal inflammation was recently

reported to increase the risk of children ADHD diagnosis by approximately 10 years old^{406,407}. Remarkably, hyperactivity and anxiety were totally abrogated in *Tcrd*^{-/-} mice surviving neonatal meningitis (Manuscript 2), indicating that the neonatal IL-17-driven inflammation due to meningeal $\gamma\delta$ T cells causes long-term behavioural alterations consistent with ADHD.

In summary, these results provide compelling evidence that immune responses in the neonatal meninges can shape brain development, potentially predisposing individuals to neurodevelopmental disorders such as ADHD. This work adds to the growing literature implicating meningeal immunity in both homeostatic brain development and long-term neurological outcomes.

While this study establishes a direct link between neonatal meningeal $\gamma\delta$ T cells and behavioural sequelae, it leaves important questions open regarding the precise mechanisms by which IL-17 and $\gamma\delta$ T cells impact neuronal circuits and behaviour. Moreover, the sex-specific nature of these findings, as indicated by our results in male survivors, requires further investigation to determine why females are not similarly affected despite presenting the same neonatal immune outcomes.

Future studies should focus on dissecting the molecular and cellular pathways through which meningeal $\gamma\delta$ T cells influence neurodevelopment, with particular attention to the role of IL-17 in modulating neuronal plasticity and synaptic function. Exploring these

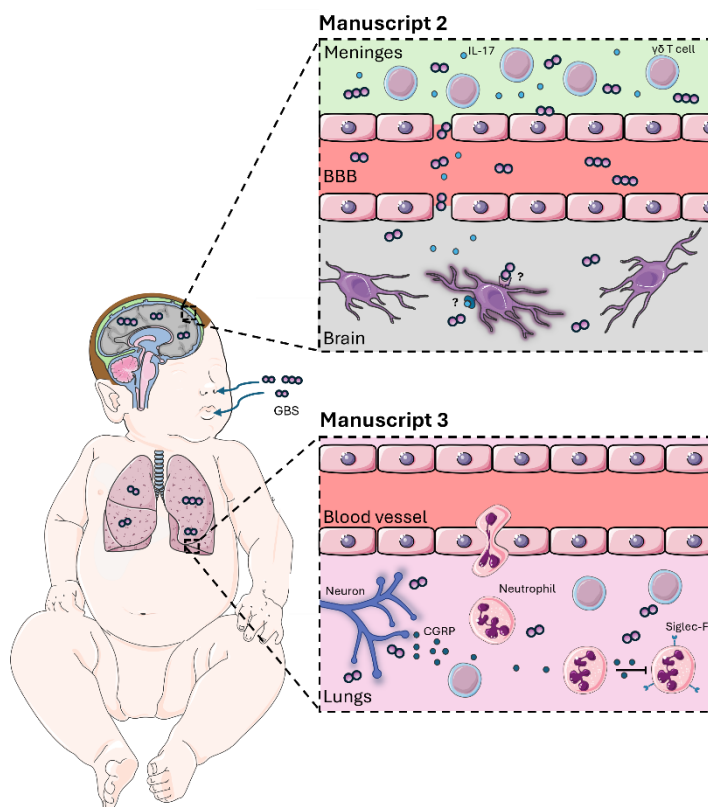


Figure 1. Thesis summary. This thesis uncovers one mechanism of neurodevelopmental impairment caused by neonatal Group B *Streptococcus* (GBS) meningitis (Manuscript 2). Briefly, the accumulation of $\gamma\delta$ T cells in the meninges leads to an enrichment in IL-17, resulting in BBB leakage and GBS parenchymal invasion. In the brain, microglia are activated. Adult survivors of neonatal meningitis present ADHD-like behaviours. Additionally, we uncover neuroimmune interactions during neonatal GBS infection. Pulmonary nociceptors produce the neuropeptide CGRP in response to GBS, inhibiting neutrophils maturation into the SiglecF^{hi} phenotype. These neutrophils present enhanced phagocytic ability.

mechanisms may uncover novel therapeutic targets to mitigate the long-term neurological impacts of neonatal meningitis.

CONCLUDING REMARKS

In this thesis, we have contributed to knowledge and the elucidation of neonatal meningitis development, associated immune alterations and short- and long-term consequences of this infection, successfully achieving all the proposed aims of the project. The most relevant achievements of this work are:

- 1- Validation of our mouse model of infection that relevantly mimics human disease and demonstrates no parenchymal infiltration by immune cells as a cause of neuronal damage;
- 2- Identification of a sustained increase in $\gamma\delta 17$ T cells in the meninges of infected pups throughout their lifespan;
- 3- Demonstration that IL-17 produced by meningeal $\gamma\delta$ T cells disrupts the BBB;
- 4- Evidence that meningeal $\gamma\delta 17$ T cells contribute to ADHD-like behaviours in neonatal meningitis survivors, as Tcrd^{-/-} survivors show abrogated anxiety and hyperactivity;
- 5- Observation that neonatal pneumonia leads to severity disease-specific outcomes;
- 6- Discovery that severely sick pups present increased SiglecF expression on neutrophils with concomitant decreased GBS phagocytosis ability;
- 7- Elucidation of a GBS immune evasion mechanism whereby neuronal production of CGRP inhibits neutrophil activation, facilitating bacterial survival.

Altogether, these results demonstrate the complexity of GBS neonatal infection and the multifaceted immune responses it elicits. Moreover, they illustrate the inter-systems communication, specifically the neuroimmune interactions in different organs, and their specific communication within those organs. This work emphasises the importance of using appropriate mouse models to study complex human diseases to avoid the oversimplification of single pathology studies without accounting for the whole-body reaction.

We strongly believe that the data gained from this thesis significantly advance our understanding of the mechanism leading to the neurodevelopmental impairment observed in survivors of neonatal GBS meningitis. This knowledge paves the way for the development of new therapeutic approaches aimed at mitigating the long-term consequences of this devastating infection.

REFERENCES

1. Perin, J. *et al.* Global, regional, and national causes of under-5 mortality in 2000–19: an updated systematic analysis with implications for the Sustainable Development Goals. *Lancet Child Adolesc. Health* **6**, 106–115 (2022).
2. Shane, A. L. & Stoll, B. J. Neonatal sepsis: Progress towards improved outcomes. *J. Infect.* **68**, S24–S32 (2014).
3. Mahtab, S. *et al.* Causes of death identified in neonates enrolled through Child Health and Mortality Prevention Surveillance (CHAMPS), December 2016 –December 2021. *PLOS Glob. Public Health* **3**, e0001612 (2023).
4. Cassini, A. *et al.* Global Report on the epidemiology and burden on sepsis: current evidence, identifying gaps and future directions. *Global Report on the epidemiology and burden on sepsis: current evidence, identifying gaps and future directions*. <https://www.who.int/publications/i/item/9789240010789> (2020).
5. Milton, R. *et al.* Neonatal sepsis and mortality in low-income and middle-income countries from a facility-based birth cohort: an international multisite prospective observational study. *Lancet Glob. Health* **10**, e661–e672 (2022).
6. Lehtonen, L., Gimeno, A., Parra-Llorca, A. & Vento, M. Early neonatal death: A challenge worldwide. *Semin. Fetal. Neonatal Med.* **22**, 153–160 (2017).
7. Li, J., Shen, L. & Qian, K. Global, regional, and national incidence and mortality of neonatal sepsis and other neonatal infections, 1990–2019. *Front. Public Health* **11**, (2023).
8. Griselet, V. *et al.* Human Chorionic Gonadotrophin: New Pleiotropic Functions for an “Old” Hormone During Pregnancy. *Front. Immunol.* **11**, (2020).
9. Schumacher, A. *et al.* Human chorionic gonadotropin attracts regulatory T cells into the fetal-maternal interface during early human pregnancy. *J. Immunol. Baltim. Md 1950* **182**, 5488–5497 (2009).

10. Silasi, M. *et al.* Human Chorionic Gonadotropin modulates CXCL10 Expression through Histone Methylation in human decidua. *Sci. Rep.* **10**, 5785 (2020).
11. Lentz, L. S. *et al.* Human chorionic gonadotropin promotes murine Treg cells and restricts pregnancy-harmful proinflammatory Th17 responses. *Front. Immunol.* **13**, (2022).
12. Dauven, D., Ehrentraut, S., Langwisch, S., Zenclussen, A. C. & Schumacher, A. Immune Modulatory Effects of Human Chorionic Gonadotropin on Dendritic Cells Supporting Fetal Survival in Murine Pregnancy. *Front. Endocrinol.* **7**, 146 (2016).
13. Schumacher, A., Poloski, E., Spörke, D. & Zenclussen, A. C. Luteinizing hormone contributes to fetal tolerance by regulating adaptive immune responses. *Am. J. Reprod. Immunol. N. Y. N 1989* **71**, 434–440 (2014).
14. Fettke, F. *et al.* Maternal and Fetal Mechanisms of B Cell Regulation during Pregnancy: Human Chorionic Gonadotropin Stimulates B Cells to Produce IL-10 While Alpha-Fetoprotein Drives Them into Apoptosis. *Front. Immunol.* **7**, 495 (2016).
15. Sauss, K., Ehrentraut, S., Zenclussen, A. C. & Schumacher, A. The pregnancy hormone human chorionic gonadotropin differentially regulates plasmacytoid and myeloid blood dendritic cell subsets. *Am. J. Reprod. Immunol. N. Y. N 1989* **79**, e12837 (2018).
16. Kwon, J.-Y. *et al.* Relevance of placental type I interferon beta regulation for pregnancy success. *Cell. Mol. Immunol.* **15**, 1010–1026 (2018).
17. Zhang, Y.-H. *et al.* Trophoblast-secreted soluble-PD-L1 modulates macrophage polarization and function. *J. Leukoc. Biol.* **108**, 983–998 (2020).
18. Bayer, A. *et al.* Human trophoblasts confer resistance to viruses implicated in perinatal infection. *Am. J. Obstet. Gynecol.* **212**, 71.e1-71.e8 (2015).
19. Koi, H. *et al.* Syncytiotrophoblast is a barrier to maternal-fetal transmission of herpes simplex virus. *Biol. Reprod.* **67**, 1572–1579 (2002).

20. Robbins, J. R., Skrzypczynska, K. M., Zeldovich, V. B., Kapidzic, M. & Bakardjiev, A. I. Placental syncytiotrophoblast constitutes a major barrier to vertical transmission of *Listeria monocytogenes*. *PLoS Pathog.* **6**, e1000732 (2010).
21. Battersby, A. J., Khara, J., Wright, V. J., Levy, O. & Kampmann, B. Antimicrobial Proteins and Peptides in Early Life: Ontogeny and Translational Opportunities. *Front. Immunol.* **7**, (2016).
22. Goldenberg, R. L., Culhane, J. F., Iams, J. D. & Romero, R. Epidemiology and causes of preterm birth. *The Lancet* **371**, 75–84 (2008).
23. Flenady, V. *et al.* Stillbirths: recall to action in high-income countries. *Lancet Lond. Engl.* **387**, 691–702 (2016).
24. Goldenberg, R. L., McClure, E. M., Saleem, S. & Reddy, U. M. Infection-related stillbirths. *The Lancet* **375**, 1482–1490 (2010).
25. Chu, D. M. *et al.* Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery. *Nat. Med.* **23**, 314–326 (2017).
26. Makino, H. *et al.* Transmission of Intestinal *Bifidobacterium longum* subsp. *longum* Strains from Mother to Infant, Determined by Multilocus Sequencing Typing and Amplified Fragment Length Polymorphism. *Appl. Environ. Microbiol.* **77**, 6788–6793 (2011).
27. Matsumiya, Y., Kato, N., Watanabe, K. & Kato, H. Molecular epidemiological study of vertical transmission of vaginal *Lactobacillus* species from mothers to newborn infants in Japanese, by arbitrarily primed polymerase chain reaction. *J. Infect. Chemother.* **8**, 43–49 (2002).
28. Martín, R. *et al.* Human milk is a source of lactic acid bacteria for the infant gut. *J. Pediatr.* **143**, 754–758 (2003).
29. Pickens, W. L., Warner, R. R., Boissy, Y. L., Boissy, R. E. & Hoath, S. B. Characterization of Vernix Caseosa: Water Content, Morphology, and Elemental Analysis. *J. Invest. Dermatol.* **115**, 875–881 (2000).

30. Tollin, M. *et al.* Vernix caseosa as a multi-component defence system based on polypeptides, lipids and their interactions. *Cell. Mol. Life Sci. CMLS* **62**, 2390–2399 (2005).
31. Maynard, C. L., Elson, C. O., Hatton, R. D. & Weaver, C. T. Reciprocal interactions of the intestinal microbiota and immune system. *Nature* **489**, 231–241 (2012).
32. Kollmann, T. R., Kampmann, B., Mazmanian, S. K., Marchant, A. & Levy, O. Protecting the Newborn and Young Infant from Infectious Diseases: Lessons from Immune Ontogeny. *Immunity* **46**, 350–363 (2017).
33. Garofoli, F., Civardi, E., Pisoni, C., Angelini, M. & Ghirardello, S. Anti-Inflammatory and Anti-Allergic Properties of Colostrum from Mothers of Full-Term and Preterm Babies: The Importance of Maternal Lactation in the First Days. *Nutrients* **15**, 4249 (2023).
34. Xu, D. *et al.* Complement in breast milk modifies offspring gut microbiota to promote infant health. *Cell* **187**, 750-763.e20 (2024).
35. De Kleer, I., Willems, F., Lambrecht, B. & Goriely, S. Ontogeny of Myeloid Cells. *Front. Immunol.* **5**, (2014).
36. Adkins, B., Leclerc, C. & Marshall-Clarke, S. Neonatal adaptive immunity comes of age. *Nat. Rev. Immunol.* **4**, 553–564 (2004).
37. Ygberg, S. & Nilsson, A. The developing immune system - from foetus to toddler. *Acta Paediatr. Oslo Nor. 1992* **101**, 120–127 (2012).
38. Darmawan, D., Raychaudhuri, S., Lakshminrusimha, S. & Dimitriades, V. R. Hypogammaglobulinemia in neonates: illustrative cases and review of the literature. *J. Perinatol.* **44**, 929–934 (2024).
39. Rijkers, G. T., Sanders, E. A., Breukels, M. A. & Zegers, B. J. Infant B cell responses to polysaccharide determinants. *Vaccine* **16**, 1396–1400 (1998).
40. Sadarangani, M. Protection Against Invasive Infections in Children Caused by Encapsulated Bacteria. *Front. Immunol.* **9**, (2018).

41. Timens, W., Boes, A., Rozeboom-Uiterwijk, T. & Poppema, S. Immaturity of the human splenic marginal zone in infancy. Possible contribution to the deficient infant immune response. *J. Immunol. Baltim. Md 1950* **143**, 3200–3206 (1989).
42. Lamagni, T. L. *et al.* Emerging Trends in the Epidemiology of Invasive Group B Streptococcal Disease in England and Wales, 1991–2010. *Clin. Infect. Dis.* **57**, 682–688 (2013).
43. Martin, N. G., Sadarangani, M., Pollard, A. J. & Goldacre, M. J. Hospital admission rates for meningitis and septicaemia caused by *Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumoniae* in children in England over five decades: a population-based observational study. *Lancet Infect. Dis.* **14**, 397–405 (2014).
44. Simister, N. E. Placental transport of immunoglobulin G. *Vaccine* **21**, 3365–3369 (2003).
45. Van de Perre, P. Transfer of antibody via mother's milk. *Vaccine* **21**, 3374–3376 (2003).
46. Levy, O. Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nat. Rev. Immunol.* **7**, 379–390 (2007).
47. Lawrence, S. M., Corriden, R. & Nizet, V. Age-Appropriate Functions and Dysfunctions of the Neonatal Neutrophil. *Front. Pediatr.* **5**, (2017).
48. Köstlin, N. *et al.* Granulocytic myeloid derived suppressor cells expand in human pregnancy and modulate T-cell responses. *Eur. J. Immunol.* **44**, 2582–2591 (2014).
49. Gervassi, A. *et al.* Myeloid Derived Suppressor Cells Are Present at High Frequency in Neonates and Suppress In Vitro T Cell Responses. *PLOS ONE* **9**, e107816 (2014).
50. Nair, R. R., Sinha, P., Khanna, A. & Singh, K. Reduced Myeloid-derived Suppressor Cells in the Blood and Endometrium is Associated with Early Miscarriage. *Am. J. Reprod. Immunol. N. Y. N 1989* **73**, 479–486 (2015).
51. Köstlin-Gille, N. & Gille, C. Myeloid-Derived Suppressor Cells in Pregnancy and the Neonatal Period. *Front. Immunol.* **11**, (2020).

52. Schwarz, J. *et al.* Granulocytic myeloid-derived suppressor cells (GR-MDSC) accumulate in cord blood of preterm infants and remain elevated during the neonatal period. *Clin. Exp. Immunol.* **191**, 328–337 (2018).
53. Grumach, A. S., Ceccon, M. E., Rutz, R., Fertig, A. & Kirschfink, M. Complement profile in neonates of different gestational ages. *Scand. J. Immunol.* **79**, 276–281 (2014).
54. Moraes-Pinto, M. I. de, Suano-Souza, F. & Aranda, C. S. Immune system: development and acquisition of immunological competence. *J. Pediatr. (Rio J.)* **97**, S59–S66 (2020).
55. Ygberg, S. & Nilsson, A. The developing immune system - from foetus to toddler. *Acta Paediatr. Oslo Nor. 1992* **101**, 120–127 (2012).
56. Basha, S., Surendran, N. & Pichichero, M. Immune responses in neonates. *Expert Rev. Clin. Immunol.* **10**, 1171–1184 (2014).
57. Rudd, B. D. Neonatal T Cells: A Reinterpretation. *Annu. Rev. Immunol.* **38**, 229 (2020).
58. Davenport, M. P., Smith, N. L. & Rudd, B. D. Building a T cell compartment: how immune cell development shapes function. *Nat. Rev. Immunol.* **20**, 499–506 (2020).
59. Montecino-Rodriguez, E. & Dorshkind, K. B-1 B Cell Development in the Fetus and Adult. *Immunity* **36**, 13–21 (2012).
60. Glaesener, S. *et al.* Decreased production of class-switched antibodies in neonatal B cells is associated with increased expression of miR-181b. *PLoS ONE* **13**, e0192230 (2018).
61. Siegrist, C.-A. & Aspinall, R. B-cell responses to vaccination at the extremes of age. *Nat. Rev. Immunol.* **9**, 185–194 (2009).
62. Sarvaria, A. *et al.* IL-10⁺ regulatory B cells are enriched in cord blood and may protect against cGVHD after cord blood transplantation. *Blood* **128**, 1346–1361 (2016).
63. Esteve-Solé, A. *et al.* Characterization of the Highly Prevalent Regulatory CD24^{hi}CD38^{hi} B-Cell Population in Human Cord Blood. *Front. Immunol.* **8**, 201 (2017).

64. Blair, P. A. *et al.* CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity* **32**, 129–140 (2010).
65. Kwambana-Adams, B. Global burden of meningitis and implications for strategy. *Lancet Neurol.* **22**, 646–648 (2023).
66. Gilsdorf, J. R. *Continual Raving: A History of Meningitis and the People Who Conquered It.* (Oxford University Press, 2019). doi:10.1093/oso/9780190677312.001.0001.
67. Wijdicks, E. F. M. The Clinicians Who Recognized Meningitis. *Neurocrit. Care* **35**, 271–273 (2021).
68. Abercrombie, J. *Pathological and Practical Researches on Diseases of the Brain and the Spinal Cord.* (Edinburgh, Waugh and Innes, 1828).
69. Herpin, F. Méningitis, ou inflammation, des membranes de l'encéphale ; précédé de quelques considérations physiologiques sur ces membranes, et suivi de plusieurs observations recueillies à l'armée du Rhin. (Cand. F. Herpin). (Paris, 2).
70. Vergnano, S., Sharland, M., Kazembe, P., Mwansambo, C. & Heath, P. Neonatal sepsis: an international perspective. *Arch. Dis. Child. Fetal Neonatal Ed.* **90**, F220–F224 (2005).
71. Wilson, C. B., Nizet, V., Maldonado, Y., Remington, J. S. & Klein, J. O. *Remington and Klein's Infectious Diseases of the Fetus and Newborn Infant.* (Elsevier Health Sciences, 2015).
72. Gonçalves, B. P. *et al.* Group B streptococcus infection during pregnancy and infancy: estimates of regional and global burden. *Lancet Glob. Health* **10**, e807–e819 (2022).
73. Zaidi, A. K. M., Thaver, D., Ali, S. A. & Khan, T. A. Pathogens associated with sepsis in newborns and young infants in developing countries. *Pediatr. Infect. Dis. J.* **28**, S10–18 (2009).
74. Hasbun, R. Progress and Challenges in Bacterial Meningitis: A Review. *JAMA* **328**, 2147–2154 (2022).

75. Ku, L. C., Boggess, K. A. & Cohen-Wolkowicz, M. Bacterial meningitis in infants. *Clin. Perinatol.* **42**, 29–45, vii–viii (2015).
76. Neonatal Meningitis Treatment & Management: Pharmacologic and Supportive Therapy, Ventriculostomy, Prevention. (2024).
77. van de Beek, D. *et al.* Community-acquired bacterial meningitis. *Nat. Rev. Dis. Primer* **2**, 1–20 (2016).
78. Lamont, R. F. *et al.* Listeriosis in Human Pregnancy: a systematic review. *J. Perinat. Med.* **39**, 227–236 (2011).
79. Engelhardt, B. & Sorokin, L. The blood-brain and the blood-cerebrospinal fluid barriers: function and dysfunction. *Semin. Immunopathol.* **31**, 497–511 (2009).
80. Bisht, K. *et al.* Capillary-associated microglia regulate vascular structure and function through PANX1-P2RY12 coupling in mice. *Nat. Commun.* **12**, 5289 (2021).
81. Kim, K. S. Mechanisms of microbial traversal of the blood–brain barrier. *Nat. Rev. Microbiol.* **6**, 625–634 (2008).
82. Coureuil, M., Lécuyer, H., Bourdoulous, S. & Nassif, X. A journey into the brain: insight into how bacterial pathogens cross blood–brain barriers. *Nat. Rev. Microbiol.* **15**, 149–159 (2017).
83. Wolburg, H. & Lippoldt, A. Tight junctions of the blood-brain barrier: development, composition and regulation. *Vascul. Pharmacol.* **38**, 323–337 (2002).
84. Barichello, T. *et al.* Pathophysiology of neonatal acute bacterial meningitis. *J. Med. Microbiol.* **62**, 1781–1789 (2013).
85. Gerber, J. & Nau, R. Mechanisms of injury in bacterial meningitis. *Curr. Opin. Neurol.* **23**, 312–318 (2010).
86. İpek, M. Ş. Neonatal Bacterial Meningitis. in *Neonatal Medicine* (IntechOpen, 2019). doi:10.5772/intechopen.87118.
87. Hoffman, O. & Weber, R. J. Pathophysiology and Treatment of Bacterial Meningitis. *Ther. Adv. Neurol. Disord.* **2**, 1–7 (2009).

88. Pierre, W. C. *et al.* Neonatal microglia: The cornerstone of brain fate. *Brain. Behav. Immun.* **59**, 333–345 (2017).
89. Nau, R. & Brück, W. Neuronal injury in bacterial meningitis: mechanisms and implications for therapy. *Trends Neurosci.* **25**, 38–45 (2002).
90. Thigpen, M. C. *et al.* Bacterial meningitis in the United States, 1998-2007. *N. Engl. J. Med.* **364**, 2016–2025 (2011).
91. Nocard, E. & Mollereau, H. Sur une mammite contagieuse des vaches laitières. (1884).
92. Lehmann, K. B. & Neumann, R. O. *Atlas und Grundriss der Bakteriologie und Lehrbuch der speziellen bakteriologischen Diagnostik.* (J.F. Lehmann, München, 1896).
93. Lancefield, R. C. A SEROLOGICAL DIFFERENTIATION OF HUMAN AND OTHER GROUPS OF HEMOLYTIC STREPTOCOCCI. *J. Exp. Med.* **57**, 571–595 (1933).
94. Facklam, R. What happened to the streptococci: overview of taxonomic and nomenclature changes. *Clin. Microbiol. Rev.* **15**, 613–630 (2002).
95. Lancefield, R. C. A SEROLOGICAL DIFFERENTIATION OF SPECIFIC TYPES OF BOVINE HEMOLYTIC STREPTOCOCCI (GROUP B). *J. Exp. Med.* **59**, 441–458 (1934).
96. Lancefield, R. C. & Hare, R. THE SEROLOGICAL DIFFERENTIATION OF PATHOGENIC AND NON-PATHOGENIC STRAINS OF HEMOLYTIC STREPTOCOCCI FROM PARTURIENT WOMEN. *J. Exp. Med.* **61**, 335–349 (1935).
97. Lo, C.-W. *et al.* Serotype distribution and clinical correlation of *Streptococcus agalactiae* causing invasive disease in infants and children in Taiwan. *J. Microbiol. Immunol. Infect.* **52**, 578–584 (2019).
98. Edmond, K. M. *et al.* Group B streptococcal disease in infants aged younger than 3 months: systematic review and meta-analysis. *The Lancet* **379**, 547–556 (2012).

99. Tettelin, H. *et al.* Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V *Streptococcus agalactiae*. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 12391–12396 (2002).
100. Davies, H. D. *et al.* Multilocus sequence typing of serotype III group B streptococcus and correlation with pathogenic potential. *J. Infect. Dis.* **189**, 1097–1102 (2004).
101. Jones, N. *et al.* Multilocus sequence typing system for group B streptococcus. *J. Clin. Microbiol.* **41**, 2530–2536 (2003).
102. Fry, R. M. FATAL INFECTIONS BY HÆMOLYTIC STREPTOCOCCUS GROUP B. *The Lancet* **231**, 199–201 (1938).
103. Hill, A. M. & Butler, H. M. Hæmolytic Streptococcal Infections Following Childbirth and Abortion. II: Clinical Features, with Special Reference to Infections Due to Streptococci of Groups Other Than A. *Med. J. Aust.* **1**, 293–299 (1940).
104. Rantz, L. A. & Kirby, W. M. M. Hemolytic Streptococcus Bacteremia. *N. Engl. J. Med.* **227**, 730–733 (1942).
105. Eickhoff, T. C., Klein, J. O., Daly, A. K., Ingall, D. & Finland, M. NEONATAL SEPSIS AND OTHER INFECTIONS DUE TO GROUP B BETA-HEMOLYTIC STREPTOCOCCI. *N. Engl. J. Med.* **271**, 1221–1228 (1964).
106. Harper, I. A. The importance of group B streptococci as human pathogens in the British Isles. *J. Clin. Pathol.* **24**, 438–441 (1971).
107. Hood, M., Janney, A. & Dameron, G. Beta hemolytic streptococcus group B associated with problems of the perinatal period. *Am. J. Obstet. Gynecol.* **82**, 809–818 (1961).
108. Randis, T. M., Baker, J. A. & Ratner, A. J. Group B Streptococcal Infections. *Pediatr. Rev.* **38**, 254–262 (2017).
109. Wadilo, F. *et al.* Prevalence of Group B Streptococcus maternal colonization, serotype distribution, and antimicrobial resistance in Sub-Saharan Africa: A systematic review and meta-analysis. *J. Glob. Antimicrob. Resist.* **32**, 134–144 (2023).
110. Badri, M. S. *et al.* Rectal colonization with group B streptococcus: relation to vaginal colonization of pregnant women. *J. Infect. Dis.* **135**, 308–312 (1977).

111. Dillon, H. C., Gray, E., Pass, M. A. & Gray, B. M. Anorectal and vaginal carriage of group B streptococci during pregnancy. *J. Infect. Dis.* **145**, 794–799 (1982).
112. Kwatra, G. *et al.* Prevalence of maternal colonisation with group B streptococcus: a systematic review and meta-analysis. *Lancet Infect. Dis.* **16**, 1076–1084 (2016).
113. Borges, S., Silva, J. & Teixeira, P. Survival and biofilm formation by Group B streptococci in simulated vaginal fluid at different pHs. *Antonie Van Leeuwenhoek* **101**, 677–682 (2012).
114. Verani, J. R., McGee, L., Schrag, S. J., & Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention (CDC). Prevention of perinatal group B streptococcal disease--revised guidelines from CDC, 2010. *MMWR Recomm. Rep. Morb. Mortal. Wkly. Rep. Recomm. Rep.* **59**, 1–36 (2010).
115. Madrid, L. *et al.* Infant Group B Streptococcal Disease Incidence and Serotypes Worldwide: Systematic Review and Meta-analyses. *Clin. Infect. Dis.* **65**, S160–S172 (2017).
116. Johri, A. K. *et al.* Group B Streptococcus: global incidence and vaccine development. *Nat. Rev. Microbiol.* **4**, 932–942 (2006).
117. Furuta, A. *et al.* Bacterial and Host Determinants of Group B Streptococcal Infection of the Neonate and Infant. *Front. Microbiol.* **13**, (2022).
118. Doran, K. S. & Nizet, V. Molecular pathogenesis of neonatal group B streptococcal infection: no longer in its infancy. *Mol. Microbiol.* **54**, 23–31 (2004).
119. Melin, P. Neonatal group B streptococcal disease: from pathogenesis to preventive strategies. *Clin. Microbiol. Infect.* **17**, 1294–1303 (2011).
120. Ferrieri, P., Cleary, P. P. & Seeds, A. E. Epidemiology of group-B streptococcal carriage in pregnant women and newborn infants. *J. Med. Microbiol.* **10**, 103–114 (1977).
121. Pass, M. A., Gray, B. M., Khare, S. & Dillon, H. C. Prospective studies of group B streptococcal infections in infants. *J. Pediatr.* **95**, 437–443 (1979).

122. Imperi, M. *et al.* Invasive neonatal GBS infections from an area-based surveillance study in Italy. *Clin. Microbiol. Infect.* **17**, 1834–1839 (2011).
123. Berardi, A. *et al.* Group B Streptococcus Late-Onset Disease: 2003–2010. *Pediatrics* **131**, e361–e368 (2013).
124. Davanzo, R. *et al.* To feed or not to feed? Case presentation and best practice guidance for human milk feeding and group B streptococcus in developed countries. *J. Hum. Lact. Off. J. Int. Lact. Consult. Assoc.* **29**, 452–457 (2013).
125. Ager, E. P. C. *et al.* Hypervirulent Streptococcus agalactiae septicemia in twin ex-premature infants transmitted by breast milk: report of source detection and isolate characterization using commonly available molecular diagnostic methods. *Ann. Clin. Microbiol. Antimicrob.* **19**, 55 (2020).
126. Ackerman, D. L. *et al.* Antimicrobial and Antibiofilm Activity of Human Milk Oligosaccharides against Streptococcus agalactiae, Staphylococcus aureus, and Acinetobacter baumannii. *ACS Infect. Dis.* **4**, 315–324 (2018).
127. Kim, H. J. *et al.* Outbreak of late-onset group B streptococcal infections in healthy newborn infants after discharge from a maternity hospital: a case report. *J. Korean Med. Sci.* **21**, 347–350 (2006).
128. Collin, S. M. *et al.* Hospital clusters of invasive Group B Streptococcal disease: A systematic review. *J. Infect.* **79**, 521–527 (2019).
129. Hansen, S. M., Uldbjerg, N., Kilian, M. & Sørensen, U. B. S. Dynamics of Streptococcus agalactiae colonization in women during and after pregnancy and in their infants. *J. Clin. Microbiol.* **42**, 83–89 (2004).
130. Miselli, F. *et al.* Transmission of Group B Streptococcus in late-onset neonatal disease: a narrative review of current evidence. *Ther. Adv. Infect. Dis.* **9**, 20499361221142732 (2022).
131. Fluegge, K., Supper, S., Siedler, A. & Berner, R. Serotype Distribution of Invasive Group B Streptococcal Isolates in Infants: Results from a Nationwide Active Laboratory Surveillance Study over 2 Years in Germany. *Clin. Infect. Dis.* **40**, 760–763 (2005).

132. Poyart, C. *et al.* Invasive Group B Streptococcal Infections in Infants, France. *Emerg. Infect. Dis.* **14**, 1647 (2008).
133. Soriano, M. *et al.* Group B Streptococcus Crosses Human Epithelial Cells by a Paracellular Route. *J. Infect. Dis.* **193**, 241–250 (2006).
134. Domínguez, K., Lindon, A. K., Gibbons, J., Darch, S. E. & Randis, T. M. Group B Streptococcus Drives Major Transcriptomic Changes in the Colonic Epithelium. *Infect. Immun.* **91**, e0003523 (2023).
135. Gibson, R. L., Nizet, V. & Rubens, C. E. Group B streptococcal beta-hemolysin promotes injury of lung microvascular endothelial cells. *Pediatr. Res.* **45**, 626–634 (1999).
136. Travier, L. *et al.* Neonatal susceptibility to meningitis results from the immaturity of epithelial barriers and gut microbiota. *Cell Rep.* **35**, (2021).
137. Tazi, A. *et al.* The surface protein HvgA mediates group B streptococcus hypervirulence and meningeal tropism in neonates. *J. Exp. Med.* **207**, 2313–2322 (2010).
138. Hays, C. *et al.* Perinatal hormones favor CC17 group B Streptococcus intestinal translocation through M cells and hypervirulence in neonates. *eLife* **8**, e48772 (2019).
139. Pellegrini, A. *et al.* The serine-rich repeat glycoprotein Srr2 mediates Streptococcus agalactiae interaction with host fibronectin. *BMC Microbiol.* **24**, 221 (2024).
140. Rubens, C. E., Wessels, M. R., Heggen, L. M. & Kasper, D. L. Transposon mutagenesis of type III group B Streptococcus: correlation of capsule expression with virulence. *Proc. Natl. Acad. Sci. U. S. A.* **84**, 7208–7212 (1987).
141. Patterson, H. *et al.* Adult zebrafish model of bacterial meningitis in Streptococcus agalactiae infection. *Dev. Comp. Immunol.* **38**, 447–455 (2012).
142. Maisey, H. C., Doran, K. S. & Nizet, V. Recent advances in understanding the molecular basis of group B Streptococcus virulence. *Expert Rev. Mol. Med.* **10**, e27 (2008).

143. Cambronne, R. D. de *et al.* CC17 group B *Streptococcus* exploits integrins for neonatal meningitis development. *J. Clin. Invest.* **131**, (2024).
144. Aznar, E. *et al.* The hypervirulent Group B *Streptococcus* HvgA adhesin promotes central nervous system invasion through transcellular crossing of the choroid plexus. *Fluids Barriers CNS* **21**, 66 (2024).
145. Kim, B. J. *et al.* Bacterial induction of Snail1 contributes to blood-brain barrier disruption. *J. Clin. Invest.* **125**, 2473–2483 (2015).
146. Campeau, A. *et al.* Multidimensional Proteome Profiling of Blood-Brain Barrier Perturbation by Group B *Streptococcus*. *mSystems* **5**, e00368-20 (2020).
147. Benmimoun, B. *et al.* An original infection model identifies host lipoprotein import as a route for blood-brain barrier crossing. *Nat. Commun.* **11**, 6106 (2020).
148. Zhu, N. *et al.* Therapeutic development of group B *Streptococcus* meningitis by targeting a host cell signaling network involving EGFR. *EMBO Mol. Med.* **13**, e12651 (2021).
149. Prevention of Group B *Streptococcal* Early-Onset Disease in Newborns: ACOG Committee Opinion, Number 797. *Obstet. Gynecol.* **135**, e51–e72 (2020).
150. Dhudasia, M. B., Flannery, D. D., Pfeifer, M. R. & Puopolo, K. M. Updated Guidance: Prevention and Management of Perinatal Group B *Streptococcus* Infection. *NeoReviews* **22**, e177–e188 (2021).
151. Afsari, M., White, A. & Adhikari, E. H. Group B *Streptococcus* and Intraamniotic Inflammation and Infection. *Clin. Obstet. Gynecol.* **67**, 576 (2024).
152. Puopolo, K. M., Lynfield, R., Cummings, J. J., COMMITTEE ON FETUS AND NEWBORN, & COMMITTEE ON INFECTIOUS DISEASES. Management of Infants at Risk for Group B *Streptococcal* Disease. *Pediatrics* **144**, e20191881 (2019).
153. Bekker, V., Bijlsma, M. W., van de Beek, D., Kuijpers, T. W. & van der Ende, A. Incidence of invasive group B streptococcal disease and pathogen genotype distribution in newborn babies in the Netherlands over 25 years: a nationwide surveillance study. *Lancet Infect. Dis.* **14**, 1083–1089 (2014).

154. Group B streptococcus - UK National Screening Committee (UK NSC) - GOV.UK.
<https://view-health-screening-recommendations.service.gov.uk/group-b-streptococcus/>.
155. Rao, G. G. & Khanna, P. To screen or not to screen women for Group B Streptococcus (*Streptococcus agalactiae*) to prevent early onset sepsis in newborns: recent advances in the unresolved debate. *Ther. Adv. Infect. Dis.* **7**, 2049936120942424 (2020).
156. Lohrmann, F. *et al.* Maternal *Streptococcus agalactiae* colonization in Europe: data from the multi-center DEVANI study. *Infection* (2024) doi:10.1007/s15010-024-02380-0.
157. Jordan, H. T. *et al.* Revisiting the need for vaccine prevention of late-onset neonatal group B streptococcal disease: a multistate, population-based analysis. *Pediatr. Infect. Dis. J.* **27**, 1057–1064 (2008).
158. Le Doare, K. & Heath, P. T. An overview of global GBS epidemiology. *Vaccine* **31 Suppl 4**, D7-12 (2013).
159. Glasgow, T. S. *et al.* Association of intrapartum antibiotic exposure and late-onset serious bacterial infections in infants. *Pediatrics* **116**, 696–702 (2005).
160. Stoll, B. J. *et al.* Changes in pathogens causing early-onset sepsis in very-low-birth-weight infants. *N. Engl. J. Med.* **347**, 240–247 (2002).
161. Corvaglia, L. *et al.* Influence of Intrapartum Antibiotic Prophylaxis for Group B Streptococcus on Gut Microbiota in the First Month of Life. *J. Pediatr. Gastroenterol. Nutr.* **62**, 304–308 (2016).
162. Mazzola, G. *et al.* Early Gut Microbiota Perturbations Following Intrapartum Antibiotic Prophylaxis to Prevent Group B Streptococcal Disease. *PloS One* **11**, e0157527 (2016).
163. Nogacka, A. *et al.* Impact of intrapartum antimicrobial prophylaxis upon the intestinal microbiota and the prevalence of antibiotic resistance genes in vaginally delivered full-term neonates. *Microbiome* **5**, 93 (2017).

164. Wang, B., Yao, M., Lv, L., Ling, Z. & Li, L. The Human Microbiota in Health and Disease. *Engineering* **3**, 71–82 (2017).
165. Defeating meningitis by 2030: a global road map. <https://www.who.int/publications/i/item/9789240026407>.
166. Ghannam, J. Y. & Al Kharazi, K. A. Neuroanatomy, Cranial Meninges. in *StatPearls* (Treasure Island (FL), 2020).
167. Wilkins, R. H. Neurosurgical classic—XVII. *J. Neurosurg.* **21**, 240–244 (1964).
168. Zhang, J., Smith, D., Yamamoto, M., Ma, L. & McCaffery, P. The meninges is a source of retinoic acid for the late-developing hindbrain. *J. Neurosci.* **23**, 7610–7620 (2003).
169. Bifari, F. *et al.* Novel stem/progenitor cells with neuronal differentiation potential reside in the leptomeningeal niche. *J. Cell. Mol. Med.* **13**, 3195–3208 (2009).
170. Siegenthaler, J. A. *et al.* Retinoic acid from the meninges regulates cortical neuron generation. *Cell* **139**, 597–609 (2009).
171. Zarbalis, K., Choe, Y., Siegenthaler, J. A., Orosco, L. A. & Pleasure, S. J. Meningeal defects alter the tangential migration of cortical interneurons in *Foxc1^{hith/hith}* mice. *Neural Develop.* **7**, 2 (2012).
172. Choe, Y., Huynh, T. & Pleasure, S. J. Migration of Oligodendrocyte Progenitor Cells Is Controlled by Transforming Growth Factor β Family Proteins during Corticogenesis. *J. Neurosci.* **34**, 14973–14983 (2014).
173. Mishra, S., Choe, Y., Pleasure, S. J. & Siegenthaler, J. A. Cerebrovascular defects in *Foxc1* mutants correlate with aberrant WNT and VEGF-A pathways downstream of retinoic acid from the meninges. *Dev Biol* **420**, 148–165 (2016).
174. Tischfield, M. A. *et al.* Cerebral Vein Malformations Result from Loss of Twist1 Expression and BMP Signaling from Skull Progenitor Cells and Dura. *Dev Cell* **42**, 445–461.e5 (2017).
175. Dasgupta, K. & Jeong, J. Developmental biology of the meninges. *genesis* **57**, e23288 (2019).

176. Etchevers, H. C., Couly, G., Vincent, C. & Le Douarin, N. M. Anterior cephalic neural crest is required for forebrain viability. *Development* **126**, 3533–43 (1999).
177. Siegenthaler, J. A. & Pleasure, S. J. We have got you ‘covered’: how the meninges control brain development. *Curr. Opin. Genet. Dev.* **21**, 249–255 (2011).
178. Decimo, I. *et al.* Meninges: A Widespread Niche of Neural Progenitors for the Brain. *The Neuroscientist* 1073858420954826 (2020) doi:10.1177/1073858420954826.
179. Alves de Lima, K., Rustenhoven, J. & Kipnis, J. Meningeal Immunity and Its Function in Maintenance of the Central Nervous System in Health and Disease. *Annu. Rev. Immunol.* **38**, 597–620 (2020).
180. Rua, R. & McGavern, D. B. Advances in Meningeal Immunity. *Trends Mol Med* **24**, 542–559 (2018).
181. Coles, J. A., Myburgh, E., Brewer, J. M. & McMenamin, P. G. Where are we? The anatomy of the murine cortical meninges revisited for intravital imaging, immunology, and clearance of waste from the brain. *Prog. Neurobiol.* **156**, 107–148 (2017).
182. Møllgård, K. *et al.* A mesothelium divides the subarachnoid space into functional compartments. *Science* **379**, 84–88 (2023).
183. Liliequist, B. The anatomy of the subarachnoid cisterns. *Acta Radiol.* **46**, 61–71 (1956).
184. Yasargil, M. G., Kasdaglis, K., Jain, K. K. & Weber, H. P. Anatomical observations of the subarachnoid cisterns of the brain during surgery. *J. Neurosurg.* **44**, 298–302 (1976).
185. Plá, V. *et al.* Structural characterization of SLYM—a 4th meningeal membrane. *Fluids Barriers CNS* **20**, 93 (2023).
186. Mapunda, J. A. *et al.* VE-cadherin in arachnoid and pia mater cells serves as a suitable landmark for in vivo imaging of CNS immune surveillance and inflammation. *Nat. Commun.* **14**, 5837 (2023).
187. Betsholtz, C. *et al.* Advances and controversies in meningeal biology. *Nat. Neurosci.* **27**, 2056–2072 (2024).

188. Waggener, J. D. & Beggs, J. The membranous coverings of neural tissues: an electron microscopy study. *J. Neuropathol. Exp. Neurol.* **26**, 412–426 (1967).
189. Nabeshima, S., Reese, T. S., Landis, D. M. & Brightman, M. W. Junctions in the meninges and marginal glia. *J. Comp. Neurol.* **164**, 127–169 (1975).
190. Rascol, M. M. & Izard, J. Y. The subdural neurothelium of the cranial meninges in man. *Anat. Rec.* **186**, 429–436 (1976).
191. Schachenmayr, W. & Friede, R. L. The origin of subdural neomembranes. I. Fine structure of the dura-arachnoid interface in man. *Am. J. Pathol.* **92**, 53–68 (1978).
192. Frederickson, R. G. The subdural space interpreted as a cellular layer of meninges. *Anat. Rec.* **230**, 38–51 (1991).
193. Haines, D. E., Harkey, H. L. & al-Mefty, O. The ‘subdural’ space: a new look at an outdated concept. *Neurosurgery* **32**, 111–120 (1993).
194. Mack, J., Squier, W. & Eastman, J. T. Anatomy and development of the meninges: implications for subdural collections and CSF circulation. *Pediatr. Radiol.* **39**, 200–210 (2009).
195. Balin, B. J., Broadwell, R. D., Salzman, M. & el-Kalliny, M. Avenues for entry of peripherally administered protein to the central nervous system in mouse, rat, and squirrel monkey. *J Comp Neurol* **251**, 260–80 (1986).
196. Absinta, M. *et al.* Human and nonhuman primate meninges harbor lymphatic vessels that can be visualized noninvasively by MRI. **6**, e29738 (2017).
197. Antila, S. *et al.* Development and plasticity of meningeal lymphatic vessels. *J. Exp. Med.* **214**, 3645–3667 (2017).
198. Aspelund, A. *et al.* A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. *J. Exp. Med.* **212**, 991–999 (2015).
199. Jani, R. H. & Sekula Jr, R. F. %J N. Magnetic resonance imaging of human dural meningeal lymphatics. **83**, E10–E12 (2018).
200. Louveau, A. *et al.* CNS lymphatic drainage and neuroinflammation are regulated by meningeal lymphatic vasculature. *Nat. Neurosci.* **21**, 1380–1391 (2018).

201. Hagan, C. E., Bolon, B. & Keene, C. D. 20 - Nervous System. in *Comparative Anatomy and Histology* (eds. Treuting, P. M. & Dintzis, S. M.) 339–394 (Academic Press, San Diego, 2012).
202. Reiter, R. J., Tan, D. X., Kim, S. J. & Cruz, M. H. C. Delivery of pineal melatonin to the brain and SCN: role of canaliculi, cerebrospinal fluid, tanycytes and Virchow–Robin perivascular spaces. *Brain Struct. Funct.* **219**, 1873–1887 (2014).
203. Izen, R. M., Yamazaki, T., Nishinaka-Arai, Y., Hong, Y. K. & Mukouyama, Y. S. Postnatal development of lymphatic vasculature in the brain meninges. *Dev Dyn* **247**, 741–753 (2018).
204. Sato, T., Konishi, H., Tamada, H., Nishiwaki, K. & Kiyama, H. Morphology, localization, and postnatal development of dural macrophages. *Cell Tissue Res.* (2021) doi:10.1007/s00441-020-03346-y.
205. Goldmann, T. *et al.* Origin, fate and dynamics of macrophages at central nervous system interfaces. *Nat. Immunol.* **17**, 797–805 (2016).
206. Kim, Y.-C. *et al.* Immaturity of immune cells around the dural venous sinuses contributes to viral meningoencephalitis in neonates. *Sci. Immunol.* **8**, eadg6155 (2023).
207. Rebejac, J. *et al.* Meningeal macrophages protect against viral neuroinfection. *Immunity* **55**, 2103–2117.e10 (2022).
208. Tanabe, S. & Yamashita, T. B-1a lymphocytes promote oligodendrogenesis during brain development. *Nat Neurosci* **21**, 506–516 (2018).
209. Wang, J., Rattner, A. & Nathans, J. Bacterial meningitis in the early postnatal mouse studied at single-cell resolution. *eLife* **12**, e86130 (2023).
210. Wang, Y. *et al.* Early developing B cells undergo negative selection by central nervous system-specific antigens in the meninges. *Immunity* **54**, 2784–2794.e6 (2021).
211. Alves de Lima, K. *et al.* Meningeal $\gamma\delta$ T cells regulate anxiety-like behavior via IL-17a signaling in neurons. *Nat. Immunol.* **21**, 1421–1429 (2020).

212. Ribeiro, M. *et al.* Meningeal gammadelta T cell-derived IL-17 controls synaptic plasticity and short-term memory. *Sci Immunol* **4**, (2019).
213. Nazmi, A. *et al.* Lymphocytes Contribute to the Pathophysiology of Neonatal Brain Injury. *Front Neurol* **9**, 159 (2018).
214. Albertsson, A.-M. *et al.* $\gamma\delta$ T Cells Contribute to Injury in the Developing Brain. *Am J Pathol* **188**, 757–767 (2018).
215. Fitzpatrick, Z. *et al.* Gut-educated IgA plasma cells defend the meningeal venous sinuses. *Nature* **587**, 472–476 (2020).
216. Bauler, T. J. *et al.* Salmonella Meningitis Associated with Monocyte Infiltration in Mice. *Am J Pathol* **187**, 187–199 (2017).
217. Chimelli, L. *et al.* The spectrum of neuropathological changes associated with congenital Zika virus infection. *Acta Neuropathol* **133**, 983–999 (2017).
218. Coles, J. A. *et al.* Intravital imaging of a massive lymphocyte response in the cortical dura of mice after peripheral infection by trypanosomes. *PLoS Negl Trop Dis* **9**, e0003714 (2015).
219. Kang, S. S. & McGavern, D. B. Lymphocytic choriomeningitis infection of the central nervous system. *Front Biosci* **13**, 4529–43 (2008).
220. Kang, S. S. & McGavern, D. B. Microbial Induction of Vascular Pathology in the CNS. *J. Neuroimmune Pharmacol.* **5**, 370–386 (2010).
221. Koopmans, M. M. *et al.* Characterization of a *Listeria monocytogenes* meningitis mouse model. *J. Neuroinflammation* **15**, 257 (2018).
222. Lamers, S. L., Gray, R. R., Salemi, M., Huysentruyt, L. C. & McGrath, M. S. HIV-1 phylogenetic analysis shows HIV-1 transits through the meninges to brain and peripheral tissues. *Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis.* **11**, 31–37 (2011).
223. Mogk, S. *et al.* African trypanosomes and brain infection - the unsolved question. *Biol Rev Camb Philos Soc* **92**, 1675–1687 (2017).

224. Poltera, A. A., Hochmann, A., Rudin, W. & Lambert, P. H. Trypanosoma brucei brucei: a model for cerebral trypanosomiasis in mice--an immunological, histological and electronmicroscopic study. *Clin Exp Immunol* **40**, 496–507 (1980).
225. Sjölander, H. & Jonsson, A. B. Olfactory nerve--a novel invasion route of Neisseria meningitidis to reach the meninges. *PLoS One* **5**, e14034 (2010).
226. Azevedo, R. S. S. *et al.* In situ immune response and mechanisms of cell damage in central nervous system of fatal cases microcephaly by Zika virus. *Sci Rep* **8**, 1 (2018).
227. Kim, J. V., Kang, S. S., Dustin, M. L. & McGavern, D. B. Myelomonocytic cell recruitment causes fatal CNS vascular injury during acute viral meningitis. *Nature* **457**, 191–5 (2009).
228. Rua, R. *et al.* Infection drives meningeal engraftment by inflammatory monocytes that impairs CNS immunity. *Nat. Immunol.* **20**, 407–419 (2019).
229. Peters, C. W., Kruse, U., Pollwein, R., Grzeschik, K. H. & Sippel, A. E. The human lysozyme gene. Sequence organization and chromosomal localization. *Eur. J. Biochem.* **182**, 507–516 (1989).
230. Cross, M. & Renkawitz, R. Repetitive sequence involvement in the duplication and divergence of mouse lysozyme genes. *EMBO J.* **9**, 1283–1288 (1990).
231. Wen, J.-H., Li, D.-Y., Liang, S. & Tang, J.-X. Is LysM-Cre a good candidate Cre for knocking out Atg5 gene in mice? *Front. Immunol.* **13**, 964496 (2022).
232. De Vlaminc, K. *et al.* Differential plasticity and fate of brain-resident and recruited macrophages during the onset and resolution of neuroinflammation. *Immunity* **55**, 2085-2102.e9 (2022).
233. O'Brien, C. A. *et al.* CD11c-Expressing Cells Affect Regulatory T Cell Behavior in the Meninges during Central Nervous System Infection. *J Immunol* **198**, 4054–4061 (2017).
234. Cervantes-Barragán, L. *et al.* Regulatory T cells selectively preserve immune privilege of self-antigens during viral central nervous system infection. *J Immunol* **188**, 3678–85 (2012).

235. de Aquino, M. T. *et al.* Role of CD25(+) CD4(+) T cells in acute and persistent coronavirus infection of the central nervous system. *Virology* **447**, 112–20 (2013).
236. Lanteri, M. C. *et al.* Tregs control the development of symptomatic West Nile virus infection in humans and mice. *J Clin Invest* **119**, 3266–77 (2009).
237. Smigielski, K. S. *et al.* CCR7 provides localized access to IL-2 and defines homeostatically distinct regulatory T cell subsets. *J Exp Med* **211**, 121–36 (2014).
238. Maudet, C. *et al.* Bacterial inhibition of Fas-mediated killing promotes neuroinvasion and persistence. *Nature* **603**, 900–906 (2022).
239. Christodoulides, M. *et al.* Interaction of *Neisseria meningitidis* with human meningeal cells induces the secretion of a distinct group of chemotactic, proinflammatory, and growth-factor cytokines. *Infect Immun* **70**, 4035–44 (2002).
240. Fowler, M. I., Yin, K. Y., Humphries, H. E., Heckels, J. E. & Christodoulides, M. Comparison of the inflammatory responses of human meningeal cells following challenge with *Neisseria lactamica* and with *Neisseria meningitidis*. *Infect Immun* **74**, 6467–78 (2006).
241. Hardy, S. J., Christodoulides, M., Weller, R. O. & Heckels, J. E. Interactions of *Neisseria meningitidis* with cells of the human meninges. *Mol Microbiol* **36**, 817–29 (2000).
242. Oordt-Speets, A. M., Bolijn, R., van Hoorn, R. C., Bhavsar, A. & Kyaw, M. H. %J P. one. Global etiology of bacterial meningitis: a systematic review and meta-analysis. **13**, e0198772 (2018).
243. Pinho-Ribeiro, F. A. *et al.* Bacteria hijack a meningeal neuroimmune axis to facilitate brain invasion. *Nature* **615**, 472–481 (2023).
244. Audshasai, T. *et al.* *Streptococcus pneumoniae* Rapidly Translocate from the Nasopharynx through the Cribriform Plate to Invade the Outer Meninges. *mBio* **13**, e01024-22 (2022).

245. Gres, V. *et al.* Dynamic role of monocytes and meningeal macrophages in bacterial meningoencephalitis. 2024.02.27.582183 Preprint at <https://doi.org/10.1101/2024.02.27.582183> (2024).
246. Rankin, L. C. & Artis, D. Beyond Host Defense: Emerging Functions of the Immune System in Regulating Complex Tissue Physiology. *Cell* **173**, 554–567 (2018).
247. Huh, J. R. & Veiga-Fernandes, H. Neuroimmune circuits in inter-organ communication. *Nat. Rev. Immunol.* **20**, 217–228 (2020).
248. Lipowski, Z. J. Psychosomatic medicine: past and present. Part I. Historical background. *Can. J. Psychiatry Rev. Can. Psychiatr.* **31**, 2–7 (1986).
249. Besedovsky, H. *et al.* The immune response evokes changes in brain noradrenergic neurons. *Science* **221**, 564–566 (1983).
250. Stead, R. H. *et al.* Intestinal mucosal mast cells in normal and nematode-infected rat intestines are in intimate contact with peptidergic nerves. *Proc. Natl. Acad. Sci. U. S. A.* **84**, 2975–2979 (1987).
251. Gottfried, C., Bambini-Junior, V., Francis, F., Riesgo, R. & Savino, W. The Impact of Neuroimmune Alterations in Autism Spectrum Disorder. *Front. Psychiatry* **6**, (2015).
252. Yang, J.-X. *et al.* Potential Neuroimmune Interaction in Chronic Pain: A Review on Immune Cells in Peripheral and Central Sensitization. *Front. Pain Res.* **3**, 946846 (2022).
253. Charabati, M., Wheeler, M. A., Weiner, H. L. & Quintana, F. J. Multiple sclerosis: Neuroimmune crosstalk and therapeutic targeting. *Cell* **186**, 1309–1327 (2023).
254. Chen, B.-R., Wu, T., Chen, T.-H. & Wang, Y. Neuroimmune interactions and their roles in neurodegenerative diseases. *Fundam. Res.* **4**, 251–261 (2024).
255. Khanmammadova, N., Islam, S., Sharma, P. & Amit, M. Neuro-immune interactions and immuno-oncology. *Trends Cancer* **9**, 636–649 (2023).
256. Vacharasin, J. M. *et al.* Neuroimmune mechanisms in autism etiology - untangling a complex problem using human cellular models. *Oxf. Open Neurosci.* **3**, kvae003 (2024).

257. Godinho-Silva, C., Cardoso, F. & Veiga-Fernandes, H. Neuro-Immune Cell Units: A New Paradigm in Physiology. *Annu. Rev. Immunol.* **37**, 19–46 (2019).
258. Kraus, A., Buckley, K. M. & Salinas, I. Sensing the world and its dangers: An evolutionary perspective in neuroimmunology. *eLife* **10**, e66706 (2021).
259. Wiertsema, S. P., van Bergenhenegouwen, J., Garssen, J. & Knippels, L. M. J. The Interplay between the Gut Microbiome and the Immune System in the Context of Infectious Diseases throughout Life and the Role of Nutrition in Optimizing Treatment Strategies. *Nutrients* **13**, 886 (2021).
260. Furness, J. B., Callaghan, B. P., Rivera, L. R. & Cho, H.-J. The enteric nervous system and gastrointestinal innervation: integrated local and central control. *Adv. Exp. Med. Biol.* **817**, 39–71 (2014).
261. Jacobson, A., Yang, D., Vella, M. & Chiu, I. M. The intestinal neuro-immune axis: crosstalk between neurons, immune cells, and microbes. *Mucosal Immunol.* **14**, 555–565 (2021).
262. Waxenbaum, J. A., Reddy, V. & Varacallo, M. Anatomy, Autonomic Nervous System. in *StatPearls* (StatPearls Publishing, Treasure Island (FL), 2024).
263. Koop, L. K. & Tadi, P. Neuroanatomy, Sensory Nerves. in *StatPearls* (StatPearls Publishing, Treasure Island (FL), 2024).
264. Muller, P. A. *et al.* Crosstalk between muscularis macrophages and enteric neurons regulates gastrointestinal motility. *Cell* **158**, 300–313 (2014).
265. van Diest, S. A., Stanisor, O. I., Boeckxstaens, G. E., de Jonge, W. J. & van den Wijngaard, R. M. Relevance of mast cell–nerve interactions in intestinal nociception. *Biochim. Biophys. Acta BBA - Mol. Basis Dis.* **1822**, 74–84 (2012).
266. Hu, D. *et al.* Immunofluorescence characterization of innervation and nerve-immune cell interactions in mouse lymph nodes. *Eur. J. Histochem. EJH* **63**, 3059 (2019).
267. Bost, K. L. & Pascual, D. W. Substance P: a late-acting B lymphocyte differentiation cofactor. *Am. J. Physiol.-Cell Physiol.* **262**, C537–C545 (1992).

268. Kasproicz, D. J. *et al.* Stimulation of the B Cell Receptor, CD86 (B7-2), and the β 2-Adrenergic Receptor Intrinsically Modulates the Level of IgG1 and IgE Produced per B Cell1. *J. Immunol.* **165**, 680–690 (2000).
269. Vicentini, F. A. *et al.* Intestinal microbiota shapes gut physiology and regulates enteric neurons and glia. *Microbiome* **9**, 210 (2021).
270. Erny, D. *et al.* Host microbiota constantly control maturation and function of microglia in the CNS. *Nat. Neurosci.* **18**, 965–977 (2015).
271. Haghighia, A. *et al.* Dietary Fatty Acids Directly Impact Central Nervous System Autoimmunity via the Small Intestine. *Immunity* **43**, 817–829 (2015).
272. Becher, B., Spath, S. & Goverman, J. Cytokine networks in neuroinflammation. *Nat. Rev. Immunol.* **17**, 49–59 (2017).
273. Kim, T.-K. *et al.* Local Interleukin-18 System in the Basolateral Amygdala Regulates Susceptibility to Chronic Stress. *Mol. Neurobiol.* **54**, 5347–5358 (2017).
274. Stellwagen, D. & Malenka, R. C. Synaptic scaling mediated by glial TNF- α . *Nature* **440**, 1054–1059 (2006).
275. Zhu, P. J. *et al.* Suppression of PKR Promotes Network Excitability and Enhanced Cognition by Interferon- γ -Mediated Disinhibition. *Cell* **147**, 1384–1396 (2011).
276. Singh, V. *et al.* Microbiota Dysbiosis Controls the Neuroinflammatory Response after Stroke. *J. Neurosci.* **36**, 7428–7440 (2016).
277. Benakis, C. *et al.* Commensal microbiota affects ischemic stroke outcome by regulating intestinal $\gamma\delta$ T cells. *Nat. Med.* **22**, 516–523 (2016).
278. Xu, D. *et al.* Peripherally derived T regulatory and $\gamma\delta$ T cells have opposing roles in the pathogenesis of intractable pediatric epilepsy. *J. Exp. Med.* **215**, 1169–1186 (2018).
279. Buffington, S. A. *et al.* Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring. *Cell* **165**, 1762–1775 (2016).
280. Leclercq, S. *et al.* Low-dose penicillin in early life induces long-term changes in murine gut microbiota, brain cytokines and behavior. *Nat. Commun.* **8**, 15062 (2017).

281. Gomez de Agüero, M. *et al.* The maternal microbiota drives early postnatal innate immune development. *Science* **351**, 1296–1302 (2016).
282. Aven, L. & Ai, X. Mechanisms of respiratory innervation during embryonic development. *Organogenesis* **9**, 194–198 (2013).
283. Lommel, A. V. Pulmonary neuroendocrine cells (PNEC) and neuroepithelial bodies (NEB): chemoreceptors and regulators of lung development. *Paediatr. Respir. Rev.* **2**, 171–176 (2001).
284. Cyphert, J. M. *et al.* Co-operation between mast cells and neurons is essential for antigen-mediated bronchoconstriction. *J. Immunol. Baltim. Md 1950* **182**, 7430–7439 (2009).
285. Ansel, J. C., Brown, J. R., Payan, D. G. & Brown, M. A. Substance P selectively activates TNF-alpha gene expression in murine mast cells. *J. Immunol. Baltim. Md 1950* **150**, 4478–4485 (1993).
286. Kulka, M., Sheen, C. H., Tancowny, B. P., Grammer, L. C. & Schleimer, R. P. Neuropeptides activate human mast cell degranulation and chemokine production. *Immunology* **123**, 398–410 (2008).
287. Nussbaum, J. C. *et al.* Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature* **502**, 245–248 (2013).
288. Talbot, S. *et al.* Silencing nociceptor neurons reduces allergic airway inflammation. *Neuron* **87**, 341–354 (2015).
289. Kamei, J., Takahashi, Y., Yoshikawa, Y. & Saitoh, A. Involvement of P2X receptor subtypes in ATP-induced enhancement of the cough reflex sensitivity. *Eur. J. Pharmacol.* **528**, 158–161 (2005).
290. Arzola-Martínez, L. *et al.* Blocking ATP-releasing channels prevents high extracellular ATP levels and airway hyperreactivity in an asthmatic mouse model. *Am. J. Physiol.-Lung Cell. Mol. Physiol.* **321**, L466–L476 (2021).
291. Yu, J., Deng, Z. & Roman, J. Neuro-immune Mechanisms and Pulmonary Fibrosis. *FASEB J.* **33**, 740.2-740.2 (2019).

292. Sawatzky, D. A. *et al.* Eosinophil adhesion to cholinergic nerves via ICAM-1 and VCAM-1 and associated eosinophil degranulation. *Am. J. Physiol.-Lung Cell. Mol. Physiol.* **282**, L1279–L1288 (2002).
293. Nie, Z., Nelson, C. S., Jacoby, D. B. & Fryer, A. D. Expression and regulation of intercellular adhesion molecule-1 on airway parasympathetic nerves. *J. Allergy Clin. Immunol.* **119**, 1415–1422 (2007).
294. Fryer, A. D. *et al.* Neuronal eotaxin and the effects of CCR3 antagonist on airway hyperreactivity and M2 receptor dysfunction. *J. Clin. Invest.* **116**, 228–236 (2006).
295. Sui, P. *et al.* Pulmonary Neuroendocrine Cells Amplify Allergic Asthma Responses. *Science* **360**, eaan8546 (2018).
296. Gabanyi, I. *et al.* Neuro-immune interactions drive tissue programming in intestinal macrophages. *Cell* **164**, 378–391 (2016).
297. Matheis, F. *et al.* Adrenergic Signaling in Muscularis Macrophages Limits Infection-Induced Neuronal Loss. *Cell* **180**, 64-78.e16 (2020).
298. Cardoso, V. *et al.* Neuronal regulation of type 2 innate lymphoid cells via neuromedin U. *Nature* **549**, 277–281 (2017).
299. Xu, H. *et al.* Transcriptional Atlas of Intestinal Immune Cells Reveals that Neuropeptide α -CGRP Modulates Group 2 Innate Lymphoid Cell Responses. *Immunity* **51**, 696-708.e9 (2019).
300. Moriyama, S. *et al.* β 2-adrenergic receptor-mediated negative regulation of group 2 innate lymphoid cell responses. *Science* **359**, 1056–1061 (2018).
301. Ibiza, S. *et al.* Glial-cell-derived neuroregulators control type 3 innate lymphoid cells and gut defence. *Nature* **535**, 440–443 (2016).
302. Talbot, J. *et al.* Feeding-dependent VIP neuron–ILC3 circuit regulates the intestinal barrier. *Nature* **579**, 575–580 (2020).
303. Darby, M. *et al.* The M3 Muscarinic Receptor Is Required for Optimal Adaptive Immunity to Helminth and Bacterial Infection. *PLOS Pathog.* **11**, e1004636 (2015).

304. Ramirez, V. T. *et al.* T-cell derived acetylcholine aids host defenses during enteric bacterial infection with *Citrobacter rodentium*. *PLOS Pathog.* **15**, e1007719 (2019).
305. Olofsson, P. S. *et al.* $\alpha 7$ Nicotinic Acetylcholine Receptor ($\alpha 7$ nAChR) Expression in Bone Marrow-Derived Non-T Cells Is Required for the Inflammatory Reflex. *Mol. Med.* **18**, 539–543 (2012).
306. Cox, M. A. *et al.* Choline acetyltransferase-expressing T cells are required to control chronic viral infection. *Science* **363**, 639–644 (2019).
307. White, J. P. *et al.* Intestinal Dysmotility Syndromes following Systemic Infection by Flaviviruses. *Cell* **175**, 1198-1212.e12 (2018).
308. Sanchez-Ruiz, M. *et al.* Enteric Murine Ganglionitis Induced by Autoimmune CD8 T Cells Mimics Human Gastrointestinal Dysmotility. *Am. J. Pathol.* **189**, 540–551 (2019).
309. Su, X., Matthay, M. A. & Malik, A. B. Requisite Role of the Cholinergic $\alpha 7$ Nicotinic Acetylcholine Receptor Pathway in Suppressing Gram-Negative Sepsis-Induced Acute Lung Inflammatory Injury. *J. Immunol.* **184**, 401–410 (2009).
310. Liu, T. *et al.* Local sympathetic innervations modulate the lung innate immune responses. *Sci. Adv.* **6**, eaay1497 (2020).
311. Baral, P. *et al.* Nociceptor sensory neurons suppress neutrophil and $\gamma \delta$ T cell responses in bacterial lung infections and lethal pneumonia. *Nat. Med.* **24**, 417–426 (2018).
312. Ruhl, C. R. *et al.* Mycobacterium tuberculosis Sulfolipid-1 Activates Nociceptive Neurons and Induces Cough. *Cell* **181**, 293-305.e11 (2020).
313. Grebe, K. M. *et al.* Cutting Edge: Sympathetic Nervous System Increases Proinflammatory Cytokines and Exacerbates Influenza A Virus Pathogenesis. *J. Immunol.* **184**, 540–544 (2009).
314. Gao, Z. *et al.* Vagal- $\alpha 7$ nAChR signaling is required for lung anti-inflammatory responses and arginase 1 expression during an influenza infection. *Acta Pharmacol. Sin.* **42**, 1642–1652 (2021).

315. Verzele, N. A. J. *et al.* The impact of influenza pulmonary infection and inflammation on vagal bronchopulmonary sensory neurons. *FASEB J.* **35**, e21320 (2021).
316. Horkowitz, A. P. *et al.* Acetylcholine Regulates Pulmonary Pathology During Viral Infection and Recovery. *ImmunoTargets Ther.* **9**, 333–350 (2020).
317. Shi, L., Fatemi, S. H., Sidwell, R. W. & Patterson, P. H. Maternal Influenza Infection Causes Marked Behavioral and Pharmacological Changes in the Offspring. *J. Neurosci.* **23**, 297–302 (2003).
318. Malkova, N. V., Yu, C. Z., Hsiao, E. Y., Moore, M. J. & Patterson, P. H. Maternal immune activation yields offspring displaying mouse versions of the three core symptoms of autism. *Brain. Behav. Immun.* **26**, 607–616 (2012).
319. Choi, G. B. *et al.* The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. *Science* **351**, 933–939 (2016).
320. Shin Yim, Y. *et al.* Reversing behavioural abnormalities in mice exposed to maternal inflammation. *Nature* **549**, 482–487 (2017).
321. Kim, S. *et al.* Maternal gut bacteria promote neurodevelopmental abnormalities in mouse offspring. *Nature* **549**, 528–532 (2017).
322. Lammert, C. R. *et al.* Cutting Edge: Critical Roles for Microbiota-Mediated Regulation of the Immune System in a Prenatal Immune Activation Model of Autism. *J. Immunol. Baltim. Md 1950* **201**, 845–850 (2018).
323. Lorga, I. *et al.* Development of bioluminescent Group B streptococcal strains for longitudinal infection studies. *Sci. Rep.* **14**, 24439 (2024).
324. Nanduri, S. A. *et al.* Epidemiology of Invasive Early-Onset and Late-Onset Group B Streptococcal Disease in the United States, 2006 to 2015: Multistate Laboratory and Population-Based Surveillance. *JAMA Pediatr.* **173**, 224–233 (2019).
325. Plainvert, C. *et al.* Multidrug-Resistant Hypervirulent Group B Streptococcus in Neonatal Invasive Infections, France, 2007-2019. *Emerg. Infect. Dis.* **26**, 2721–2724 (2020).

326. Jamrozny, D. *et al.* Increasing incidence of group B streptococcus neonatal infections in the Netherlands is associated with clonal expansion of CC17 and CC23. *Sci. Rep.* **10**, 9539 (2020).
327. Wiswell, T. E., Baumgart, S., Gannon, C. M. & Spitzer, A. R. No lumbar puncture in the evaluation for early neonatal sepsis: will meningitis be missed? *Pediatrics* **95**, 803–806 (1995).
328. Gordon, S. M., Srinivasan, L. & Harris, M. C. Neonatal Meningitis: Overcoming Challenges in Diagnosis, Prognosis, and Treatment with Omics. *Front. Pediatr.* **5**, (2017).
329. Heath, P. T., Okike, I. O. & Oeser, C. Neonatal meningitis: can we do better? *Adv. Exp. Med. Biol.* **719**, 11–24 (2011).
330. Greenberg, R. G. & Herrera, T. I. Chapter 8 - When to Perform Lumbar Puncture in Infants at Risk for Meningitis in the Neonatal Intensive Care Unit. in *Infectious Disease and Pharmacology* (eds. Benitz, W. E. & Smith, P. B.) 87–102 (Elsevier, Philadelphia, 2019). doi:10.1016/B978-0-323-54391-0.00008-4.
331. Andrade, E. B. *et al.* A mouse model reproducing the pathophysiology of neonatal group B streptococcal infection. *Nat. Commun.* **9**, 3138 (2018).
332. Vollman, J. H., Smith, W. L., Ballard, E. T. & Light, I. J. Early onset group B streptococcal disease: clinical, roentgenographic, and pathologic features. *J. Pediatr.* **89**, 199–203 (1976).
333. Barichello, T. *et al.* Evaluation of the brain-derived neurotrophic factor, nerve growth factor and memory in adult rats survivors of the neonatal meningitis by *Streptococcus agalactiae*. *Brain Res. Bull.* **92**, 56–59 (2013).
334. Ferrieri, P., Burke, B. & Nelson, J. Production of bacteremia and meningitis in infant rats with group B streptococcal serotypes. *Infect. Immun.* **27**, 1023–1032 (1980).
335. Mancuso, G. *et al.* Beneficial effects of interleukin-6 in neonatal mouse models of group B streptococcal disease. *Infect. Immun.* **62**, 4997–5002 (1994).

336. Zhao, Y. *et al.* Protective Role of $\gamma\delta$ T Cells in Different Pathogen Infections and Its Potential Clinical Application. *J. Immunol. Res.* **2018**, 5081634 (2018).
337. Kang, I., Kim, Y. & Lee, H. K. Double-edged sword: $\gamma\delta$ T cells in mucosal homeostasis and disease. *Exp. Mol. Med.* **55**, 1895–1904 (2023).
338. Hu, Y. *et al.* $\gamma\delta$ T cells: origin and fate, subsets, diseases and immunotherapy. *Signal Transduct. Target. Ther.* **8**, 434 (2023).
339. Pietschmann, K. *et al.* Toll-like receptor expression and function in subsets of human gammadelta T lymphocytes. *Scand. J. Immunol.* **70**, 245–255 (2009).
340. Sutton, C. E. *et al.* Interleukin-1 and IL-23 Induce Innate IL-17 Production from $\gamma\delta$ T Cells, Amplifying Th17 Responses and Autoimmunity. *Immunity* **31**, 331–341 (2009).
341. He, Y. *et al.* $\gamma\delta$ T Cell and Other Immune Cells Crosstalk in Cellular Immunity. *J. Immunol. Res.* **2014**, 960252 (2014).
342. Khairallah, C., Chu, T. H. & Sheridan, B. S. Tissue Adaptations of Memory and Tissue-Resident Gamma Delta T Cells. *Front. Immunol.* **9**, (2018).
343. Munoz, L. D., Sweeney, M. J. & Jameson, J. M. Skin Resident $\gamma\delta$ T Cell Function and Regulation in Wound Repair. *Int. J. Mol. Sci.* **21**, 9286 (2020).
344. Shibata, K., Yamada, H., Hara, H., Kishihara, K. & Yoshikai, Y. Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production. *J. Immunol. Baltim. Md 1950* **178**, 4466–4472 (2007).
345. Cho, J. S. *et al.* IL-17 is essential for host defense against cutaneous Staphylococcus aureus infection in mice. *J. Clin. Invest.* **120**, 1762–1773 (2010).
346. Jameson, J. *et al.* A Role for Skin $\gamma\delta$ T Cells in Wound Repair. *Science* **296**, 747–749 (2002).
347. Kirby, A., Newton, D., Carding, S. & Kaye, P. Pulmonary dendritic cells and alveolar macrophages are regulated by $\gamma\delta$ T cells during the resolution of S. pneumoniae-induced inflammation. *J. Pathol.* **212**, 29–37 (2007).
348. Conti, H. R. *et al.* Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J. Exp. Med.* **206**, 299–311 (2009).

349. Liu, R. *et al.* Interleukin-17 Promotes Neutrophil-Mediated Immunity by Activating Microvascular Pericytes and Not Endothelium. *J. Immunol. Baltim. Md 1950* **197**, 2400–2408 (2016).
350. Archer, N. K. *et al.* Interleukin-17A (IL-17A) and IL-17F Are Critical for Antimicrobial Peptide Production and Clearance of *Staphylococcus aureus* Nasal Colonization. *Infect. Immun.* **84**, 3575–3583 (2016).
351. Luchtman, D. W., Ellwardt, E., Larochelle, C. & Zipp, F. IL-17 and related cytokines involved in the pathology and immunotherapy of multiple sclerosis: Current and future developments. *Cytokine Growth Factor Rev.* **25**, 403–413 (2014).
352. Lu, Y., Zhang, P., Xu, F., Zheng, Y. & Zhao, H. Advances in the study of IL-17 in neurological diseases and mental disorders. *Front. Neurol.* **14**, 1284304 (2023).
353. Setiadi, A. F. *et al.* IL-17A is associated with the breakdown of the blood-brain barrier in relapsing-remitting multiple sclerosis. *J. Neuroimmunol.* **332**, 147–154 (2019).
354. Kebir, H. *et al.* Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat. Med.* **13**, 1173–1175 (2007).
355. Huppert, J. *et al.* Cellular mechanisms of IL-17-induced blood-brain barrier disruption. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **24**, 1023–1034 (2010).
356. Kawanokuchi, J. *et al.* Production and functions of IL-17 in microglia. *J. Neuroimmunol.* **194**, 54–61 (2008).
357. Di Filippo, M. *et al.* Interleukin-17 affects synaptic plasticity and cognition in an experimental model of multiple sclerosis. *Cell Rep.* **37**, 110094 (2021).
358. Milovanovic, J. *et al.* Interleukin-17 in Chronic Inflammatory Neurological Diseases. *Front. Immunol.* **11**, (2020).
359. Lock, C. *et al.* Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat. Med.* **8**, 500–508 (2002).
360. Tzartos, J. S. *et al.* Interleukin-17 Production in Central Nervous System-Infiltrating T Cells and Glial Cells Is Associated with Active Disease in Multiple Sclerosis. *Am. J. Pathol.* **172**, 146–155 (2008).

361. Sarma, J. D. *et al.* Functional interleukin-17 receptor A is expressed in central nervous system glia and upregulated in experimental autoimmune encephalomyelitis. *J. Neuroinflammation* **6**, 14 (2009).
362. Schirmer, L., Rothhammer, V., Hemmer, B. & Korn, T. Enriched CD161^{high} CCR6⁺ $\gamma\delta$ T Cells in the Cerebrospinal Fluid of Patients With Multiple Sclerosis. *JAMA Neurol.* **70**, 345–351 (2013).
363. Asano, T. *et al.* IL-17 is elevated in cerebrospinal fluids in bacterial meningitis in children. *Cytokine* **51**, 101–106 (2010).
364. Gelderblom, M. *et al.* Neutralization of the IL-17 axis diminishes neutrophil invasion and protects from ischemic stroke. *Blood* **120**, 3793–3802 (2012).
365. Li, G.-Z. *et al.* Expression of Interleukin-17 in Ischemic Brain Tissue. *Scand. J. Immunol.* **62**, 481–486 (2005).
366. Wang, D. *et al.* IL-17 potentiates neuronal injury induced by oxygen–glucose deprivation and affects neuronal IL-17 receptor expression. *J. Neuroimmunol.* **212**, 17–25 (2009).
367. Shichita, T. *et al.* Pivotal role of cerebral interleukin-17–producing $\gamma\delta$ T cells in the delayed phase of ischemic brain injury. *Nat. Med.* **15**, 946–950 (2009).
368. Reed, M. D. *et al.* IL-17a promotes sociability in mouse models of neurodevelopmental disorders. *Nature* **577**, 249–253 (2020).
369. Lu, Y.-Z. *et al.* CGRP sensory neurons promote tissue healing via neutrophils and macrophages. *Nature* **628**, 604–611 (2024).
370. Zarbock, A., Allegretti, M. & Ley, K. Therapeutic inhibition of CXCR2 by Reparixin attenuates acute lung injury in mice. *Br. J. Pharmacol.* **155**, 357–364 (2008).
371. Zheng, Z. *et al.* Donor pulmonary intravascular nonclassical monocytes recruit recipient neutrophils and mediate primary lung allograft dysfunction. *Sci. Transl. Med.* **9**, eaal4508 (2017).
372. Reutershan, J. *et al.* Critical role of endothelial CXCR2 in LPS-induced neutrophil migration into the lung. *J. Clin. Invest.* **116**, 695–702 (2006).

373. Andrade, E. B. *et al.* TLR2-Induced IL-10 Production Impairs Neutrophil Recruitment to Infected Tissues during Neonatal Bacterial Sepsis. *J. Immunol.* **191**, 4759–4768 (2013).
374. Carlson, T., Kroenke, M., Rao, P., Lane, T. E. & Segal, B. The Th17–ELR+ CXC chemokine pathway is essential for the development of central nervous system autoimmune disease. *J. Exp. Med.* **205**, 811–823 (2008).
375. Zenaro, E. *et al.* Neutrophils promote Alzheimer’s disease–like pathology and cognitive decline via LFA-1 integrin. *Nat. Med.* **21**, 880–886 (2015).
376. Steffen, B. J., Butcher, E. C. & Engelhardt, B. Evidence for involvement of ICAM-1 and VCAM-1 in lymphocyte interaction with endothelium in experimental autoimmune encephalomyelitis in the central nervous system in the SJL/J mouse. *Am. J. Pathol.* **145**, 189 (1994).
377. Roebuck, K. A. & Finnegan, A. Regulation of intercellular adhesion molecule-1 (CD54) gene expression. *J. Leukoc. Biol.* **66**, 876–888 (1999).
378. Madureira, P. *et al.* Inhibition of IL-10 Production by Maternal Antibodies against Group B Streptococcus GAPDH Confers Immunity to Offspring by Favoring Neutrophil Recruitment. *PLoS Pathog.* **7**, e1002363 (2011).
379. Kim, N. D. & Luster, A. D. The role of tissue resident cells in neutrophil recruitment. *Trends Immunol.* **36**, 547–555 (2015).
380. Jupelli, M. *et al.* Neonatal chlamydial pneumonia induces altered respiratory structure and function lasting into adult life. *Lab. Invest.* **91**, 1530–1539 (2011).
381. WATKINS, C. J. *et al.* Doctor Diagnosis and Maternal Recall of Lower Respiratory Illness. *Int. J. Epidemiol.* **11**, 62–66 (1982).
382. Johnston, I. D. A., Strachan, D. P. & Anderson, H. R. Effect of Pneumonia and Whooping Cough in Childhood on Adult Lung Function. *N. Engl. J. Med.* **338**, 581–587 (1998).
383. Edmond, K. *et al.* Long Term Sequelae from Childhood Pneumonia; Systematic Review and Meta-Analysis. *PLoS ONE* **7**, e31239 (2012).

384. Kadekar, D. *et al.* The neonatal microenvironment programs innate $\gamma\delta$ T cells through the transcription factor STAT5. *J. Clin. Invest.* **130**, 2496–2508 (2020).
385. van Kassel, M. N. *et al.* Sex Differences in Long-term Outcomes After Group B Streptococcal Infections During Infancy in Denmark and the Netherlands: National Cohort Studies of Neurodevelopmental Impairments and Mortality. *Clin. Infect. Dis.* **74**, S54–S63 (2022).
386. Mesquita, A. R. *et al.* Neurodevelopment milestone abnormalities in rats exposed to stress in early life. *Neuroscience* **147**, 1022–1033 (2007).
387. Walker, F. R., Knott, B. & Hodgson, D. M. Neonatal endotoxin exposure modifies the acoustic startle response and circulating levels of corticosterone in the adult rat but only following acute stress. *J. Psychiatr. Res.* **42**, 1094–1103 (2008).
388. Moriceau, S., Shionoya, K., Jakubs, K. & Sullivan, R. M. Early-life stress disrupts attachment learning: the role of amygdala corticosterone, locus ceruleus corticotropin releasing hormone, and olfactory bulb norepinephrine. *J. Neurosci. Off. J. Soc. Neurosci.* **29**, 15745–15755 (2009).
389. Brunjes, P. C. & Alberts, J. R. Olfactory stimulation induces filial preferences for huddling in rat pups. *J. Comp. Physiol. Psychol.* **93**, 548–555 (1979).
390. Galef, B. G. & Kaner, H. C. Establishment and maintenance of preference for natural and artificial olfactory stimuli in juvenile rats. *J. Comp. Physiol. Psychol.* **94**, 588–595 (1980).
391. Moriceau, S. & Sullivan, R. M. Unique Neural Circuitry for Neonatal Olfactory Learning. *J. Neurosci.* **24**, 1182–1189 (2004).
392. Naeem, N. *et al.* The Neurobiology of Infant Attachment-Trauma and Disruption of Parent–Infant Interactions. *Front. Behav. Neurosci.* **16**, 882464 (2022).
393. Heim, C., Newport, D. J., Bonsall, R., Miller, A. H. & Nemeroff, C. B. Altered pituitary-adrenal axis responses to provocative challenge tests in adult survivors of childhood abuse. *Am. J. Psychiatry* **158**, 575–581 (2001).
394. Nestler, E. J. *et al.* Neurobiology of depression. *Neuron* **34**, 13–25 (2002).

395. Teicher, M. H. *et al.* The neurobiological consequences of early stress and childhood maltreatment. *Neurosci. Biobehav. Rev.* **27**, 33–44 (2003).
396. Gunnar, M. R., Frenn, K., Wewerka, S. S. & Van Ryzin, M. J. Moderate versus severe early life stress: associations with stress reactivity and regulation in 10-12-year-old children. *Psychoneuroendocrinology* **34**, 62–75 (2009).
397. Lupien, S. J., McEwen, B. S., Gunnar, M. R. & Heim, C. Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nat. Rev. Neurosci.* **10**, 434–445 (2009).
398. Tottenham, N. *et al.* Prolonged institutional rearing is associated with atypically large amygdala volume and difficulties in emotion regulation. *Dev. Sci.* **13**, 46–61 (2010).
399. Lupien, S. J. *et al.* Larger amygdala but no change in hippocampal volume in 10-year-old children exposed to maternal depressive symptomatology since birth. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 14324–14329 (2011).
400. Gee, D. G. *et al.* Early developmental emergence of human amygdala-prefrontal connectivity after maternal deprivation. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 15638–15643 (2013).
401. Ribot, J. C., Lopes, N. & Silva-Santos, B. $\gamma\delta$ T cells in tissue physiology and surveillance. *Nat. Rev. Immunol.* **21**, 221–232 (2021).
402. Jarrett, M. A. & Ollendick, T. H. A conceptual review of the comorbidity of attention-deficit/hyperactivity disorder and anxiety: Implications for future research and practice. *Clin. Psychol. Rev.* **28**, 1266–1280 (2008).
403. Reimherr, F. W., Marchant, B. K., Gift, T. E. & Steans, T. A. ADHD and Anxiety: Clinical Significance and Treatment Implications. *Curr. Psychiatry Rep.* **19**, 109 (2017).
404. Penteado, S. H. W. *et al.* Prenatal lipopolysaccharide disrupts maternal behavior, reduces nest odor preference in pups, and induces anxiety: studies of F1 and F2 generations. *Eur. J. Pharmacol.* **738**, 342–351 (2014).

405. Makinson, R. *et al.* Intrauterine inflammation induces sex-specific effects on neuroinflammation, white matter, and behavior. *Brain. Behav. Immun.* **66**, 277–288 (2017).
406. Takahashi, N. *et al.* Interaction of genetic liability for attention deficit hyperactivity disorder (ADHD) and perinatal inflammation contributes to ADHD symptoms in children. *Brain Behav. Immun. - Health* **30**, 100630 (2023).
407. Rosenberg, J. B. *et al.* Maternal inflammation during pregnancy is associated with risk of ADHD in children at age 10. *Brain. Behav. Immun.* **115**, 450–457 (2024).

ANNEXES

ANNEX 1

Meningeal immunity: anatomy, function, and neonatal unique features

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Abstract

The central nervous system (CNS) is responsible for controlling the entire body functions and actions. The meninges, long seen as physical protection of the CNS, have been recently studied from a completely different perspective, given their unique location and structural composition, as well as the functional features of the vast immune cell repertoire that they comprise. Over the past decade, research has demonstrated the key roles of meningeal immunity in the CNS, either in steady state or pathological conditions. In this review, we provide an overview of meningeal immunity, including its anatomical structure, immune cell composition, and functional dynamics under normal physiological conditions. Moreover, we discuss recent evidence on the involvement of meningeal immunity in neuroinflammation, particularly in the context of infection. Finally, we provide insights into a relatively understudied area: neonatal meninges and their unique immunological features.

Keywords

Meninges; development; meningeal immunity; inflammation; infection; neonatal meninges

1. Introduction

The central nervous system (CNS) is a highly complex, sensitive, and dynamic system responsible for controlling the entire body's actions. The meninges were long thought to be only a protective barrier, surrounding the brain and spinal cord, conferring physical protection from impacts¹. Despite being first reported in an Ancient Egyptian medical text around ca. 1600 BCE², it has only been over the last two decades, particularly with the (re)discovery of meningeal lymphatic vessels, that

the meninges have gained attention and their role has been redefined beyond mere a physical barrier. The meninges are strategically located at the interface between the periphery and the brain, and their known functions have been continuously expanding. The meninges regulate the development of the forebrain and hindbrain, skull and brain blood vessels³⁻¹⁰. Additionally, they contribute to neural survival, migration, and position in the brain, and in the maintenance of the pial basement membrane^{5,9,11,12}. Notably, a stem cell niche with the ability of neural differentiation was also discovered to reside in the meninges, being already present during the embryonic period. These cells can be triggered by an injury, such as tissue damage and inflammatory signals, proliferating and migrating to the CNS to activate parenchymal response^{3,4,13}. Furthermore, a vast immune cell repertoire was found in the meninges. Meningeal immunity has been linked to central roles in CNS immunosurveillance and repairment, lymphatic drainage and brain homeostasis¹⁴. Meningeal inflammation has also been found to precede the onset of neurological disease, even in non-infectious conditions¹⁵. Thus, the meninges remind an immunological barrier, comparable to those found in the skin and intestine¹⁴. Indeed, a disruption in the meningeal immune homeostasis may result in inflammation or cytokine milieu dysregulation, potentially leading to brain dysfunction and/or pathology. While increasing knowledge of meningeal immunity has been accumulating in understanding meningeal immunity in adults, very little is known regarding the neonatal period. Here, we will focus on the meninges surrounding the brain parenchyma and review the current advances in meningeal immunity in physiological and infection conditions. Additionally, we provide insights into the scarce but emerging knowledge regarding the unique features of neonatal meninges and meningeal immunity.

2. Meningeal anatomy

2.1. Meningeal structure

The meninges are classically composed of three cellular layers: the dura mater (adjacent to the skull), the arachnoid mater and the pia mater (the closest to the parenchyma) (Figure 1)¹⁶. The arachnoid and pia mater form the leptomeninges. Between these two layers is the subarachnoid space (SAS), where the cerebrospinal fluid (CSF) produced by the choroid plexus flows, being extremely important in protecting the brain from physical trauma¹⁶. Recently, a novel meningeal layer, the subarachnoid lymphatic-like membrane (SLYM) was proposed¹⁷, in line with last century studies stating the SAS subdivide by thin membranes^{18,19}. The SLYM is located between the arachnoid and the pia mater, dividing the SAS into outer (near the dura) and inner (closer to the pia) compartments^{17,20}. This layer is, however, controversial in the meningeal field, as other authors contest it as the inner arachnoid instead of a fourth meningeal layer^{21,22}. These results also align with studies from the second half of the last century²³⁻²⁹.

The dura mater is a thick, dense, collagenous membrane, composed of two layers: the periosteal layer, closest to the skull, and the meningeal layer¹. Both layers are composed of collagen fibres and fibroblasts, being these cells flattened in the inner layer of the dura mater¹. These layers are mostly fused, widening to harbour the dural vessels and separating at the longitudinal fissure that defines the hemispheres. Here, only the meningeal layer enters the brain to form the falx cerebri, whereas the periosteal layer remains attached to the skull^{1,16}. The dura mater is highly innervated with sympathetic, parasympathetic and sensory fibres¹⁶.

Dural vascularisation is also extensive, being its blood vessels fenestrated lacking tight junctions, allowing the passage of relatively large molecules (~45 kDa)³⁰. Moreover, the dura mater harbours conventional and functional lymphatic vessels (LVs) unevenly distributed throughout the brain, in both mice and humans³¹⁻³⁴. While the superior portions scarcely present LVs, the base of the skull harbours a large LV network³³. Recent studies have revealed a role for the dural LVs, located parallel to the venous sinus (Figure 1), in continuous drainage and clearance of CSF, macromolecules and immune cells from the brain into the cervical lymph nodes (CLN)^{33,35}.

The central meningeal membrane is the arachnoid layer. This thin, avascular structure is composed of cells connected by tight junctions, forming an impermeable layer, important to regulate the entry of dural blood-derived molecules into the CSF^{1,16}. The arachnoid fibroblasts, together with the connective intercellular tissue, protrude the subarachnoid space forming the trabeculae, a vast spiderweb-like network connecting the arachnoid and pia mater³⁶. The subarachnoid space is filled with the CSF and the most important brain arteries¹.

The recently proposed SLYM is composed of a mesothelium membrane covering the vessels in the SAS¹⁷. The SLYM divides the SAS, creating a barrier between the two compartments for solutes of or bigger than 3 kDa, guiding and selecting the CSF that reaches the brain parenchyma¹⁷.

The pia mater, the innermost layer, consists of two sub-layers: the epipial layer and the intima pia. The epipial layer is composed of collagen fibres attached to the arachnoid by the trabeculae, while the inner layer comprises reticular fibres and is connected to the external layer of the parenchyma, the glial membrane¹. As in the

dura mater, the pial membrane is highly innervated¹⁶. The pia also harbours arteries, arterioles, veins, and venules, forming sheaths around the vessels that run from the subarachnoid space into the deep brain parenchyma, creating an interstitial fluid (ISF)/CSF-filled space, the Virchow-Robin space, important for the brain waste clearance^{1,16,37}. Both subarachnoid and pial vessels are composed of epithelial cells connected by tight junctions, forming the impermeable blood-meningeal barrier³⁰.

2.2. Steady-state meningeal immune composition

The meninges harbour a diverse repertoire of immune cells³⁸⁻⁴⁰, with each layer featuring a different milieu of immune populations, with the potential to regulate brain homeostasis (Figure 1). The dura mater is the most heterogeneous and enriched in immune cells, containing macrophages, monocytes, dendritic cells (DCs), mast cells, natural killer (NK) cells, B and T cells, and innate lymphoid cells⁴¹. The arachnoid mater mainly presents DCs¹⁶, while the pia mater is populated by mast cells, macrophages and DCs, although to a lesser extent when compared to the dura mater^{15,16}. As a highly vascularised structure, the meninges harbour resident and infiltrating cells. Among these, meningeal macrophages are the most abundant immune cells⁴². Recent studies combining RNA-sequencing and fate-mapping animals revealed that border-associated macrophages (BAMs) in steady-state dural and leptomeningeal meninges exhibited mixed ontogeny, and subsets displayed distinct capacities for self-renewal. Mast cells and DCs were also identified as meningeal resident cells^{43,44}. Regarding the lymphoid compartment, B cells were recently found in the dural meninges, particularly (~60%) naïve B cells⁴⁵. T cells are among the most extensively studied meningeal immune cells and have emerged as key regulators for CNS immunosurveillance and homeostasis⁴⁶. Studies with parabiotic mouse model have demonstrated that CD4⁺ T cells enter the meninges

via blood vessels⁴⁷, whereas the lymphatic vasculature is important for their egress, as surgical removal of the deep CLN (dCLN) leads to meningeal accumulation of CD4⁺ T cells⁴⁷. These studies point to the existence of two different subsets of T cells: tissue-resident and circulating cells. Nevertheless, the resident *versus* trafficking profile of these cells in steady-state conditions is not yet fully understood. Also, these studies indicate that meninges are relatively permeable to immune cell trafficking, specifically CD4⁺ T cells, and serve as a site of continuous surveillance by circulating leukocytes before entering the lymphatic system.

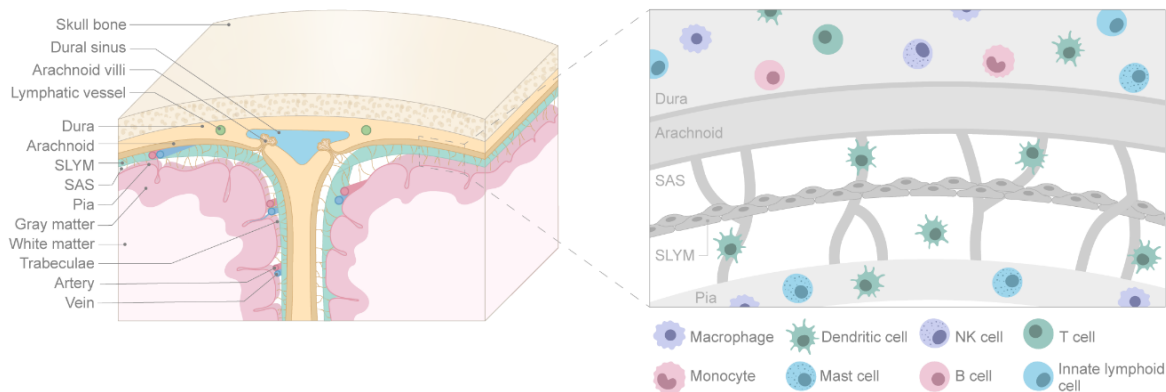


Figure 1. Steady-state meninges and meningeal immune composition. The meninges consist of the following layers: the dura mater (adjacent to the skull), the arachnoid mater and the pia mater. A fourth meningeal layer has been proposed, the Subarachnoid Lymphatic-like Membrane (SLYM), dividing the subarachnoid space (SAS). The dura mater contains blood and lymphatic vessels, and the largest meningeal immune repertoire (dural macrophages, monocytes, dendritic cells (DCs), B and T cells, mast cells, natural killer (NK) cells, and innate lymphoid cells). The arachnoid is avascular and composed of cells connected by tight junctions, representing an impermeable barrier. This layer's immunity is mainly composed of DCs. The arachnoid fibroblasts form trabeculae connecting the arachnoid and pia mater. The SLYM is composed of a mesothelium membrane covering the vessels in the SAS and creates a permeability barrier in this space. The pia mater covers the

brain parenchyma and form sheaths around the vessels that run from the subarachnoid space to the deep brain parenchyma (Virchow-Robin spaces), important for the clearance of brain waste products and cellular debris. The pia mater presents pial macrophages, DCs and mast cells. The SAS is settled between the arachnoid and pia mater and is where the cerebrospinal fluid flows. The major arteries are also present in this space.

3. Meningeal immunity in homeostasis and infection

3.1. Meningeal immunity and CNS surveillance

Meningeal immune cells play an important role in CNS surveillance, patrolling for the existence of tissue damage and the presence of foreign antigens. The innate immune cells are strategically positioned throughout the meninges to sense and contain pathogens before they invade the brain parenchyma¹⁵. These cells can also efficiently recruit circulating leucocytes and reprogram them to enter the parenchyma¹⁵. Studies regarding the anatomic features of the CNS and the immune cells' composition and trafficking found the SAS as the base for CNS immune surveillance⁴⁸.

Macrophages are abundant within the meninges and patrol both the CSF and the meningeal blood vessels, acting as guardians against infection and tissue damage. These meningeal macrophages sense the surrounding environment, adjusting their phenotype (either anti- or pro-inflammatory) to mediate the resulting immune response⁴⁹. Perivascular macrophages are strategically positioned to scan for foreign antigens and tissue damage in the blood vessels, CSF, and CNS parenchyma, thus functioning as a gate between the periphery and the CNS parenchyma⁴⁹. Both meningeal and perivascular macrophages express higher

levels of MHC and present increased phagocytic activity compared to microglia, highlighting their role in immune surveillance and homeostasis⁵⁰. DCs, well-known APCs, also patrol the meninges for harmful agents and present antigens to T cells which are themselves monitoring the tissue⁵¹. Interferon- γ (IFN- γ) produced by meningeal NK cells leads to the expression of the death receptor ligand TRAIL in astrocytes, important in controlling inflammation⁵².

Regarding lymphoid cells, it has been recently shown that IgA-secreting plasma cells, partially educated in the gut, are present in both human and mouse meninges⁴⁵. These cells are located adjacent to the dural venous sinuses, where they play a crucial role in protecting the meninges, and lately the parenchyma, from the entrance of blood-borne pathogens⁴⁵. As previously mentioned, T cells are essential in CNS physiological conditions and immunosurveillance⁴⁶. These cells enter the SAS through the leptomeningeal blood vessels, where they screen the CSF for pathogenic antigens⁴⁶. In case of recognition of their cognate antigen, T cells become reactivated and determine the fate of the immune response by triggering an inflammatory cascade, being able to breach the blood-brain barrier (BBB), and recruiting additional phagocytic cells⁵³. Antigen reconfirmation within the subarachnoid space is the most important step for T cells to cross the BBB, ultimately determining the consequent immune response⁵³. Contrary to this barrier, the endothelial cells of the leptomeningeal blood vessels are permeable to all T cells, independent of their antigen specificity. However, only reactivated T cells can invade the parenchyma⁵³. Non-CNS-antigen-specific T cells follow the CSF flow and are drained to the meningeal LVs, eventually returning to the periphery^{46,53}.

3.2. Meningeal immunity and brain function

Over the past two decades, there has been mounting evidence of the importance of meningeal immunity to brain function⁵⁴⁻⁵⁷. As meningeal immune cells do not penetrate the brain parenchyma under steady-state conditions, their influence on brain function and homeostasis occur mainly through the secretion of cytokines from the meninges³⁸.

Meningeal CD4⁺ T cells play important roles in cognitive functions, such as spatial learning^{47,58} and memory^{47,57}, and anxiety-like behaviour^{55,59}. Remarkably, mice deficient in the recombination-activating genes (RAG) enzymes RAG-1 and RAG-2 (which lack B and T cells), OT-II transgenic mice (which have a restricted T cell repertoire), severe combined immunodeficient (SCID) mice (that lack mature B and T cells) and mice in which meningeal T cells were depleted all show decreased spatial learning and memory, evaluated through the Morris water maze (MWM) performance^{58,60}. These cognitive deficits were reverted when mice were replenished with CD4⁺ T cells, but not when CD4⁺ T cells lacking interleukin (IL)-4 were used⁵⁸. Moreover, cognitive tasks' performance led to an accumulation of IL-4-producing T cells in the meninges immediately after MWM training⁵⁸. This study showed, for the first time, the utmost importance of the cytokine content in the meninges for brain physiology⁵⁸. Both IL-4-deficient mice and mice depleted of CD4⁺ T cells exhibited cognitive deficits^{58,61}, through a mechanism involving reduced production of brain-derived neurotrophic factor (BDNF) and decreased hippocampal neurogenesis⁶². Moreover, astrocytes treated *in vitro* with IL-4 presented increased expression of BDNF mRNA, and the hippocampus of mice trained in the MWM had significantly higher levels of BDNF mRNA after the task performance, which was absent in mice deficient in IL-4⁵⁸. These findings suggest that during cognitive tasks,

CD4⁺ T cells produce IL-4 which further leads to the production of BDNF and neurogenesis in the hippocampus. However, researchers found that OT-II transgenic mice presented impaired hippocampal neurogenesis and cognitive performance, despite the elevated levels of IL-4 in the CNS⁶³. In fact, *in vitro* treatment of hippocampal neural stem cells with IL-4 inhibited their proliferation⁶³. Therefore, the definitive role of IL-4-producing CD4⁺ T cells in cognitive tasks, such as learning and memory, remains unclear. Another cytokine tightly involved in cognitive functions is IL-13 produced by CD4⁺ T cells⁶¹. Similar to IL-4-deficient mice, IL-13-deficient mice failed to accumulate CD4⁺ T cells and IL-13 in the meninges and presented impaired capacity to perform the MWM task⁶¹. Moreover, like IL-4, IL-13 regulates cognitive function by stimulating meningeal and hippocampal astrocytes to produce BDNF, thereby enhancing cognitive ability⁶¹.

Meningeal $\gamma\delta$ T cells are strongly biased towards IL-17 production, being involved in cognition and regulation of anxious behaviour. Mice lacking $\gamma\delta$ T cells showed impaired short-term memory⁵⁷. Local neutralisation of IL-17, by administering antibodies via the intracerebroventricular route, leads to similar cognitive impairment⁵⁷. The absence of IL-17 reduces glutamatergic synaptic plasticity, resulting in reduced long-term hippocampal potentiation⁵⁷. However, restoring local IL-17 in deficient mice (by intracerebroventricular injection) enhances BDNF production by glial cells, rescuing the synaptic plasticity and short-term working memory⁵⁷. Thus, meningeal $\gamma\delta$ T cell producing IL-17 seems to be central to brain function.

Besides cognition, T cells also play an important role in behaviour. RAG1^{-/-} mice exhibit compulsive behaviours and impaired nest building⁵⁹. Interestingly, these behavioural alterations do not occur when CD4⁺ T cells are depleted in wild-

type mice, nor when RAG1^{-/-} are replenished with these cells⁵⁹. However, the anxiety-like behaviour was reverted in RAG1^{-/-}/OT-II transgenic mice, indicating that emotional behaviour is influenced by lifelong rather than transient immunodeficiency conditions in mice⁵⁹. Meningeal $\gamma\delta$ T cell-derived IL-17 is also associated with anxiety-like behaviour⁵⁵. Mice deficient in $\gamma\delta$ T cells or treated with anti-TCR $\gamma\delta$ antibodies in the CSF exhibit reduced anxiety, as indicated by increased time spent in the open arms of the elevated plus maze and the centre area of the open-field test⁵⁵. IL-17-deficient mice and those treated with IL-17 neutralising antibodies recapitulated the behaviour abnormalities, showing the link between $\gamma\delta$ T cells, production of IL-17, and brain homeostasis⁵⁵. Moreover, abnormal behaviour was associated with the signalling of IL-17 to cortical neurons, as a genetic depletion of the IL-17a receptor decreased anxiety-like behaviour⁵⁵. IFN- γ produced by CD4⁺ T cells has also been linked to social behaviour⁶⁴. When analysing the mouse preference for either another mouse or an object, SCID mice, unlike wild-type controls, show no social preference. This behaviour is reverted by the adoptive transfer of wild-type lymphocytes, suggesting the importance of these cells in social behaviour. Curiously, IFN- γ -deficient mice also exhibit impaired social impairment, leading to the assumption that IFN- γ producing T cells promote social behaviour⁴⁷. Deleting the IFN- γ receptor from prefrontal cortex neurons, also led to the dampening of social behaviour⁶⁴, indicating that inhibitory neurons respond to IFN- γ . Interestingly, socially isolated rodents lose the IFN- γ transcription signature observed in animals housed in groups, further supporting the link between social behaviour and IFN- γ signalling⁶⁴.

Although less studied, B cells also play a role in meningeal and brain homeostasis⁶⁵. B cells were found to be decreased in the meninges upon

psychosocial stress⁶⁵. Moreover, single-cell RNA sequencing analysis reveals increased expression of genes associated with myeloid compartment activation following stress, showing a role for these lymphocytes in brain function⁶⁵. In fact, B cells have a niche in the meningeal compartment originating from the calvaria bone marrow⁶⁶, highlighting the importance of these cells in brain homeostasis. These B cells reach the meninges through specific vascular skull channels and mature *in situ*⁶⁶. Moreover, they acquire a tolerogenic phenotype against CNS antigens, so the meninges have specialised and CNS antigen-educated B cells⁶⁶.

The myeloid meningeal compartment, particularly macrophages, also contributes to CNS homeostasis and brain function. T cell-deficient mice exhibit increased pro-inflammatory phenotype in the meningeal CD11b⁺ myeloid cells, which was linked to cognitive impairment⁵⁸. When SCID mice were injected with *in vitro*-activated anti-inflammatory macrophages into the ventricular spaces or intravenously, the MWM performance surpassed that of the control mice⁶⁷. Thus, the pro- and anti-inflammatory profile of meningeal myeloid cells also plays a role in regulating brain function and is independent of the adaptive immune system.

Considering the anatomical barriers of the meninges, it raises the intriguing question of how cytokines within meninges and CSF can transmit their signals to the brain parenchyma. A potential route for cytokine transmission is through the glymphatic system, an astrocyte-dependent system facilitating fluids and small molecules movement between CSF and ISF, particularly important for the clearance of waste products and debris⁶⁸. Despite some controversy, it has been proposed that cytokines present at the SAS may reach the Virchow-Robin spaces, and diffuse into the brain parenchyma through the glial limitans⁶⁸. How they interact and cross glial limitans is unknown, but cytokines may either cross through paravascular

routes, exerting their function directly on neurons, glia and stroma cells, or by indirect pathways where cytokines are recognised by glial cells [astrocytes, microglia, oligodendrocyte precursor cells (OPCs), and oligodendrocytes] that, in turn, release molecules capable of modulating neuronal function^{69,70}. Another unsolved question is how the molecules flow within the meninges, particularly how they travel from the dura mater to the CSF to further signal to the brain parenchyma. The recently proposed SLYM layer might bring new insights into the CSF flow organisation.

3.3. Meningeal infection and immunity

As meninges harbour blood and lymphatic vessels, they may serve as a gateway for microorganisms' entrance with concomitant inflammation, disease, and later neurological impairment. A few studies already reported the presence of neurotropic pathogenic microbes in the meninges upon infection^{45,71-80}. A phylogenetic study showed that the meninges are the primary transport tissue for HIV-1 infection, with the virus initially migrating from the meninges to the brain within monocytes/macrophages, then freely migrating to the brain, as the BBB disrupts. In addition, the virus may traffic from the meninges back to the periphery⁷⁷. Therefore, not only do meninges can act as an entry point for pathogens, but they are also a gateway out into the periphery⁷⁷.

Further supporting the role of the meninges as an entry point for pathogens, several studies have observed microbes and inflammatory cells in the meninges even without or prior to brain parenchyma infiltration^{72,74,79}. For example, the Zika virus was found in the meninges of human neonates without evidence of parenchyma invasion⁷². Inflammatory lymphocytes and macrophages were also

observed in the meninges upon infection, particularly in the Virchow-Robin space⁸¹. The lymphocytic choriomeningitis virus (LCMV), a natural pathogen in humans and mice that causes meningitis, predominantly infects the meninges with minimal parenchymal invasion⁷⁴. In this case, fibroblast-like meningeal cells and resident macrophages are the primary infected cells^{82,83}. Within two days of infection, one-third of meningeal macrophages are infected, triggering the upregulation of antiviral cytokines⁸³. Moreover, these macrophages present upregulated genes associated with their activation (phagocytosis, endocytosis, and APCs and antiviral activities), engaging in programmed cell death around a week after infection, being cleaned and replaced after the onset of meningitis⁸³. The virus also spreads into the periphery, inducing strong CD8⁺ T cell responses⁷⁴. These cells, along with myelomonocytic cells (LysM⁺ cells, including monocytes, macrophages and granulocytes⁸⁴⁻⁸⁶), infiltrate the meninges via meningeal blood vessels around six days post-infection, causing immune-mediated pathology and brain oedema^{74,75,83}. Depletion of antiviral CD8⁺ T cells prevented fatal disease and the recruitment of myelomonocytic cells⁸², suggesting a role of CD8⁺ T cells in recruiting myelomonocytic cells, either by releasing chemoattractants or by inducing other cells to do so⁸². Notably, mice lacking CD8⁺ T cell classical effector pathways (TNF- α , IFN- γ , Fas, perforin, granzymes and degranulation) succumb to LCMV-induced meningitis, suggesting an alternative unknown pathway of CD8⁺ T cell-induced disease⁸². Intravital imaging of the meninges during disease onset showed myelomonocytic cells extravasating from meningeal blood vessels, causing severe vascular injury, including vasculature rips and endothelial leakage⁸². The detailed mechanism of vascular injury by myelomonocytic cells are still unknown. Moreover, engrafted monocytes became refractory to secondary microbial challenge and

presented a reduced ability to control inflammation⁸³. Thus, CD8⁺ T cell-mediated disease is dependent, at least in part, on the short- and long-term effects of myelomonocytic cells recruited to the meninges.

Parasites, such as *Trypanosoma brucei*, were also found in the meninges before invading the parenchyma^{79,87}. Injecting *T. brucei* directly into the brain does not result in infection, while intraperitoneal injection rapidly leads to the appearance of this parasite in the CSF and meninges⁷⁸. Moreover, a kinetic analysis shows that the parasite accumulates in the meninges approximately two weeks before entering the parenchyma⁸⁷. These results suggest that *T. brucei* likely enters the brain parenchyma through the meninges. After infection, T cells infiltrate the cortical meninges, particularly the dura mater, where the parasites are mainly confined⁷³. APCs, mainly DCs, also increase in the meninges⁷³, in accordance with the observed T cell infiltration dependent on antigen presentation. Recruited macrophages to the parenchyma outnumber the resident ones and exhibit a different phenotype, by expressing antimicrobial genes⁸⁷. In contrast, *Toxoplasma gondii* only invades the brain parenchyma but still requires meningeal immunity and even brings a new player to the game: the regulatory T cells (Tregs)⁸⁸. While effector CD4⁺ T cells infiltrate the parenchyma to encounter and fight this pathogen, recruited Tregs are less migratory, remaining in the meninges where they perform stable interactions with DCs. Reducing meningeal CD11c⁺ cells using anti-LFA-1 antibodies increases the motility of Tregs, suggesting a role of Treg-DCs interaction in regulating their local behaviour⁸⁸. Tregs are known to regulate CNS inflammation during infections⁸⁹⁻⁹¹, likely by suppressing APC function, downregulating the costimulatory signals or their cytokine production, thus fine-tuning the effector T

cells' response⁸⁸. Alternatively, DCs may provide survival signals to maintain Tregs in the brain⁹².

The fungus *Candida albicans* was also found localized within the dural sinus walls before spreading to the brain parenchyma when mice were intravenously inoculated. In this context, meningeal IgA⁺ cells act as an immunological shield, playing a decisive role in fungal entrapment in the peri-sinus region⁴⁵.

Regarding bacterial infection and meningeal immunity, our knowledge is also scarce. *Salmonella enterica*, *Listeria monocytogenes* and *Neisseria meningitidis* are a few bacteria already found to be present in the meninges^{71,76,80}. During oral infection with *S. enterica*, bacteria were detected in the meninges with little or no parenchymal invasion⁷¹. Mild meningeal inflammation due to monocyte infiltration was also detected by immunofluorescence in *S. enterica* infected mice⁷¹. *L. monocytogenes* is also capable of infecting both the meninges and the brain parenchyma⁷⁶. Moreover, following intracisternal infection, meningeal infiltration, vascular inflammation and subarachnoid haemorrhages were observed within 24 hours⁷⁶. However, the mechanisms through which these bacteria reach the meninges and eventually invade brain parenchyma, how they interact with meningeal cells and how these cells respond to this stimulus and signal to others are still poorly understood. Regarding *L. monocytogenes*, one study recently uncovered a mechanism through which it can invade the CNS. The bacterial surface protein InlB prevents monocyte infected cells from CD8⁺ Tcell Fas-mediated death⁹³. Increasing monocytes lifespan render increased survival to the bacteria itself favouring parenchymal invasion⁹³.

N. meningitidis, a human-specific pathogen, has been studied mainly through *in vitro* models⁹⁴⁻⁹⁶. Using human brain sections and meningioma cell cultures, researchers demonstrated that *N. meningitidis* preferentially adheres to the leptomeninges rather than the brain parenchyma⁹⁶. Upon challenge, meningeal cells increased the secretion of pro-inflammatory cytokines and chemokines, including IL-6, IL-8, monocyte chemoattractant protein-1, RANTES and granulocyte-macrophage colony-stimulating factor (GM-CSF), suggesting an innate immune response upon infection^{94,95}. Although no direct infection of meningeal cells was detected, prolonged *N. meningitidis* infection led to meningioma cell necrosis, likely mediated by external molecules produced and secreted by the bacteria⁹⁵. An *in vivo* mouse model of intranasal infection demonstrated that 20% of challenged mice developed lethal meningitis without detectable bacteria in the blood⁸⁰. The olfactory epithelium, which is connected to the CNS, was disrupted, with detectable bacteria along the olfactory nerve and bulb and, finally, in the meninges, particularly the SAS⁸⁰. How the bacteria induce mucosal epithelium damage is still unclear, as well as the mechanisms of immune cell activation leading to the secretion of pro-inflammatory cytokines and chemokines.

Several other pathogens are known to cause meningitis, such as *Streptococcus pneumoniae*, *Escherichia coli* and *Streptococcus agalactiae*⁹⁷. However, to our knowledge, detailed information on the immune mechanisms triggered by these pathogens in the meninges remains limited. Intravenous infection with *S. pneumoniae* and *S. agalactiae* revealed the presence of bacteria in the meninges as soon as 6 and 12 h after infection, respectively⁹⁸. The presence of bacteria in this tissue is dependent on meningeal macrophages, as the polarisation of its transcriptional profile led to reduced dural antimicrobial defences⁹⁸. Nasal infection

with *S. pneumoniae* also demonstrated the presence of bacteria in the dura mater up to at least 72 h post inhalation⁹⁹. Interestingly, depletion of the nociceptor Nav1.8, present in a subset of sensory neurons, significantly reduced meningeal and brain bacterial invasion 24 h after infection with either species⁹⁸. Both bacteria secrete toxins that activate nociceptors which, in turn, stimulate the release of calcitonin gene-related peptide (CGRP). *S. pneumoniae* infection ultimately led to meningeal immunosuppression, dampening host defences⁹⁸. Additionally, intravenous infection with *S. pneumoniae* revealed that bacterial presence was predominantly restricted to the meningeal compartment¹⁰⁰. In response, meningeal macrophages and monocytes are highly activated, with skull marrow presenting phenotypically different monocyte progenitors¹⁰⁰. These are more immature for emergency myelopoiesis for high production of monocytes to fight the infection¹⁰⁰.

4. Neonatal meninges in health and pathology

Structurally, the neonatal meninges resemble those of adults. The three-layered structure (as SLYM remains to be confirmed in the neonatal period) starts to develop during the foetal stage, more precisely at gestation week 5 in humans and embryonic day 9.5 in mice⁴. At birth, the three layers, as well as the SAS and arachnoid trabeculae are already developed⁴. Regarding vascularisation, blood vessels are already established at birth¹⁰¹. However, recent research in mice reveals that the blood vessel network in the dura mater changes during the neonatal period¹⁰². During the first week of life, the outer layer of the meninges presents a large number of fine and complex branching points¹⁰². As the neonate matures, these vessels become larger and the vascular network simpler¹⁰². The lymphatic system, however, only starts to develop at birth (Figure 2)^{101,103}. Studies using

whole-mounts of mouse meninges show that the lymphatic system develops alongside blood vasculature and cranial and spinal nerves during the first month of life¹⁰³. Lymphatic vessels first appear just before birth at the base of the skull, near the foramen magnum (FM) and the pterygopalatine artery (PPA)¹⁰³. At birth, LVs also started developing at the base of the middle meningeal artery (MMA)¹⁰¹. Lymphatic valves are also observed at birth, increasing until postnatal day (P) 20¹⁰³. LVs develop all around the FM during the first two weeks of life¹⁰³. LVs at PPA extend along the MMA, after P4, and reach the top of the skull, more precisely the sagittal suture, at around P24¹⁰³. Parasinusoidal LVs develop alongside the jugular vein, through sagittal, transverse and superior sagittal sinuses^{101,103}. The LVs reach the transverse sinus by P8 and the superior sagittal sinus by P16^{101,103}. From this day, LVs are also observed covering the cerebellum, alongside veins branching from the transversal sinus¹⁰³. From P16 to P24, the LVs extend over the olfactory bulb and beside the rostral rhinal veins¹⁰³. Under the olfactory bulb, LVs are detectable as early as P2 and connect to nasal mucosal LVs, draining into the dCLN¹⁰³. Currently, no corresponding studies exist for humans. Overall, the lymphatic system develops from the base of the skull to the top, consistent with the unevenly distributed meningeal vasculature already described in this review, being completely developed one month after birth^{101,103}.

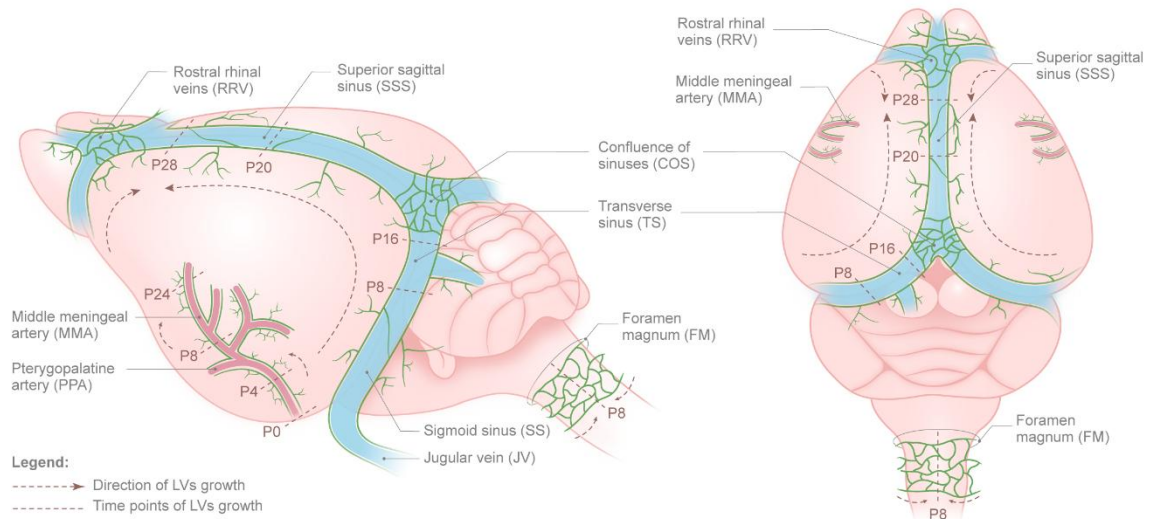


Figure 2. Neonatal mice development of the lymphatic vessels. Lateral (a) and top (b) view of mouse brain. The lymphatic network (green) develops postnatally, starting from the pterygopalatine artery (PPA), foramen magnum (FM) and meningeal artery (MMA), around birth (P0). Two weeks after birth, the lymphatic vasculature is fully developed around the FM and reaches the superior sagittal sinus (SSS). From P16 to P24 the LVs were observed overlying the olfactory bulb and beside the rostral rhinal veins (RRV). The lymphatic vasculature is fully developed around a month after birth. JV, jugular vein; SS, sagittal sinus; TS, transversal sinus; COS, confluence of the sinuses.

Little is known about neonatal meningeal immune repertoire and function. As with other resident macrophages, these cells are seeded in the meninges before birth, more precisely at embryonic day 9.5 in mice⁴². Immunohistochemistry analyses of whole-mounts meninges show that macrophages are already present alongside the sinuses at birth^{101,102}. Their number and morphology change over time, with the highest density and the most rounded morphology observed at birth¹⁰². At this early stage, meningeal macrophages are mainly MHC-II negative, potentially leading to an increased pathogenic burden at the borders of the

brain^{104,105}. As the neonate matures, macrophage density significantly decreases, and from week two after birth, their shape shifts from ameboid/round to a more rod-like form¹⁰². Moreover, by P14, macrophages show significant alignment along blood vessels¹⁰², in accordance with their patrolling and antigen presentation functions¹⁰⁶. Using a mouse model of human neonatal *E. coli* meningitis, with pups inoculated intravenously at P5, bacteria were found in the leptomeninges and dura mater 22 h after infection¹⁰⁷. Moreover, single nucleus RNA sequencing showed that infection leads to altered transcriptome in the most abundant meningeal cell types, including endothelial cells, macrophages, and fibroblasts¹⁰⁷.

B cells appear in neonatal meninges as soon as one day after birth, predominantly comprising the B-1a subset¹⁰⁶. These cells can infiltrate the brain parenchyma, through a CXCL13–CXCR5 dependent signalling, where they promote oligodendrogenesis¹⁰⁶. Neutralisation of the receptor for the Fc region of IgM secreted by B-1a cells results in impaired OPC proliferation and reduced myelinated axons in the developing brain. These data suggest that meningeal B-1a infiltration into the brain parenchyma promotes oligodendrogenesis and axon myelination by stimulating OPCs proliferation via IgM-Fc receptor signalling¹⁰⁶. Moreover, and similarly to the adult meninges, B cells in the developing mouse and non-human primate are replenished by skull marrow developing B cells, undergoing CNS antigen-dependent checkpoints to prevent self-reactivity¹⁰⁸.

T cells are also found in the neonatal meninges at birth, with different kinetics regarding their subsets^{55,57}. While $\gamma\delta$ T cells peak one week after birth and decrease over time, $\alpha\beta$ T cells tend to accumulate after weaning^{55,57}. During the neonatal period, $\gamma\delta$ T cells exhibit high proliferative activity, and parabiotic studies indicate that they possess a tissue-resident phenotype⁵⁵. Their function during the neonatal

period is, however, still unclear. Postmortem studies of preterm human infant brain with periventricular leukomalacia (PVL), the most common preterm brain injury affecting the white matter, showed the presence of both T and B cells in the meninges, contrary to the control cases¹⁰⁹. These cells seem to be involved in the induction of brain injury, as a mouse model of infection using RAG1^{-/-} mice showed protection against white matter loss¹⁰⁹. $\gamma\delta$ T cells were also found in the meninges of neonatal humans and mice with PVL, and a mouse model of preterm brain injury showed a significant increase of these cells 6 h upon infection¹¹⁰. Depletion of $\gamma\delta$ T cells conferred protection against brain injury¹¹⁰, suggesting their potential role in driving brain damage.

5. Concluding remarks and future perspectives

The meninges harbour a diverse repertoire of immune cells distinct from those found in the CNS parenchyma, contributing to a unique and complex microenvironment that remains poorly understood. Meningeal immunity plays an active role in controlling brain functions, including cognition, behaviour, neuronal activity, and CNS immune surveillance. Moreover, the meninges serve as a potential entry point for pathogens, and inflammation in this region is associated with brain injury and dysfunction, suggesting their importance in maintaining brain homeostasis. During infection, meningeal immune cells can recruit systemic leucocytes and even endow them with the capacity to infiltrate the CNS parenchyma. When referring to neonates, our knowledge is even more scarce. Although structurally similar to the adults', neonatal meninges are still maturing their lymphatic system and besides macrophages, B and T cells, other immune cell populations have yet to be fully characterised. The specific immune functions of these cells, as well as the

mechanisms underlying meningeal infection, inflammation, and neuroimmune interactions, remain largely unknown. Therapeutically, due to the barrier restrictions of the CNS and our poor knowledge about meningeal immunity, it has been challenging to develop efficient strategies. Future research is needed to elucidate the dynamics of neonatal meningeal immunity in both health and disease. Combining conventional techniques such as flow cytometry, with cutting-edge approaches, like intravital imaging, meningeal whole-mount immunohistochemistry, and single-cell RNA sequencing will be key to identifying novel therapeutic targets. Such insights could pave the way for the development of neuroprotective strategies.

References

- 1 Ghannam, J. Y. & Al Kharazi, K. A. in *StatPearls* (2020).
- 2 Wilkins, R. H. Neurosurgical classic—XVII. *Journal of Neurosurgery* 21, 240-244 (1964).
- 3 Bifari, F. *et al.* Novel stem/progenitor cells with neuronal differentiation potential reside in the leptomeningeal niche. *Journal of Cellular and Molecular Medicine* 13, 3195-3208, doi:<https://doi.org/10.1111/j.1582-4934.2009.00706.x> (2009).
- 4 Dasgupta, K. & Jeong, J. Developmental biology of the meninges. *genesis* 57, e23288, doi:<https://doi.org/10.1002/dvg.23288> (2019).
- 5 Choe, Y., Huynh, T. & Pleasure, S. J. Migration of Oligodendrocyte Progenitor Cells Is Controlled by Transforming Growth Factor β Family Proteins during Corticogenesis. *The Journal of Neuroscience* 34, 14973-14983, doi:10.1523/jneurosci.1156-14.2014 (2014).

- 6 Mishra, S., Choe, Y., Pleasure, S. J. & Siegenthaler, J. A. Cerebrovascular defects in *Foxc1* mutants correlate with aberrant WNT and VEGF-A pathways downstream of retinoic acid from the meninges. *Dev Biol* 420, 148-165, doi:10.1016/j.ydbio.2016.09.019 (2016).
- 7 Siegenthaler, J. A. *et al.* Retinoic acid from the meninges regulates cortical neuron generation. *Cell* 139, 597-609 (2009).
- 8 Tischfield, M. A. *et al.* Cerebral Vein Malformations Result from Loss of Twist1 Expression and BMP Signaling from Skull Progenitor Cells and Dura. *Dev Cell* 42, 445-461.e445, doi:10.1016/j.devcel.2017.07.027 (2017).
- 9 Zarbalis, K., Choe, Y., Siegenthaler, J. A., Orosco, L. A. & Pleasure, S. J. Meningeal defects alter the tangential migration of cortical interneurons in *Foxc1*^{hith/hith} mice. *Neural Development* 7, 2, doi:10.1186/1749-8104-7-2 (2012).
- 10 Zhang, J., Smith, D., Yamamoto, M., Ma, L. & McCaffery, P. The meninges is a source of retinoic acid for the late-developing hindbrain. *Journal of Neuroscience* 23, 7610-7620 (2003).
- 11 Etchevers, H. C., Couly, G., Vincent, C. & Le Douarin, N. M. Anterior cephalic neural crest is required for forebrain viability. *Development* 126, 3533-3543 (1999).
- 12 Siegenthaler, J. A. & Pleasure, S. J. We have got you 'covered': how the meninges control brain development. *Current Opinion in Genetics & Development* 21, 249-255, doi:<https://doi.org/10.1016/j.gde.2010.12.005> (2011).
- 13 Decimo, I. *et al.* Meninges: A Widespread Niche of Neural Progenitors for the Brain. *The Neuroscientist*, 1073858420954826, doi:10.1177/1073858420954826 (2020).

- 14 Alves de Lima, K., Rustenhoven, J. & Kipnis, J. Meningeal Immunity and Its Function in Maintenance of the Central Nervous System in Health and Disease. *Annual Review of Immunology* 38, 597-620, doi:10.1146/annurev-immunol-102319-103410 (2020).
- 15 Rua, R. & McGavern, D. B. Advances in Meningeal Immunity. *Trends Mol Med* 24, 542-559, doi:10.1016/j.molmed.2018.04.003 (2018).
- 16 Coles, J. A., Myburgh, E., Brewer, J. M. & McMenamin, P. G. Where are we? The anatomy of the murine cortical meninges revisited for intravital imaging, immunology, and clearance of waste from the brain. *Progress in Neurobiology* 156, 107-148, doi:<https://doi.org/10.1016/j.pneurobio.2017.05.002> (2017).
- 17 Mollgard, K. *et al.* A mesothelium divides the subarachnoid space into functional compartments. *Science* 379, 84-88, doi:10.1126/science.adc8810 (2023).
- 18 Liliequist, B. The anatomy of the subarachnoid cisterns. *Acta radiologica* 46, 61-71, doi:10.3109/00016925609170813 (1956).
- 19 Yasargil, M. G., Kasdaglis, K., Jain, K. K. & Weber, H. P. Anatomical observations of the subarachnoid cisterns of the brain during surgery. *J Neurosurg* 44, 298-302, doi:10.3171/jns.1976.44.3.0298 (1976).
- 20 Pla, V. *et al.* Structural characterization of SLYM-a 4th meningeal membrane. *Fluids Barriers CNS* 20, 93, doi:10.1186/s12987-023-00500-w (2023).
- 21 Mapunda, J. A. *et al.* VE-cadherin in arachnoid and pia mater cells serves as a suitable landmark for in vivo imaging of CNS immune surveillance and inflammation. *Nature Communications* 14, 5837, doi:10.1038/s41467-023-41580-4 (2023).

- 22 Betsholtz, C. *et al.* Advances and controversies in meningeal biology. *Nat Neurosci* 27, 2056-2072, doi:10.1038/s41593-024-01701-8 (2024).
- 23 Waggener, J. D. & Beggs, J. The membranous coverings of neural tissues: an electron microscopy study. *Journal of neuropathology and experimental neurology* 26, 412-426, doi:10.1097/00005072-196707000-00005 (1967).
- 24 Nabeshima, S., Reese, T. S., Landis, D. M. & Brightman, M. W. Junctions in the meninges and marginal glia. *J Comp Neurol* 164, 127-169, doi:10.1002/cne.901640202 (1975).
- 25 Rascol, M. M. & Izard, J. Y. The subdural neurothelium of the cranial meninges in man. *The Anatomical record* 186, 429-436, doi:10.1002/ar.1091860308 (1976).
- 26 Schachenmayr, W. & Friede, R. L. The origin of subdural neomembranes. I. Fine structure of the dura-arachnoid interface in man. *Am J Pathol* 92, 53-68 (1978).
- 27 Frederickson, R. G. The subdural space interpreted as a cellular layer of meninges. *The Anatomical record* 230, 38-51, doi:10.1002/ar.1092300105 (1991).
- 28 Haines, D. E., Harkey, H. L. & al-Mefty, O. The "subdural" space: a new look at an outdated concept. *Neurosurgery* 32, 111-120, doi:10.1227/00006123-199301000-00017 (1993).
- 29 Mack, J., Squier, W. & Eastman, J. T. Anatomy and development of the meninges: implications for subdural collections and CSF circulation. *Pediatric radiology* 39, 200-210, doi:10.1007/s00247-008-1084-6 (2009).

- 30 Balin, B. J., Broadwell, R. D., Salcman, M. & el-Kalliny, M. Avenues for entry of peripherally administered protein to the central nervous system in mouse, rat, and squirrel monkey. *J Comp Neurol* 251, 260-280, doi:10.1002/cne.902510209 (1986).
- 31 Absinta, M. *et al.* Human and nonhuman primate meninges harbor lymphatic vessels that can be visualized noninvasively by MRI. 6, e29738 (2017).
- 32 Antila, S. *et al.* Development and plasticity of meningeal lymphatic vessels. 214, 3645-3667 (2017).
- 33 Aspelund, A. *et al.* A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. *Journal of Experimental Medicine* 212, 991-999, doi:10.1084/jem.20142290 (2015).
- 34 Jani, R. H. & Sekula Jr, R. F. J. N. Magnetic resonance imaging of human dural meningeal lymphatics. 83, E10-E12 (2018).
- 35 Louveau, A. *et al.* CNS lymphatic drainage and neuroinflammation are regulated by meningeal lymphatic vasculature. *Nature Neuroscience* 21, 1380-1391, doi:10.1038/s41593-018-0227-9 (2018).
- 36 Hagan, C. E., Bolon, B. & Keene, C. D. in *Comparative Anatomy and Histology* (eds Piper M. Treuting & Suzanne M. Dintzis) 339-394 (Academic Press, 2012).
- 37 Reiter, R. J., Tan, D. X., Kim, S. J. & Cruz, M. H. C. Delivery of pineal melatonin to the brain and SCN: role of canaliculi, cerebrospinal fluid, tanycytes and Virchow–Robin perivascular spaces. *Brain Structure and Function* 219, 1873-1887, doi:10.1007/s00429-014-0719-7 (2014).

- 38 Kipnis, J. Multifaceted interactions between adaptive immunity and the central nervous system. *Science* 353, 766-771, doi:10.1126/science.aag2638 (2016).
- 39 Korin, B. *et al.* High-dimensional, single-cell characterization of the brain's immune compartment. *Nat Neurosci* 20, 1300-1309, doi:10.1038/nn.4610 (2017).
- 40 Norris, G. T. & Kipnis, J. Immune cells and CNS physiology: Microglia and beyond. *J Exp Med* 216, 60-70, doi:10.1084/jem.20180199 (2019).
- 41 Van Hove, H. *et al.* A single-cell atlas of mouse brain macrophages reveals unique transcriptional identities shaped by ontogeny and tissue environment. *Nat Neurosci* 22, 1021-1035, doi:10.1038/s41593-019-0393-4 (2019).
- 42 Goldmann, T. *et al.* Origin, fate and dynamics of macrophages at central nervous system interfaces. *Nature Immunology* 17, 797-805, doi:10.1038/ni.3423 (2016).
- 43 Giles, D. A., Duncker, P. C., Wilkinson, N. M., Washnock-Schmid, J. M. & Segal, B. M. CNS-resident classical DCs play a critical role in CNS autoimmune disease. *J Clin Invest* 128, 5322-5334, doi:10.1172/jci123708 (2018).
- 44 Dropp, J. J. Mast cells in mammalian brain. *Acta Anat (Basel)* 94, 1-21, doi:10.1159/000144540 (1976).
- 45 Fitzpatrick, Z. *et al.* Gut-educated IgA plasma cells defend the meningeal venous sinuses. *Nature* 587, 472-476, doi:10.1038/s41586-020-2886-4 (2020).

- 46 Ellwardt, E., Walsh, J. T., Kipnis, J. & Zipp, F. Understanding the Role of T Cells in CNS Homeostasis. *Trends in Immunology* 37, 154-165, doi:<https://doi.org/10.1016/j.it.2015.12.008> (2016).
- 47 Radjavi, A., Smirnov, I., Derecki, N. & Kipnis, J. Dynamics of the meningeal CD4(+) T-cell repertoire are defined by the cervical lymph nodes and facilitate cognitive task performance in mice. *Mol Psychiatry* 19, 531-533, doi:10.1038/mp.2013.79 (2014).
- 48 Ransohoff, R. M. & Engelhardt, B. The anatomical and cellular basis of immune surveillance in the central nervous system. *Nature Reviews Immunology* 12, 623-635, doi:10.1038/nri3265 (2012).
- 49 Herz, J., Filiano, A. J., Smith, A., Yogev, N. & Kipnis, J. Myeloid Cells in the Central Nervous System. *Immunity* 46, 943-956, doi:<https://doi.org/10.1016/j.immuni.2017.06.007> (2017).
- 50 Zhang, G. X., Li, J., Ventura, E. & Rostami, A. Parenchymal microglia of naïve adult C57BL/6J mice express high levels of B7.1, B7.2, and MHC class II. *Exp Mol Pathol* 73, 35-45, doi:10.1006/exmp.2002.2441 (2002).
- 51 Ousman, S. S. & Kubes, P. Immune surveillance in the central nervous system. *Nature Neuroscience* 15, 1096-1101, doi:10.1038/nn.3161 (2012).
- 52 Sanmarco, L. M. *et al.* Gut-licensed IFN γ + NK cells drive LAMP1+TRAIL+ anti-inflammatory astrocytes. *Nature* 590, 473-479, doi:10.1038/s41586-020-03116-4 (2021).
- 53 Mundt, S., Greter, M., Flügel, A. & Becher, B. The CNS Immune Landscape from the Viewpoint of a T Cell. *Trends in Neurosciences* 42, 667-679, doi:<https://doi.org/10.1016/j.tins.2019.07.008> (2019).

- 54 Brynskikh, A., Warren, T., Zhu, J. & Kipnis, J. Adaptive immunity affects learning behavior in mice. *Brain, Behavior, and Immunity* 22, 861-869, doi:<https://doi.org/10.1016/j.bbi.2007.12.008> (2008).
- 55 Alves de Lima, K. *et al.* Meningeal $\gamma\delta$ T cells regulate anxiety-like behavior via IL-17a signaling in neurons. *Nature Immunology* 21, 1421-1429, doi:10.1038/s41590-020-0776-4 (2020).
- 56 Kipnis, J., Cohen, H., Cardon, M., Ziv, Y. & Schwartz, M. T cell deficiency leads to cognitive dysfunction: Implications for therapeutic vaccination for schizophrenia and other psychiatric conditions. *Proceedings of the National Academy of Sciences of the United States of America* 101, 8180, doi:10.1073/pnas.0402268101 (2004).
- 57 Ribeiro, M. *et al.* Meningeal gammadelta T cell-derived IL-17 controls synaptic plasticity and short-term memory. *Sci Immunol* 4, doi:10.1126/sciimmunol.aay5199 (2019).
- 58 Derecki, N. C. *et al.* Regulation of learning and memory by meningeal immunity: a key role for IL-4. *J Exp Med* 207, 1067-1080, doi:10.1084/jem.20091419 (2010).
- 59 Rattazzi, L. *et al.* CD4⁺ but not CD8⁺ T cells revert the impaired emotional behavior of immunocompromised RAG-1-deficient mice. *Translational Psychiatry* 3, e280-e280, doi:10.1038/tp.2013.54 (2013).
- 60 Radjavi, A., Smirnov, I. & Kipnis, J. Brain antigen-reactive CD4⁺ T cells are sufficient to support learning behavior in mice with limited T cell repertoire. *Brain, Behavior, and Immunity* 35, 58-63, doi:<https://doi.org/10.1016/j.bbi.2013.08.013> (2014).

- 61 Brombacher, T. M. *et al.* IL-13-Mediated Regulation of Learning and Memory. *J Immunol* 198, 2681-2688, doi:10.4049/jimmunol.1601546 (2017).
- 62 Wolf, S. A. *et al.* CD4-Positive T Lymphocytes Provide a Neuroimmunological Link in the Control of Adult Hippocampal Neurogenesis. *The Journal of Immunology* 182, 3979, doi:10.4049/jimmunol.0801218 (2009).
- 63 Jeon, S. G. *et al.* Impaired Memory in OT-II Transgenic Mice Is Associated with Decreased Adult Hippocampal Neurogenesis Possibly Induced by Alteration in Th2 Cytokine Levels. *Mol Cells* 39, 603-610, doi:10.14348/molcells.2016.0072 (2016).
- 64 Filiano, A. J. *et al.* Unexpected role of interferon- γ in regulating neuronal connectivity and social behaviour. *Nature* 535, 425-429, doi:10.1038/nature18626 (2016).
- 65 Lynall, M. E. *et al.* B-cells are abnormal in psychosocial stress and regulate meningeal myeloid cell activation. *Brain Behav Immun* 97, 226-238, doi:10.1016/j.bbi.2021.08.002 (2021).
- 66 Brioschi, S. *et al.* Heterogeneity of meningeal B cells reveals a lymphopoietic niche at the CNS borders. *Science* 373, doi:10.1126/science.abf9277 (2021).
- 67 Derecki, N. C., Quinlins, K. M. & Kipnis, J. Alternatively activated myeloid (M2) cells enhance cognitive function in immune compromised mice. *Brain, behavior, and immunity* 25, 379-385, doi:10.1016/j.bbi.2010.11.009 (2011).
- 68 Da Mesquita, S., Fu, Z. & Kipnis, J. The Meningeal Lymphatic System: A New Player in Neurophysiology. *Neuron* 100, 375-388, doi:10.1016/j.neuron.2018.09.022 (2018).

- 69 Alves de Lima, K., Rustenhoven, J. & Kipnis, J. Meningeal Immunity and Its Function in Maintenance of the Central Nervous System in Health and Disease. *Annu Rev Immunol* 38, 597-620, doi:10.1146/annurev-immunol-102319-103410 (2020).
- 70 Salvador, A. F., de Lima, K. A. & Kipnis, J. Neuromodulation by the immune system: a focus on cytokines. *Nature Reviews Immunology*, doi:10.1038/s41577-021-00508-z (2021).
- 71 Bauler, T. J. *et al.* Salmonella Meningitis Associated with Monocyte Infiltration in Mice. *Am J Pathol* 187, 187-199, doi:10.1016/j.ajpath.2016.09.002 (2017).
- 72 Chimelli, L. *et al.* The spectrum of neuropathological changes associated with congenital Zika virus infection. *Acta Neuropathol* 133, 983-999, doi:10.1007/s00401-017-1699-5 (2017).
- 73 Coles, J. A. *et al.* Intravital imaging of a massive lymphocyte response in the cortical dura of mice after peripheral infection by trypanosomes. *PLoS Negl Trop Dis* 9, e0003714, doi:10.1371/journal.pntd.0003714 (2015).
- 74 Kang, S. S. & McGavern, D. B. Lymphocytic choriomeningitis infection of the central nervous system. *Front Biosci* 13, 4529-4543, doi:10.2741/3021 (2008).
- 75 Kang, S. S. & McGavern, D. B. Microbial Induction of Vascular Pathology in the CNS. *Journal of Neuroimmune Pharmacology* 5, 370-386, doi:10.1007/s11481-010-9208-9 (2010).
- 76 Koopmans, M. M. *et al.* Characterization of a *Listeria monocytogenes* meningitis mouse model. *Journal of Neuroinflammation* 15, 257, doi:10.1186/s12974-018-1293-3 (2018).

- 77 Lamers, S. L., Gray, R. R., Salemi, M., Huysentruyt, L. C. & McGrath, M. S. HIV-1 phylogenetic analysis shows HIV-1 transits through the meninges to brain and peripheral tissues. *Infect Genet Evol* 11, 31-37, doi:10.1016/j.meegid.2010.10.016 (2011).
- 78 Mogk, S. *et al.* African trypanosomes and brain infection - the unsolved question. *Biol Rev Camb Philos Soc* 92, 1675-1687, doi:10.1111/brv.12301 (2017).
- 79 Poltera, A. A., Hochmann, A., Rudin, W. & Lambert, P. H. Trypanosoma brucei brucei: a model for cerebral trypanosomiasis in mice--an immunological, histological and electronmicroscopic study. *Clin Exp Immunol* 40, 496-507 (1980).
- 80 Sjölander, H. & Jonsson, A. B. Olfactory nerve--a novel invasion route of Neisseria meningitidis to reach the meninges. *PLoS One* 5, e14034, doi:10.1371/journal.pone.0014034 (2010).
- 81 Azevedo, R. S. S. *et al.* In situ immune response and mechanisms of cell damage in central nervous system of fatal cases microcephaly by Zika virus. *Sci Rep* 8, 1, doi:10.1038/s41598-017-17765-5 (2018).
- 82 Kim, J. V., Kang, S. S., Dustin, M. L. & McGavern, D. B. Myelomonocytic cell recruitment causes fatal CNS vascular injury during acute viral meningitis. *Nature* 457, 191-195, doi:10.1038/nature07591 (2009).
- 83 Rua, R. *et al.* Infection drives meningeal engraftment by inflammatory monocytes that impairs CNS immunity. *Nature Immunology* 20, 407-419, doi:10.1038/s41590-019-0344-y (2019).
- 84 Peters, C. W., Kruse, U., Pollwein, R., Grzeschik, K. H. & Sippel, A. E. The human lysozyme gene. Sequence organization and chromosomal

localization. *European journal of biochemistry* 182, 507-516,
doi:10.1111/j.1432-1033.1989.tb14857.x (1989).

- 85 Cross, M. & Renkawitz, R. Repetitive sequence involvement in the duplication and divergence of mouse lysozyme genes. *Embo j* 9, 1283-1288, doi:10.1002/j.1460-2075.1990.tb08237.x (1990).
- 86 Wen, J. H., Li, D. Y., Liang, S. & Tang, J. X. Is LysM-Cre a good candidate Cre for knocking out Atg5 gene in mice? *Frontiers in immunology* 13, 964496, doi:10.3389/fimmu.2022.964496 (2022).
- 87 De Vlaminck, K. *et al.* Differential plasticity and fate of brain-resident and recruited macrophages during the onset and resolution of neuroinflammation. *Immunity* 55, 2085-2102.e2089, doi:10.1016/j.immuni.2022.09.005 (2022).
- 88 O'Brien, C. A. *et al.* CD11c-Expressing Cells Affect Regulatory T Cell Behavior in the Meninges during Central Nervous System Infection. *J Immunol* 198, 4054-4061, doi:10.4049/jimmunol.1601581 (2017).
- 89 Cervantes-Barragán, L. *et al.* Regulatory T cells selectively preserve immune privilege of self-antigens during viral central nervous system infection. *J Immunol* 188, 3678-3685, doi:10.4049/jimmunol.1102422 (2012).
- 90 de Aquino, M. T. *et al.* Role of CD25(+) CD4(+) T cells in acute and persistent coronavirus infection of the central nervous system. *Virology* 447, 112-120, doi:10.1016/j.virol.2013.08.030 (2013).
- 91 Lanteri, M. C. *et al.* Tregs control the development of symptomatic West Nile virus infection in humans and mice. *J Clin Invest* 119, 3266-3277, doi:10.1172/jci39387 (2009).

- 92 Smigiel, K. S. *et al.* CCR7 provides localized access to IL-2 and defines homeostatically distinct regulatory T cell subsets. *J Exp Med* 211, 121-136, doi:10.1084/jem.20131142 (2014).
- 93 Maudet, C. *et al.* Bacterial inhibition of Fas-mediated killing promotes neuroinvasion and persistence. *Nature* 603, 900-906, doi:10.1038/s41586-022-04505-7 (2022).
- 94 Christodoulides, M. *et al.* Interaction of *Neisseria meningitidis* with human meningeal cells induces the secretion of a distinct group of chemotactic, proinflammatory, and growth-factor cytokines. *Infect Immun* 70, 4035-4044, doi:10.1128/iai.70.8.4035-4044.2002 (2002).
- 95 Fowler, M. I., Yin, K. Y., Humphries, H. E., Heckels, J. E. & Christodoulides, M. Comparison of the inflammatory responses of human meningeal cells following challenge with *Neisseria lactamica* and with *Neisseria meningitidis*. *Infect Immun* 74, 6467-6478, doi:10.1128/iai.00644-06 (2006).
- 96 Hardy, S. J., Christodoulides, M., Weller, R. O. & Heckels, J. E. Interactions of *Neisseria meningitidis* with cells of the human meninges. *Mol Microbiol* 36, 817-829, doi:10.1046/j.1365-2958.2000.01923.x (2000).
- 97 Oordt-Speets, A. M., Bolijn, R., van Hoorn, R. C., Bhavsar, A. & Kyaw, M. H. J. P. o. Global etiology of bacterial meningitis: a systematic review and meta-analysis. 13, e0198772 (2018).
- 98 Pinho-Ribeiro, F. A. *et al.* Bacteria hijack a meningeal neuroimmune axis to facilitate brain invasion. *Nature* 615, 472-481, doi:10.1038/s41586-023-05753-x (2023).
- 99 Audshasai, T. *et al.* *Streptococcus pneumoniae* Rapidly Translocate from the Nasopharynx through the Cribriform Plate to Invade the Outer Meninges. *mBio* 13, e0102422, doi:10.1128/mbio.01024-22 (2022).

- 100 Gres, V. *et al.* Dynamic role of monocytes and meningeal macrophages in bacterial meningoencephalitis. 2024.2002.2027.582183, doi:10.1101/2024.02.27.582183 %J bioRxiv (2024).
- 101 Izen, R. M., Yamazaki, T., Nishinaka-Arai, Y., Hong, Y. K. & Mukouyama, Y. S. Postnatal development of lymphatic vasculature in the brain meninges. *Dev Dyn* 247, 741-753, doi:10.1002/dvdy.24624 (2018).
- 102 Sato, T., Konishi, H., Tamada, H., Nishiwaki, K. & Kiyama, H. Morphology, localization, and postnatal development of dural macrophages. *Cell and Tissue Research*, doi:10.1007/s00441-020-03346-y (2021).
- 103 Antila, S. *et al.* Development and plasticity of meningeal lymphatic vessels. *J Exp Med* 214, 3645-3667, doi:10.1084/jem.20170391 (2017).
- 104 Kim, Y. C. *et al.* Immaturity of immune cells around the dural venous sinuses contributes to viral meningoencephalitis in neonates. *Sci Immunol* 8, eadg6155, doi:10.1126/sciimmunol.adg6155 (2023).
- 105 Rebejac, J. *et al.* Meningeal macrophages protect against viral neuroinfection. *Immunity* 55, 2103-2117.e2110, doi:10.1016/j.immuni.2022.10.005 (2022).
- 106 Tanabe, S. & Yamashita, T. B-1a lymphocytes promote oligodendrogenesis during brain development. *Nat Neurosci* 21, 506-516, doi:10.1038/s41593-018-0106-4 (2018).
- 107 Wang, J., Rattner, A. & Nathans, J. Bacterial meningitis in the early postnatal mouse studied at single-cell resolution. *eLife* 12, e86130, doi:10.7554/eLife.86130 (2023).

- 108 Wang, Y. *et al.* Early developing B cells undergo negative selection by central nervous system-specific antigens in the meninges. *Immunity* 54, 2784-2794 e2786, doi:10.1016/j.immuni.2021.09.016 (2021).
- 109 Nazmi, A. *et al.* Lymphocytes Contribute to the Pathophysiology of Neonatal Brain Injury. *Front Neurol* 9, 159, doi:10.3389/fneur.2018.00159 (2018).
- 110 Albertsson, A.-M. *et al.* $\gamma\delta$ T Cells Contribute to Injury in the Developing Brain. *The American journal of pathology* 188, 757-767, doi:10.1016/j.ajpath.2017.11.012 (2018).

Declaration of Competing Interest

The authors declare no personal or financial competing interests.

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ANNEX 2



OPEN Development of bioluminescent Group B streptococcal strains for longitudinal infection studies

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Group B *Streptococcus* (GBS) remains the leading bacterial cause of invasive neonatal disease, resulting in substantial morbidity and mortality. New therapeutic approaches beyond antibacterial treatment to prevent neonatal disease outcomes are urgent. One significant limitation in studying GBS disease and progression is the lack of non-invasive technologies for longitudinal studies. Here, we develop and compare three bioluminescent GBS strains for *in vivo* pathogenic analysis. Bioluminescence is based on the *luxABCDE* operon on a replicative vector (*lux*GBS-CC17), and the red-shifted firefly luciferase on a replicative vector (*ffluc*GBS-CC17) or integrated in the genome (*gluc*GBS-CC17). We show that *lux*GBS-CC17 is suitable for *in vitro* analysis but does not produce a significant bioluminescent signal in infected pups. In contrast, the *ffluc*GBS-CC17 results in a strong bioluminescent signal proportional to the organ colonisation level. However, the stability of the replicative vector depends on the route of infection, especially when pups acquire the bacteria from infected vaginal mucosa. Stable chromosomal integration of luciferase in *gluc*GBS-CC17 leads to significant bioluminescence in both haematological and vertical infection models associated with high systemic colonisation. These strains will allow the preclinical evaluation of treatment efficacy against GBS invasive disease using whole-mouse bioluminescence imaging.

Group B *Streptococcus* (GBS) remains the leading bacterial pathogen responsible for invasive disease and death in infants under two months old¹. GBS colonises the lower genital tract of pregnant women and can be transmitted vertically to the newborn *in utero* or during birth, leading to early-onset disease (EOD), mostly associated with sepsis and pneumonia, or late-onset disease (LOD), frequently presented as meningitis². Invasive GBS disease results in a mortality rate of 10–15% and life-long neurodevelopment impairment (NDI) in up to 50% of survivors³. GBS meningitis is particularly concerning, as its incidence has been increasing over the last decade^{4–6}. Moreover, no neuroprotective treatment is currently available, and *intrapartum* antibiotic prophylaxis is ineffective to prevent LOD meningitis⁷, highlighting the necessity for developing new preventive and therapeutic solutions.

Over the past decades, significant efforts have been made to elucidate the pathophysiology and long-term NDI resulting from GBS neonatal disease, leading to the establishment of several animal models, including hematogenous, oral and direct brain infections^{8–11}. Recently, our group developed an animal mouse model that mimics the natural human route of infection, *i.e.* mother-to-progeny transmission, through intra-vaginal colonisation of pregnant females¹². However, these models still do not provide a complete understanding of the disease establishment and progression^{2,12}. Traditional colony-forming unit (CFU) counting methods have limitations, including the need to euthanise animals to assess organ colonisation¹³. Using non-invasive bioluminescence imaging (BLI) would enable longitudinal *in vivo* studies, enhancing our understanding of the pathophysiology of GBS neonatal disease. Additionally, BLI would facilitate testing the efficacy of novel prophylactic and therapeutic treatments. Moreover, this technique aligns with the ethical principles of the 3Rs in animal experiments by reducing and refining animal usage, allowing each animal to be studied across multiple time points and organs, rather than sacrificing one animal per time point for limited organ studies^{13,14}.

Bioluminescent reporter systems have been developed in several pathogenic streptococcal species^{15–18}. In GBS, luminescence has been obtained with a replicative vector containing the luciferase gene (*luc*) from the firefly *Photinus pyralis*¹⁵. The firefly luciferase converts D-luciferin, its substrate, to oxyluciferin, emitting light

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with a maximum intensity of 560 nm¹⁹. The replicative vector is stabilised by antibiotic selection and a toxin-antitoxin (TA) system plasmid stability for up to 10 days *in vitro* even without the antibiotic selective pressure¹⁵. However, this system was not evaluated *in vivo*, in animal models of infection. Group A *Streptococcus* (GAS) has been the most studied streptococcal species with bioluminescent reporters^{15–18}. Initially, the bacterial luciferin-luciferase operon (*luxABCDE*) was used, which has the advantage of not requiring an external luciferin substrate for bioluminescent light emission (maximum intensity at 490 nm)¹⁸. Nevertheless, this system produces a weaker signal, and the vector is less stable¹⁸. To improve sensitivity *in vivo*, a variant using the red-shifted firefly luciferase (*ffluc*), which emits light at 610 nm, was created in GAS¹⁸. Bioluminescent streptococcal strains have been used for studying disease progression, antimicrobial compounds, vaccine efficacy or even pathogenic synergism^{20–23}, highlighting the importance and wide-range applicability of BLI for enhanced *in vivo* studies.

In the present study, we aimed to develop bioluminescent GBS strains to further improve our knowledge of neonatal GBS invasive disease. We found that vector-based approaches are unstable and inefficient for longitudinal studies with GBS, even with a stable *ffluc* plasmid carrying a TA system. However, genomic insertion of the red-shifted firefly luciferase gene into GBS creates a stable bioluminescent strain suitable for evaluating severe GBS disease using whole-mouse live imaging.

Results

Development and assessment of a *lux* bioluminescent reporter system in GBS

To generate a GBS bioluminescent reporter strain, we used the BM110 strain, of capsular serotype III and belonging to the CC-17 hypervirulent lineage responsible for more than 80% of meningitis cases^{12,24,25}. We first tested an available bioluminescent vector-based system containing the *luxABCDE* operon, which encodes the luciferase and its aldehyde substrate, thereby eliminating the need for an exogenous substrate^{13,26}. To this end, we introduced the replicative vector pSL101P₃₂, containing the constitutively active P32 promoter^{27,28} to generate the GBS strain *lux*GBS-CC17.

To evaluate whether light emission could be expressed as a function of cell growth, bacteria from the mid-log phase were serially diluted, and the luminescence (emission OD_{490nm}), optical density (OD_{600nm}), and cell counts (CFU/mL) were determined. The bioluminescent signal of *lux*GBS-CC17 positively correlated with both OD_{600nm} and the number of CFU, indicating that the bioluminescent signal reliably predicts bacterial count (Fig. 1a). Moreover, the pSL101P₃₂ vector does not have a fitness cost since the growth curve was equivalent to the one of wild-type (WT) GBS (GBS-CC17) under standard conditions in TH (Fig. 1b). To assess the stability of the pSL101P₃₂ vector, we grew serial cultures of *lux*GBS-CC17 with or without the selective antibiotic (spectinomycin) for 8 days. Bacteria were quantified each 24 h, by evaluating CFU on selective and non-selective media (total *versus* spectinomycin resistant bacteria). No significant differences were found between total and spectinomycin resistant GBS strains, even when grown without selective pressure, indicating that the plasmid was stable *in vitro* (Fig. 1c and d).

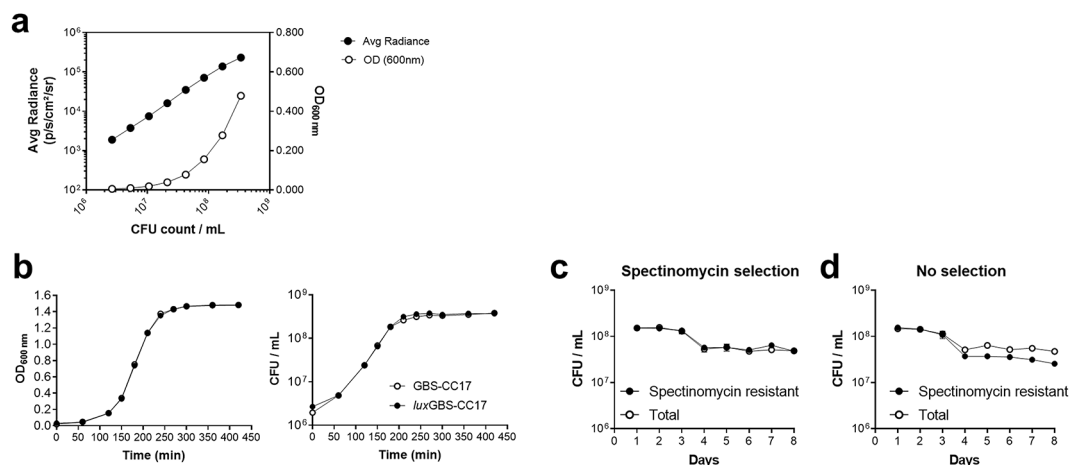


Fig. 1. *In vitro* bioluminescence with the pSL101P₃₂ replicative vector in GBS. The pSL101P₃₂ vector was introduced in the BM110 wild-type strain (WT) to give origin to the strain *lux*GBS-CC17. **(a)** Correlation of bioluminescent signal, optical density (OD) and CFU count. *lux*GBS-CC17 was grown until the mid-log phase and serially diluted in 96-well plate. Bioluminescence, OD and CFU quantified. Field symbols represent radiance, whereas open symbols OD. Bioluminescent signal plotted as radiance (photons/seconds/cm²/sr). Results represent data from 3 independent experiments. **(b)** Growth curves of WT and *lux*GBS-CC-17 strains. Optical density (left) measured every 30–60 min for 7 h and the corresponding GBS numbers (right). Mean values $\pm$ SEM data pooled from 3 independent experiments. Comparisons were performed by two-way ANOVA. **(c–d)** The *lux*GBS-CC-17 strain was daily diluted 1:1000 into fresh TH medium (with or without spectinomycin) and grown for 24 h, for 8 consecutive days. **(c)** CFU enumeration of both strains plated on TH medium with or **(d)** without selection for CFU count. Mean values $\pm$ SEM data pooled from 3 independent experiments. Comparisons were performed by two-way ANOVA.

To evaluate the infectivity of the bioluminescent strain, we tested its adhesion and invasion properties on human Caco-2 cells. No significant differences were observed between *lux*GBS-CC17 and WT GBS-infected cells for their ability to adhere to and invade the Caco-2 enterocytes (Fig. 2a). To test the virulence of *lux*GBS-CC17, newborn mice at postnatal day (P) 2 were intraperitoneally (i.p.) infected with 5×10^5 CFU of *lux*GBS-CC17 or GBS-CC17. No differences were observed in the survival rates of the pups between groups (Fig. 2b). To investigate invasion and colonisation of internal organs, we next determined the number of CFU in the brain, lungs, and liver 18 h post-infection (hpi). No differences were observed between groups, showing that the replicative vector did not affect bacterial virulence (Fig. 2c). Moreover, all *lux*GBS-CC17 colonies recovered from infected organs are spectinomycin-resistant, as assayed by CFU counting on media with and without antibiotics, indicating that the vector is stable for the duration of the experiment (Fig. 2c). To determine whether this strain generates a robust bioluminescent signal, mice infected with lower (5×10^4 CFU) or higher (1×10^5 CFU) doses of *lux*GBS-CC17 were imaged using an IVIS Lumina LT (Fig. 2d). Unexpectedly, as shown in Fig. 2d, no signal was detected on any tested pup regardless of the infection dose, suggesting that the individual luminescence signal was not high enough to be detected. Overall, these results show that the bioluminescent *luxABCDE*-based GBS strain is not efficient for *in vivo* studies.

Development and evaluation of a firefly luciferase-expressing GBS strain for *in vivo* short-term infection studies

Aiming at developing a bioluminescent GBS strain for longitudinal studies, we next used a replicative vector containing the red-shifted firefly luciferase, pLZ12Km2-P23R: TA: *ffluc*^{15,18} and the constitutively active P23 promoter²⁹. The red-shifted firefly *ffluc* luciferase system has a peak of light emission with a longer wavelength (610 nm) which enhances tissue penetration compared to the *lux* reporter system^{18,30}. Therefore, we introduced the pLZ12Km2-P23R: TA: *ffluc* vector in BM110 by electroporation, creating the *ffluc*GBS-CC17 strain. Similarly to *lux*GBS-CC17, the bioluminescent signal of *ffluc*GBS-CC17 positively correlated with the OD_{600nm} and the number of CFU (Fig. 3a). The presence of the plasmid did not affect bacterial growth, as indicated by overlapping growth curves of the WT strain and *ffluc*GBS-CC17 (Fig. 3b). Moreover, the plasmid remained stable *in vitro* for up to 10 days without antibiotic selective pressure (Fig. 3c and d). Using the human enterocytes cell line Caco-2, we confirmed that the *ffluc*GBS-CC17 strain adheres to and invades cells similarly to the WT strain (Fig. 4a). Moreover, when assessing the infectivity *in vivo* using a neonatal mouse model of infection, no differences in survival were observed between pups infected with *ffluc*GBS-CC17 or with the WT strain (Fig. 4b). Bacterial loads in the brain, lungs and liver were comparable between groups 18 hpi (Fig. 4c). Additionally, no differences

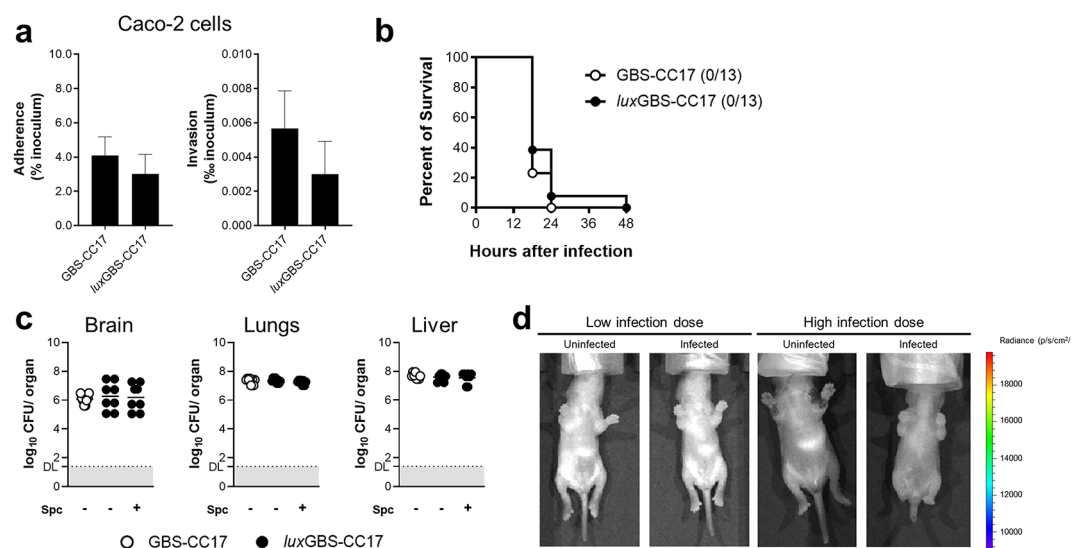


Fig. 2. Infectivity and *in vivo* bioluminescence of *lux*GBS-CC17. **(a)** Caco-2 cells were infected with WT or *lux*GBS-CC17 strains at a multiplicity of infection of 30, for 3 h, in triplicates. Bacteria adhesion and invasion were determined. Results are expressed relative to the initial inoculum. Data are shown as mean + SEM pool from 3 independent experiments. **(b-c)** Neonatal mice were inoculated intraperitoneally with 5×10^4 CFU of WT or *lux*GBS-CC17. **(b)** Kaplan-Meier survival curve monitored for 48 h. Numbers in parenthesis indicate the number of animals that survived versus the total number of infected animals ($n = 13$). Results represent data pooled from 2 independent experiments. Comparisons with the log-rank Mantel-Cox test. **(c)** Bacterial load in the brain, lungs and liver 18 h post-infection (hpi). The organs from WT infected pups were plated on TH medium, while those from *ffluc*GBS-CC17 were plated on TH with and without spectinomycin. Data are shown as means, and each symbol represents data from individual pups ($n = 8$). DL, detection limit = 1.4 (25 CFU). **(d)** Neonatal mice were inoculated intraperitoneally with 5×10^4 (low dose) or 1×10^5 (high dose) CFU of *lux*GBS-CC17 and euthanised 18 hpi. Representative images of uninfected or infected pups resulting from the superimposition of the bioluminescence signal map and a grey-scale photograph of the mice in the ventral position.

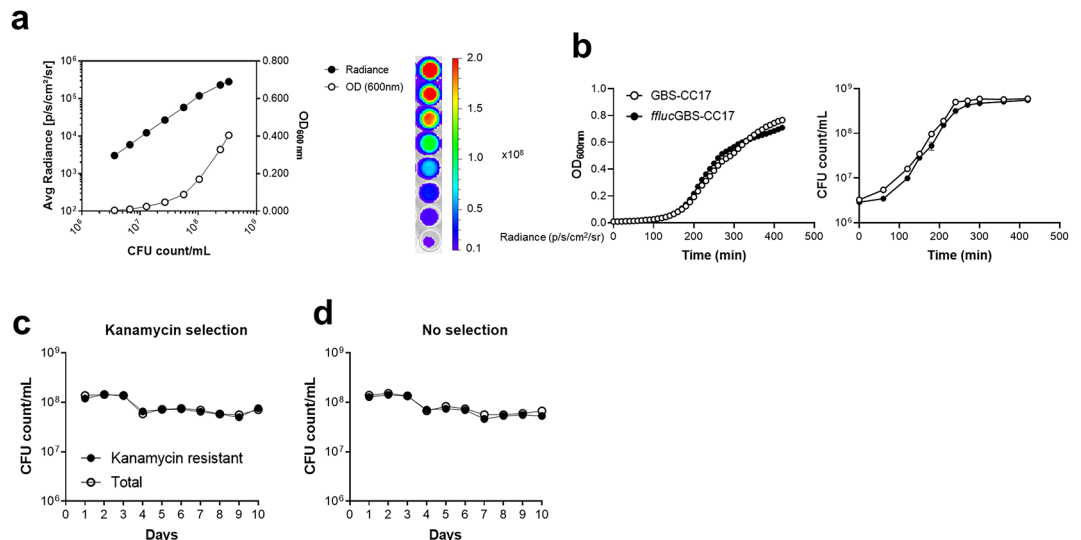


Fig. 3. *In vitro* bioluminescence with the pLZ12Km2-P23R: TA: *ffluc*RT replicative vector in GBS. The WT strain was transformed with the pLZ12Km2-P23R: TA: *ffluc*RT vector to give the *ffluc*GBS-CC17 strain. **(a)** Correlation of bioluminescent signal, optical density (OD) and CFU count. The *ffluc*GBS-CC17 strain was grown until the mid-log phase and serially diluted on a 96-well plate. Left: Bioluminescence (Radiance, 0.5 mg/mL of Luciferin) and OD quantification on a plate reader (Synergy 2), and CFU counts. Field symbols represent radiance, whereas open symbols represent OD. Right: Bioluminescence signal imaged using IVIS Lumina LT. Areas with high photon emission correspond to the red colour and the areas with lower emission are displayed in blue. The detection of bioluminescent signals by the system resulted in the generation of signal maps overlaid on a grey-scale photograph of the 96-well plate. Bioluminescent signal plotted as radiance (photons/seconds/cm²/sr). Results represent data pooled from 3 independent experiments. **(b)** Growth curves of WT and *ffluc*GBS-CC17 strains. Optical density (left) measured every 10 min for 7 h on Synergy 2. GBS numbers were counted every 30–60 min (right). Mean values $\pm$ SEM data pooled from 3 independent experiments. Comparisons were performed by two-way ANOVA. **(c–d)** WT and *ffluc*GBS-CC17 strains were daily diluted 1:1000 into fresh TH medium (with or without kanamycin) and grown for 24 h, for 10 consecutive days. Numbers of kanamycin resistant GBS and total GBS cells grown in medium with **(c)** or without **(d)** kanamycin selection were counted. Mean values $\pm$ SEM data pooled from 3 independent experiments. Comparisons were performed by two-way ANOVA.

were found when enumerating *ffluc*GBS-CC17 CFU in organs plated on selective and non-selective media, confirming plasmid stability *in vivo* (Fig. 4c).

To validate the strain for BLI, P2 pups were infected with a low (5×10^4 CFU) and high (1×10^5 CFU) doses of *ffluc*GBS-CC17. At 18 hpi, pups were administered D-Luciferin subcutaneously (s.c.) 5 minutes before BLI acquisition, and images were captured in both dorsal and ventral positions (Fig. 4d). As expected, high inoculum levels resulted in significantly ($P = 0.0003$) stronger bioluminescent signals, measured by average radiance (photons/second/cm²/steradian) (Fig. 4d–e). However, 7 out of 12 pups infected with the low dose did not exhibit bioluminescent signal (Fig. 4e). Thus, to determine whether these differences were correlated with different bacterial colonisation, GBS burden in the brain, lungs and liver were evaluated. Pups infected with the low dose presented significantly ($P = 0.0003$) lower bacterial loads than those infected with the high dose (Fig. 4f). Among the 7 pups without bioluminescent signal, only 2 had CFU counts below the detection level, suggesting that this inoculum led to colonisation below the detection threshold of the IVIS system. Importantly, a positive correlation was observed when plotting the whole-body radiance with the sum of bacterial colonisation in the lungs, liver, and brain (Fig. 4g and h). Taken together, these data show that the overall lack of bioluminescent signal intensity was due to reduced number of GBS in these organs.

To determine whether the bioluminescent *ffluc*GBS-CC17 strain could be used as a reliable bacterial quantification method in a mouse model mimicking the pathophysiology of GBS disease, newborn mice were infected vertically using our mouse model of infection¹², adapted for C57BL/6 mice. Briefly, pregnant mice were colonised with GBS-CC17 (WT or *ffluc*) in their vaginal mucosa at gestational days 16 and 17. No selective antibiotic was given to the mother or their progeny. Three-day-old pups were given D-luciferin, anaesthetised, subjected to BLI, and sacrificed for organ colonisation assessment. Unexpectedly, no bioluminescent signal was detected in any of the tested pups (data not shown). As expected, the organ colonisation in the brain, lungs and liver is lower than in the systemic infection, as our infection model usually results in decreased bacterial load (Fig. 4c and i). Contrary to what was found in the i.p. model, CFU counts in the brain differed when plated on agar-medium with and without antibiotic selection (Fig. 4i). In the brain, only 1 out of 7 pups had detectable GBS CFU on kanamycin-containing medium, while 5 out of 7 pups had no detectable CFU when using non-selective medium (Fig. 4i). Regarding the lungs, only 1 pup presented colonisation without selection, and none with antibiotic selection (Fig. 4i). To investigate if this result was due to plasmid loss, we performed a colony PCR

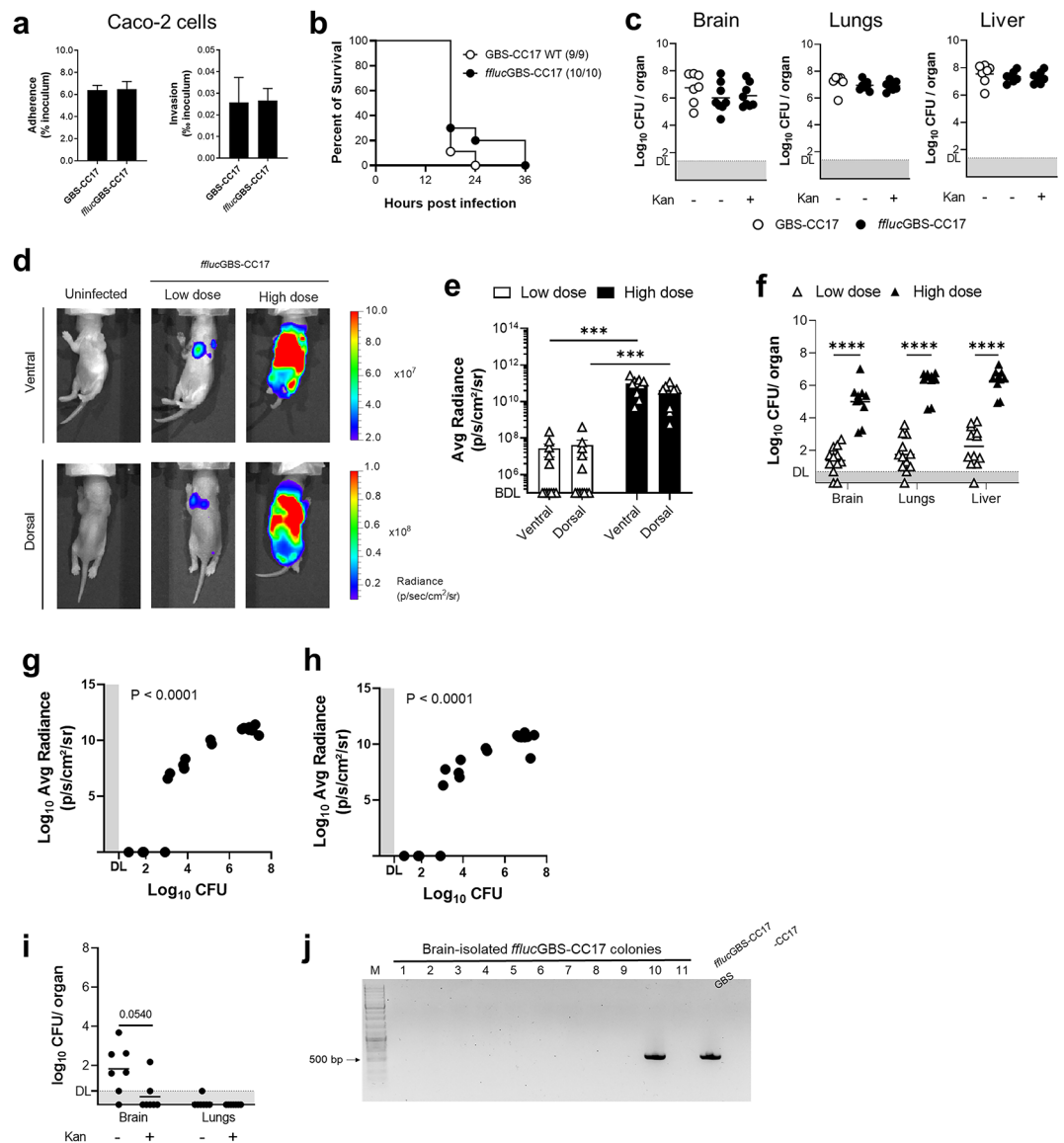


Fig. 4. Infectivity and *in vivo* bioluminescence of *ffluc*GBS-CC17. **(a)** Caco-2 cells were infected with WT or *ffluc*GBS-CC17 for 3 h. Bacteria adhesion and invasion were determined. Results are expressed relative to the initial inoculum. Data shown as mean + SEM from 3 independent experiments. **(b–c)** Neonatal mice were inoculated intraperitoneally with 5×10^4 CFU of WT or *ffluc*GBS-CC17. Data pooled from 3 independent experiments. **(b)** Kaplan-Meier survival curve monitored for 36 h. Numbers in parenthesis: survivors versus total ($n = 9$ WT; $n = 10$ *ffluc*GBS-CC17). Log-rank Mantel-Cox test comparisons. **(c)** Bacterial load in indicated organs at 18 h post-infection (hpi), plated on TH with or without kanamycin. Data shown as mean, each symbol represents one pup ($n = 7$ WT; $n = 8$ *ffluc*GBS-CC17). DL, detection limit = 1.4. **(d–h)** Pups were inoculated intraperitoneally with 5×10^4 (low dose) or 1×10^5 (high dose) CFU of *ffluc*GBS-ST17 and analysed 18 hpi. **(d)** Representative images by superimposition of the bioluminescence signal map and a grey-scale photograph. **(e)** The regions of interest (ROIs) were used to quantify the whole mouse bioluminescence signal and expressed as average radiance (photons/seconds/cm²/steradian). Mean $\pm$ SEM, each symbol represents one individual. BDL, below detection limit. **(f)** Bacterial load in the indicated organs. Data shown as mean, each symbol represents one animal ($n = 12$ Low dose; $n = 10$ High dose). Statistical analysis using unpaired Student's t-test. *** $P < 0.001$; **** $P < 0.0001$. DL, detection limit = 0.7. **(g–h)** Correlation between colonisation and average radiance in infected pups in the **(g)** ventral and **(h)** dorsal positions. Each symbol represents one animal. DL, detection limit. * $P < 0.0001$. **(i–j)** Pregnant mice were intra-vaginally inoculated with 8×10^4 *ffluc*GBS-CC17. Three-day-old pups were analysed. **(i)** Bacterial load in the indicated organs. Data shown as mean pooled from 2 independent experiments. Each symbol represents one animal ($n = 7$). Statistical analysis by unpaired Student's t-test. * $P < 0.05$. DL, detection limit = 0.7. **(j)** Representative agarose gel showing colony PCR for screening *ffluc*GBS-CC17 collected from pups' brains, using plasmid-targeted primers (495 bp amplicon). M, molecular weight marker (10 kb GeneRuler DNA ladder mix); 1–11, bacteria isolates; *ffluc*GBS-CC17 and WT GBS-CC17 as controls.

of brain CFU using *ffluc* plasmid-specific primers (Fig. 4j) and Supplementary Fig. 2). Only 1 out of 11 colonies tested retained the plasmid, indicating that GBS loses the plasmid *in vivo* over time, especially under conditions requiring extended analysis periods after infection. Overall, these data show that while the firefly luciferase-expressing GBS with the pLZ12Km2-P23R: TA: *ffluc* plasmid is a useful tool for models of infection leading to high organ colonisation, it is unsuitable for long-term studies due to plasmid instability over time *in vivo*.

A stable luciferase reporter for *in vivo* imaging in GBS infections

Integration of the firefly luciferase gene into the bacterial chromosome ensures stability and transmissibility of the reporter. Thus, we next sought to insert the red-shifted firefly luciferase into the BM110 genome. We first amplified the *ffluc* gene from the pLZ12Km2-P23R: TA: *ffluc* vector, including the P23 promoter²⁹, as well as two contiguous chromosomal GBS regions of 500 bp in a conserved intergenic region between the BQ8897_RS05445 and BQ8897_RS05450 genes, which encode a FAD-dependent oxidoreductase and L-lactate dehydrogenase, respectively (Supplementary Fig. 1). The three amplicons were combined and cloned into the pG1 integrative vector, with *ffluc* inserted into the middle of the 1 kb chromosomal sequence. Following GBS transformation, selection for homologous recombination at the targeted locus, derecombination, and vector loss, we isolated the *gluc*GBS-CC17 strain. Whole-genome sequencing of *gluc*GBS-CC17 confirmed the integration of *ffluc* at the targeted locus, with no remaining traces of the integrative vector and no other secondary mutations in the genome.

Similar to the previously developed reporter models, we started by assessing the *in vitro* luminescence signal and fitness. The optical density and CFU of *gluc*GBS-CC17 cultures reliably correlate with the radiance signal (Fig. 5a) and no differences in bacterial growth were found between the *gluc*GBS-CC17 and the WT strains (Fig. 5b). Next, we tested the infectivity and virulence *in vivo* using a haematological model of infection. No significant differences in organ colonisation 18 hpi was observed between the *gluc*GBS-CC17 and the WT strain (Fig. 6a), confirming that the integrated *ffluc* does not impact bacterial load in all tested organs. To test the *in vivo* bioluminescence, we infected mice using low (5×10^4 CFU) and high (1×10^5 CFU) inoculums of *gluc*GBS-CC17. The radiance values were significantly ($P = 0.0292$ ventral position, $P = 0.0103$ dorsal position) different and proportional to the infection doses (Fig. 6b–d). As expected, bacterial colonisation in the brain, lungs, and liver was significantly ($P < 0.0001$) higher in pups infected with the higher dose (Fig. 6d). These results indicate that this bioluminescent GBS strain is suitable for studying systemic infections. However, the *gluc*GBS-CC17

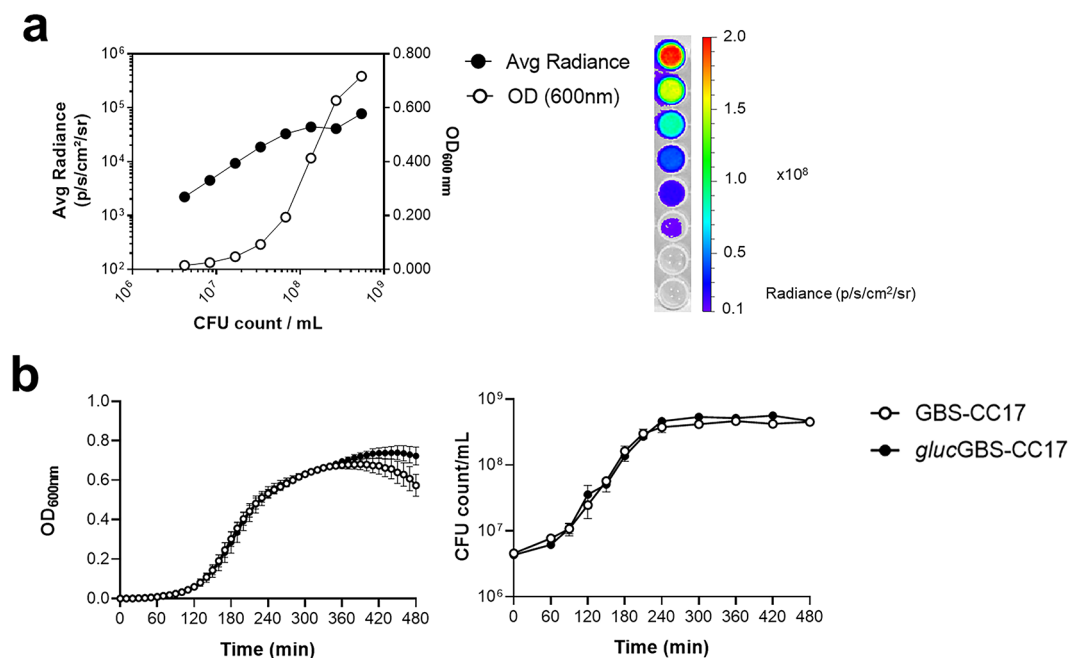


Fig. 5. *In vitro* bioluminescence of the *gluc*GBS-CC17 with a chromosomally integrated luciferase. The red-shifted firefly luciferase gene was integrated into the genome of the WT strain to give the *gluc*GBS-CC17 strain. (a) Correlation of bioluminescent signal, optical density (OD) and CFU count. The *gluc*GBS-CC17 strain was grown until the mid-log phase and serially diluted in a 96-well plate. Left: Bioluminescence (Radiance, 0.5 mg/mL of Luciferin) and OD quantification on a plate reader (Synergy 2), and corresponding CFU counts. Field symbols represent radiance, whereas open symbols represent OD. Right: Bioluminescence signal imaged using IVIS Lumina LT. The detection of bioluminescent signals by the system resulted in the generation of signal maps overlaid on a grey-scale photograph of the 96-well plate. Bioluminescent signal plotted as radiance (photons/seconds/cm²/sr). Results represent data from 3 independent experiments. (b) Growth curves of WT and *gluc*GBS-CC17 strains. Optical density (left) measured every 10 min for 7 h on Synergy 2. GBS numbers counted every 30–60 min (right). Mean values $\pm$ SEM pooled from 3 independent experiments. Comparisons by two-way ANOVA.

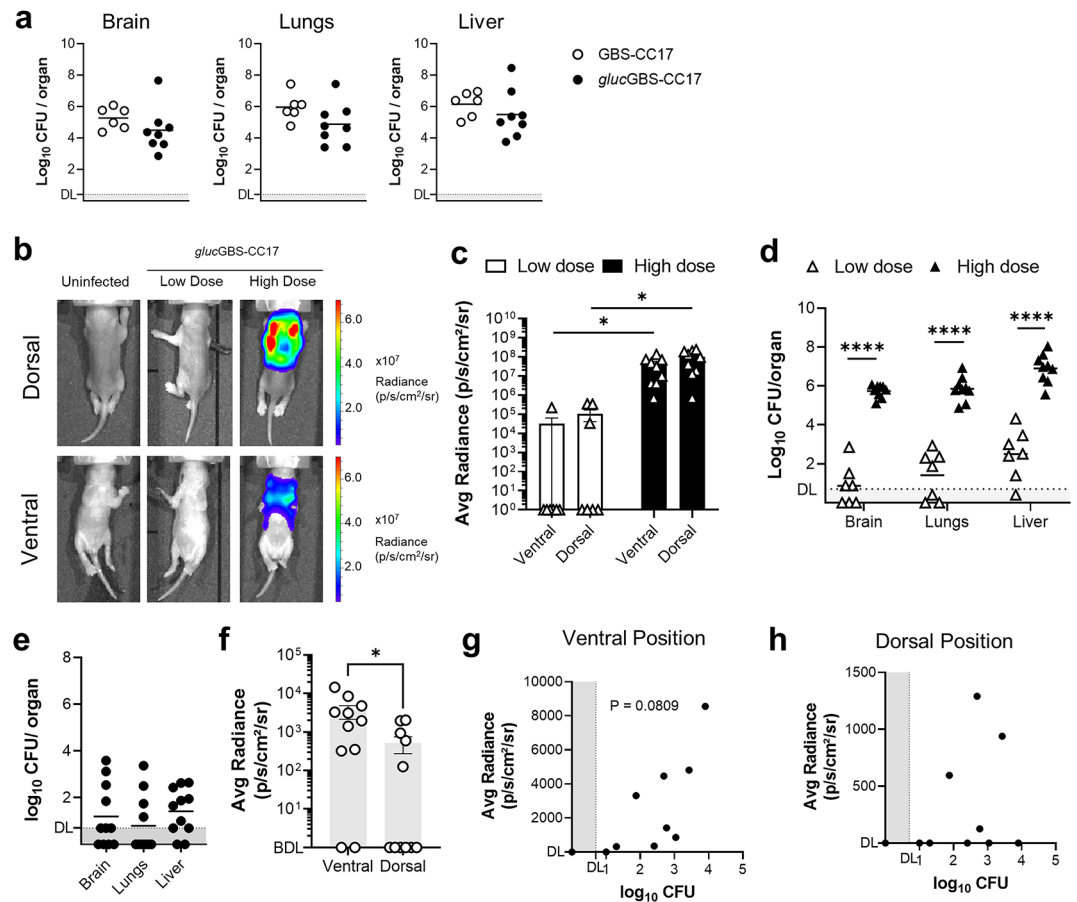


Fig. 6. Infectivity and *in vivo* bioluminescence of *glucGBS-CC17*. **(a)** Neonatal mice were inoculated intraperitoneally with 5×10^4 CFU of *glucGBS-CC17*. Bacterial load in the indicated organs at 18 hpi. Data as mean pooled from 2 independent experiments. Each symbol represents one pup ($n=6$ WT; $n=8$ *glucGBS-CC17*). DL, detection limit = 0.7. **(b–d)** Neonatal mice were inoculated intraperitoneally with 5×10^4 (low dose), 1×10^5 (high dose) CFU of *glucGBS-CC17* or left uninfected. **(b)** Representative images of pups through the superimposition of the bioluminescence signal and a grey-scale photograph in the indicated positions. **(c)** The regions of interest (ROIs) were used to quantify the bioluminescence signal, expressed as average radiance (photons/s/cm²/steradian). Bars represent the mean $\pm$ SEM. Each symbol represents one animal ($n=6$ Uninfected; $n=7$ Low dose; $n=9$ High dose). **(d)** Bacterial load in the indicated organs and infection doses. Data as mean, each symbol represents one animal ($n=7$ Low dose; $n=9$ High dose). Statistical analysis using unpaired Student's t-test. * $P < 0.05$, **** $P < 0.0001$. DL, detection limit = 0.4. **(e–h)** Pregnant mice were intravaginally inoculated with 5×10^4 CFU of *glucGBS-CC17* during gestational. Three-day-old pups were analysed. **(e)** Bacterial load in the indicated organs. Data shown as mean pooled from 2 independent experiments. Each symbol represents one animal ($n=11$). DL, detection limit = 0.7. **(f–h)** The ROIs were used to quantify the bioluminescence signal from the whole mouse, expressed as average radiance (photons/s/cm²/steradian). **(f)** Whole body average radiance in ventral and dorsal position. Mean $\pm$ SEM, each symbol represents one individual ($n=11$). BDL, below detection limit. **(g–h)** Correlation between colonisation and the average radiance in the infected pups in the **(g)** ventral and **(h)** dorsal positions. Each symbol represents one individual ($n=10$). BDL, below detection limit. * $P < 0.05$.

strain contains a single copy of the luciferase gene, resulting in a lower signal when compared to the *flucGBS-CC17* strain containing the pLZ12Km2-P23R: TA:*fluc* replicative vector (Figs. 4e and 6c).

The stable chromosomal integration of *fluc* allows testing the *glucGBS-CC17* strain in our intra-vaginal (i.vag.) infection model of mother-to-pup transmission during delivery. At P3, the brain and liver of almost all pups were colonised, while only 4 out of 11 pups showed colonisation in the lungs (Fig. 6e). Despite the relatively low bacterial burden, significant luminescence signals were detectable in the pups (Fig. 6f). To further evaluate whether the radiance values could be correlated with bacterial brain colonisation a square region (ROIs) was drawn in the anatomical area of the brain and the bioluminescent signal obtained was plotted against the respective bacterial levels. No positive correlation was found regardless of ventral or dorsal mouse position (Supplementary Fig. 3a and b). However, close to statistical significance ($P = 0.0809$) was obtained when plotting the whole-body radiance obtained in the ventral position with the sum of bacterial colonisation in the lungs, liver, and brain (Fig. 6g), sustaining the validity of this strain to study moderate and severe GBS disease. Finally,

aiming to improve sensitivity, we also tested a synthetic analogue of D-luciferin, cyclic alkylamino luciferin (CycLuc1), reported to produce a higher signal than D-luciferin^{31,32}. Pups were i.p. infected with *gluc*GBS-CC17, and at 18 hpi different concentrations of CycLuc1 and time points were tested (Supplementary Fig. 4). However, no differences in the bioluminescent signal were observed compared to standard D-luciferin, regardless of concentration or time post-injection (Supplementary Fig. 4a and b). Altogether, our results show a bioluminescent GBS strain constitutively expressing the luciferase gene stably integrated in the chromosome suitable for studying GBS invasive disease.

Discussion

Group B *Streptococcus* neonatal infections remain a major clinical problem. One limitation in studying GBS neonatal infection is that colonisation assessment entails animal euthanasia and, therefore, no longitudinal studies can be performed to study the kinetics of the infection, the disease progression, and the long-term sequelae. Here, we developed three bioluminescent GBS strains: one with the luciferase gene contained in the *lux* operon on a replicative vector, another with the *ffluc* gene also encoded on a replicative vector, and a third with the *ffluc* gene inserted into the GBS genome. Each bioluminescent strains are advantageous for specific applications: *in vitro* (*lux*GBS-CC17), *in vivo* for short term infections (*ffluc*GBS-CC17), or *in vivo* for complex infection models (*gluc*GBS-CC17). The choice between strains is based on the sensitivity (single or multi-copy) and the stability (vector-based or chromosomal integration) of the luciferase gene.

Our results suggest that the *lux*GBS-CC17 containing the *luxABCDE* operon on a replicative vector is appropriate for *in vitro* studies and not for invasive GBS infection models. *In vitro* luminescence is proportional to bacterial burden, consistent with results in other bioluminescent bacterial species¹³. The replicative vector is stable even in the absence of the antibiotic selective pressure, and no difference in growth with the WT strain is observed under standard conditions in TH broth. The main advantage is that this system does not require an exogenous substrate^{13,26}. However, no luminescence signal was detected in infected animals, even when using a high infection dose. This unexpected result might be due to the bacteria spreading throughout the body, resulting in a dispersed, undetectable signal, or being located deeper within organs. The short wavelength (490 nm) of the *lux* operon limits tissue penetration^{33,34}, especially due to absorption by hemoglobin^{33,35,36}. Miller and colleagues³⁰ showed that tissue penetration for a *Staphylococcus aureus* strain transformed with the same *lux* operon is limited up to 2 cm. Although 3-day-old C57BL/6 mice are thinner than 2 cm, their organ density is high due to their small size³⁷, which could contribute to the limited detection. While it is unlikely that the P32 promoter is inactive during infection, and thus resulting in no bioluminescence signal, the optimisation of promoters should be explored in the future.

The second *ffluc*GBS-CC17 strain used contains the red-shifted firefly luciferase (*ffluc*) on a replicative vector, a *luc* reporter derivative validated for multiple streptococcal species¹⁵ overcoming the main limit of the *luxABCDE* system. The *ffluc*GBS-CC17 strain is therefore adapted to follow invasive infection in specific models. The *ffluc* has a longer wavelength (610 nm) than *lux* (490 nm)^{18,33}, and improved bioluminescent signal and tissue penetration to detect bacteria in deeper tissues¹⁸. The replicative vector was previously used in GBS for *in vitro* bioluminescence analysis¹⁵. Here, we confirmed the *in vitro* stability of the replicative vector and validated the *in vivo* stability in GBS, as previously shown in *Streptococcus pyogenes*²³. We also show that the pLZ12Km2-P23R: TA:*ffluc* vector did not impact on *in vitro* growth under standard conditions in TH broth and does not alter GBS virulence. The *ffluc*GBS-CC17 strain demonstrated robust bioluminescence, allowing visualisation and quantification of bacterial colonisation in the brain, lungs, and liver. The two infectious doses tested are the minimum for detecting luminescent signals associated with low and high bacterial loads, respectively. The lower dose of 5×10^4 CFU is the minimum needed to visualize colonisation through bioluminescence, while doubling the dose to 10^5 CFU produced higher, more uniform colonisation across all animals. Even when using a low inoculum to ensure increased pups' survival, the BLI signal was proportional to the CFU colonisation in the organs, in both ventral and dorsal mice positions. Both radiance and CFU values were statistically significant between the lower and higher infection dose, confirming that the *ffluc*GBS-CC17 strain provides a reliable and sensitive method for tracking bacterial infection in live animals, even under non-selective antibiotic conditions.

The main limit of the *ffluc*GBS-CC17 strain emerged when tested in the vertical transmission model. Despite initial success in establishing infection, we observed plasmid loss over time, as evidenced by a lack of bioluminescent signal in later stages of infection. In this model, pregnant females are colonised in the vaginal mucosa at the gestational days 16 and 17, and allowed to deliver spontaneously¹². During birth, the pups cross the vaginal canal and may inhale or ingest contaminated fluids, becoming infected¹². As the pups are only studied after birth, the *ffluc*-containing plasmid should be stable for at least 3 to 7 days to detect luminescence during postnatal life. However, the stabilisation of the vector by the encoded toxin-antitoxin system is insufficient *in vivo* without an additional antibiotic selective pressure. Adding antibiotics to drinking water could stabilise the vector but would alter the microbiota. This plasmid instability under *in vivo* conditions suggests that while the *ffluc*GBS-CC17 strain is suitable for short-term studies and high-dose infections, it may not be ideal for long-term studies or infections acquired through natural routes.

The integration of the *ffluc* gene into the GBS genome resolves the stability issue. The *gluc*GBS-CC17 strain has a single copy of *ffluc* constitutively transcribed and integrated in a neutral intergenic region at the 3' ends of two divergent transcribed genes. The genome of the *gluc*GBS-CC17 strain is identical to the genome of the WT parental strain, except for the integrated *ffluc* which does not affect bacterial growth and virulence. The stable integration ensures the maintenance of the reporter gene over extended periods in complex infection model of infection. When tested in the haematogenous model, *gluc*GBS-CC17 allowed to visualise and quantify bacterial colonisation of key organs such as the brain, lungs, and liver. The bioluminescent signal was proportional to CFU counts, providing a reliable measure of bacterial load. Importantly, the use of the *gluc*GBS-CC17 strain facilitated the detection of bioluminescence in the pups' whole-body, even at lower infection doses.

The main limitation of the *gluc*GBS-CC17 strain is its weaker luminescence signals, linked to the presence of a single copy of the *ffluc* gene. This is especially limiting for brain colonisation in the vertical mouse model of infection. The low level of colonisation and the properties of the skull and blood-brain barrier (BBB), which impact and limit the access of D-luciferin to the parenchyma³¹, severely impacts the detection of the *gluc*GBS-CC17 strain. The luciferase substrate cyclic alkylamino luciferin (CycLuc1) has higher photon emission than D-luciferin at lower concentrations^{31,32} and increased BBB diffusion as it has increased lipophilicity^{31,38}. Previous findings showed that CycLuc1 allows the detection of bioluminescence signals in cardiovascular brain regions, including the circumventricular subfornical organ (SFO) and paraventricular nucleus of the hypothalamus (PVN)³¹. Nevertheless, in our study CycLuc1 did not show a stronger luminescent signal regardless of the administered concentration or acquisition waiting time after injection. A possible explanation for this may be the lower bacterial load and the fact that it is widespread in the pup's body, reducing the signal per area. While the introduction of CycLuc1 as a luciferase substrate initially showed promise for enhanced CNS imaging, our results highlight the complexities and limitations in achieving consistent bioluminescent signals in neonatal mice, necessitating further exploration of alternative strategies. Optimisation of the *ffluc* gene and promoter, or the use of alternative luminescence genes, could improve bioluminescent sensitivity to obtain a universal bioluminescent GBS strain.

In conclusion, we generated three GBS bioluminescent strains for pathogenic analysis, among which two containing the red-shifted firefly luciferase (*ffluc*) suitable to follow short-term and long-term invasive infections. This strain enables detailed tracking of bacterial colonisation and disease progression *in vivo*, providing better understanding of GBS pathophysiology. Moreover, it will pave the way for discovering novel approaches against neonatal GBS infections, while also allowing animal reduction and experiment refinement, implementing the ethical animal experiments 3R's.

Methods

Bacterial strains, plasmids and growth conditions

Enterococcus faecalis containing the plasmid pSL101P32 carrying the *luxABCDE* cassette (Table 1) was kindly provided by Dzung B. Diep²⁷. The pLZ12Km2-P23R: TA: *ffluc*RT plasmid was a gift from Thomas Proft (Addgene, #88901)¹⁸. *E. faecalis* and *Escherichia coli* DH5α (Table 1) were grown in Brain Heart Infusion (BHI) broth or agar (Difco Laboratories) and Luria Bertani (LB) broth or agar (GRiSP, Lda), respectively, at 37 °C. The wildtype GBS strain BM110, a CC-17 clinical isolate, was cultured at 37 °C in Todd-Hewitt (TH) broth or agar (Difco Laboratories). For strains containing vectors (Table 1), antibiotics were added as appropriate at the following concentrations: spectinomycin (EMD Millipore) at 500 µg/mL (*E. faecalis*), 70 µg/mL (*E. coli*) or 200 µg/mL (GBS); kanamycin (Kan) (Sigma Aldrich) at 50 µg/mL (*E. coli*) or 200 µg/mL (GBS); erythromycin (Erm) (Sigma Aldrich) at 150 µg/mL (*E. coli*) or 5 µg/mL (GBS).

Bacterial growth and *in vitro* luminescence

*Lux*GBS-CC17 was grown overnight (ON) and 1:100 subcultured in non-vented culture flasks in 30 mL of TH with 5 µg/mL erythromycin at 37 °C, for 7 h. The optical density (OD) at 600 nm (OD_{600 nm}) was measured every 30–60 min using a spectrophotometer (Biochrom Libra S6+). The culture was plated at the same timepoints on TH agar with 5 µg/mL erythromycin and incubated at 37 °C ON for CFU count. All the other strains were 1:100 subcultured in TH with the respective antibiotic, in transparent 96-well microplates. The cultures were maintained at 37 °C and measurements were performed every 10 min for 7 h, on Synergy 2 Plate reader (OD_{600 nm}).

For the luminescent signal, the strains were grown in non-vented culture flasks containing 30 mL of TH. An aliquot of the culture was taken at indicated timepoints and read on Synergy 2 Plate reader, using a white 96-well flat bottom microplate (settings at 37 °C, 3s shaking before reading and luminescence read with 150 of gain). In all experiments technical duplicates and biological triplicates were performed.

GBS transformation

Plasmids were extracted using commercial kits according to manufacturer's instructions. The pSL101P32 or pLZ12Km2-P23R: TA: *ffluc*RT vectors were introduced in GBS by electroporation. Electrocompetent bacteria were prepared starting from an ON culture in TH at 37 °C used to inoculate (1:10) fresh medium. The culture was grown at 37 °C until exponential growth phase (OD 600 nm around 0.9). Then, one volume of a 1 M sucrose solution and 0.05% of glycine were added, and the culture was incubated for 1 h at 37 °C with mild shaking. The

Bacterial strain	Plasmid	Selective antibiotic	Nomenclature (if applicable)
<i>Enterococcus faecalis</i>	PSL101P32	Spectinomycin	
<i>Escherichia coli</i> DH5α	PSL101P32	Spectinomycin	
<i>Escherichia coli</i>	pLZ12Km2-P23R: TA: <i>ffluc</i> RT	Kanamycin	
<i>Escherichia coli</i> XL-1 blue	pG-oxi-luc-ldh	Erythromycin	-
Group B <i>Streptococcus</i> BM110		-	GBS-CC17
Group B <i>Streptococcus</i> BM110	PSL101P32	Spectinomycin	<i>lux</i> GBS-CC17
Group B <i>Streptococcus</i> BM110	pLZ12Km2-P23R: TA: <i>ffluc</i> RT	Kanamycin	<i>ffluc</i> GBS-CC17

Table 1. Bacterial strains and plasmids nomenclature.

culture was centrifuged at 2,000 g for 10 min at 4 °C, washed twice with electroporation buffer (7 mM sodium phosphate, 500 mM saccharose, 1 mM magnesium chloride, pH 7.4), and re-suspended in electroporation buffer with 15% glycerol. The culture was stored at -80 °C until electroporation was performed. For electroporation, 6 µL of plasmid was added to 100 µL of competent bacteria, and the mixture was electroporated at 200 Ω, 2500 V / cm electric field, 25 µF. Thereafter, 900 µL of TH was immediately added and the solution was incubated for 1 h 30 min, at 37 °C. The bacteria were then plated on TH plates containing spectinomycin or kanamycin at 37 °C. Isolated GBS spectinomycin-resistant transformant containing the vector pSL101P32 was referred to as *lux*GBS-C17 and the kanamycin-resistant transformant containing the vector pLZ12Km2-P23R: TA: *ffluc*RT as *ffluc*GBS-CC17.

Polymerase chain reaction (PCR)

Colony PCR was performed using the following primers (Table 2) to target the *lux*ABCDE operon: *LuxA* sense (5' GCCTACCGATGATATTAAGTTG 3') and *LuxB* anti-sense, *LuxD* sense and *LuxE* antisense; and the following primers to target *Ffluc* gene: *Ffluc* sense and antisense. The PCR reaction comprising NZYTaq 2x Green Master Mix (NZYTech) was run under the following conditions: an initial denaturation step at 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 25 s, followed by a final extension step at 72 °C for 10 min. The PCR products were run in a 1% agarose gel, containing Green Safe Premium (NZYTech). Gel images were acquired in a gel Imaging system (Gel-Doc XR+, Bio-Rad) using Image Lab (V6.0.1, Bio-Rad) software.

Plasmid stability

GBS strains containing the luminescent plasmid (pSL101P32 or pLZ12Km2-P23R: TA: *ffluc*RT) were grown in TH ON at 37 °C, with or without antibiotics and subsequently subcultured 1:1000 in fresh TH, with or without antibiotics, and incubated at 37 °C for 24 h. This step was repeated every 24 h for 8–10 days. Subcultures were serially diluted and plated on TH agar medium, with or without selection, every day and incubated ON at 37 °C. Plasmid stability was assessed given the difference in the CFU count in the presence and absence of antibiotic selection.

Construction of pG-oxi-luc-ldh

To construct the *gluc*GBS-CC17 strain with a chromosomal *ffluc* gene, we constructed the pG-oxi-luc-ldh integrative vector (Supplementary Fig. 5). First, the the *ffluc* gene was amplified from the pLZ12Km2-P23R: TA:*ffluc* vector using high-fidelity DNA polymerase (Phusion, New England Biolabs) and primers 1690 and 1691 (Table 2). Two contiguous 500 bp region of the BM110 genome were similarly amplified using primers 1688 and 1689 and 1692 and 1695 (Table 2). The three amplicons contain overlapping sequences and were purified (QIAquick PCR purification, Qiagen) and mixed for a final PCR with the external primers 1688 and 1693 (Table 2). The final amplicon was gel purified (QIAquick gel purification, Qiagen) and used for Gibson assembly into the pG1 thermosensitive vector previously amplified with the 879 and 880 primers (Table 2).

Integration of *ffluc* into the GBS CC17 genome.
The pG-oxi-luc-ldh vector was introduced in *E. coli* XL-1 blue (Stratagene) and selected with erythromycin. After purification and sanger sequencing (Eurofins Genomics), the pG-oxi-luc-ldh was introduced in BM110 by electroporation with erythromycin selection at 30°C, the permissive temperature for vector replication. Chromosomal integration at the targeted locus was selected by growing isolated transformants at 37°C on THY agar in the presence of erythromycin. Following a second round of isolation on THY with erythromycin, a single colony was inoculated into 10 ml of THY without antibiotics, grown at 30°C, and sub-cultured four times under the same conditions. Diluted cultures ($d = 10^{-5}$) were then spread on THY agar, grown overnight at 37°C, and single colonies ($n = 48$) were inoculated into 150 µl of THY in microwell plates. After 4 hours of incubation at

Primer ID	Oligonucleotide sequence
<i>LuxA</i> (sense)	GCCTACCGATGATATTAAGTTG
<i>LuxB</i> (anti-sense)	ACCACACTAATGGATCGCTCG
<i>LuxD</i> (sense)	TGCGCGGATAGCTTATGCAA
<i>LuxE</i> (anti-sense)	ACCACACTAATGGATCGCTCG
<i>Ffluc</i> (sense)	AAGGTCCTGCCCATTTTACC
<i>Ffluc</i> (anti-sense)	GCGGCAAATGACTGGTTACG
879	GAATTCGTAATCATGTCATAGCTGT
880	GAATTCGTAATCATGTCATAGCTGT
1688	CCTGCAGGTCGACTCTAGAGGATCCGACAATTTGGGATAATTCTCGTG
1689	GAGCTTAAGAATTGCCGCTCTAGATCATCCTTTAACAAGGTCAAAAATG
1690	GTTAAGCGAAATGCTTAGCCTTTTACGAAAAGCCCTGACAACCCCTTGTTTC
1691	CATTTTTGACCTTGTTAAAGGATGATCTAGAGCGCAATTCTTAAGCTCG
1692	CGAGCTTAAGAATTGCCGCTCTAGATCATCCTTTAACAAGGTCAAAAATG
1694	GTTGATATGGTGATCTTAGCTGTTG
1695	GTTATTGGTTCAGGTACTTCACCTTG

Table 2. Oligonucleotide sequences of the utilised primers.

37°C, replicas were made using a 96-pin microplate replicator (Boekel) on THY with and without erythromycin to differentiate colonies that retained or lost the vector. Erythromycin-sensitive colonies were then tested by PCR with primers 1694 and 1695 (Table 2) to determine whether they had restored a wild-type genomic sequence (indicating loss of the integrated *lux* gene) or whether the *lux* gene had precisely integrated at the targeted genomic position. The outcome (WT back or *lux* integration) depends on the side of homologous recombination (5' or 3') between the flanking genomic regions cloned around the *lux* gene. Colonies with the integrated *lux* gene were isolated and their genomic DNA was purified (DNeasy Blood and Tissue kit, Qiagen) and sequenced (Illumina, Novogen). High-quality reads were mapped against the BM110 genome (362x genome coverage) and analyzed for SNP, indel, and genomic rearrangement (Genious Prime, Biomatters Ltd). The selected *gluc*GBS-CC17 strain, with *lux* inserted at position 1,000,921 in the BM110 genome (NCBI RefSeq NZ_LT714196) and without any secondary mutation, was then stored in 20% glycerol at -80 °C.

Adhesion and invasion assays

The human colonic epithelial cell line Caco-2¹², derived from a human colorectal adenocarcinoma (ATCC), was cultured at 37 °C in a 5% CO₂ atmosphere in DMEM high glucose with GlutaMax (HyClone) supplemented with 10% heat-inactivated FBS, 1% sodium pyruvate (Gibco) and 1% penicillin/streptomycin (6.0 µg/ mL / 10.0 µg/ mL, Biowest). The cell line was grown until cells reached 80% confluence. For infection assays, cells were seeded on 12 well-standard plates at 3 × 10⁵ cells/well for 4 days at 37 °C under a 5% CO₂ atmosphere. Briefly, cells were washed thrice and infected with GBS in media without FBS or antibiotics at a multiplicity of infection (MOI) of 30. The number of adhered cells was counted to adjust the bacterial numbers. Infection was allowed for 3 h, followed by five washes to remove nonadherent bacteria. Subsequently, the cells were lysed with TrypLE Express (Gibco) containing 0.2% Triton X-100 for 10 min at 37 °C. The bacterial adhesion was quantified by subtracting the intracellular bacteria from the total cell-associated (intracellular plus surface-adherent bacteria).

For invasion assays, 3-hour-infected cell monolayers were further incubated with cellular culture media containing 100 µg/mL gentamycin (Lonza) for 1.5 h to kill the extracellular bacteria, before cell lysis. Appropriate dilutions were plated on TH agar plates, and CFU were counted. The percentage of invasion was calculated relative to the bacterial inoculum.

Animal handling and ethics statement

C57BL/6 mice were purchased from Charles River Laboratories and housed at the Instituto de Investigação e Inovação em Saúde (i3S) animal facility under a 12 h light/dark cycle and *ad libitum* access to food and water. All animal procedures were approved by the Ethics Committee of the i3S, and the Portuguese national authority the Direção Geral de Alimentação e Veterinária (DGAV). All animal experiments were performed in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and Directive 2010/63/EU and Portuguese rules (DL 113/2013). All animal experiments were planned to minimise mice suffering and number, and always handled by licensed investigators. Additionally, all experiments were performed in accordance with relevant guidelines and regulations, including the ARRIVE guidelines for animal experiments.

In vivo mouse infection models

Overnight cultures of the different GBS strains were subcultured 1:100 on TH broth and grown until mid-log phase (OD_{600nm} around 1.000) at 37 °C. Bacteria were washed twice with PBS, and the OD_{600nm} was adjusted to $\approx 0.600 \pm 0.005$, corresponding approximately to 2.0×10^8 CFU / mL. Inocula were prepared by further dilution from this bacterial suspension.

Two-day-old C57BL/6 mice were intraperitoneally (i.p.) infected with the indicated CFU of WT GBS or luciferase-expressing strains in 40 µL of total volume. Survival curves were determined over a 36 h experimental period. To assess bacterial colonisation, mice were euthanised by decapitation following isoflurane anaesthesia. The brain, lungs and liver were aseptically removed 18 h post-infection, homogenised, serially diluted in PBS, and plated on TH agar medium with or without antibiotic selection for CFU counts.

To vertically infect neonatal mice, we adapted our previously described mouse model of infection¹². Pregnant C57BL/6 mice were intravaginally inoculated, using a micropipette, with 40 µL of GBS suspension containing 3×10^4 CFU, at the gestation (G) days 16 and 17. The presence of a vaginal plug was considered the day 1 of gestation. Females were allowed to deliver spontaneously, and infected pups were kept with their mothers throughout the experimental time. Organs were collected and plated as previously described.

Bioluminescence imaging

For the *in vitro* correlation between the bioluminescence signal of luciferase-expressing GBS strains, the CFU and the OD, bacteria were grown as described above and serially diluted 1:2 in white opaque flat clear bottom or transparent flat bottom 96-well plates. The luminescence and the absorbance at 600 nm were quantified on Synergy 2 (BioTek). For *fluc*GBS BM110, 1:1 D-Luciferin potassium salt (0.5 mg / mL, Perkin Elmer) was added to the wells 5 min before luminescence quantification. Bioluminescence imaging was assessed on the IVIS Lumina LT system (Perkin Elmer). According to the strain, bacterial dilutions were plated on TH agar, with or without selection, for CFU enumeration.

For *in vivo* studies, C57BL/6 mice infected with the different bioluminescent strains were anaesthetised with 2.5% isoflurane (O₂ flow of 1.0 L/minutes). In the case of mice infected with *fluc*GBS, 20 µL of the indicated concentrations of D-Luciferin or CycLuc1 sodium salt (AOBIOUS, AOB6377) were administered s.c. The substrate was allowed to be distributed in the body of the anaesthetised animal for 5 min, unless stated otherwise, before image acquisition. Pups were allowed to recover from the anaesthesia before returning to the respective

cage. Bioluminescence imaging was acquired on the IVIS Lumina LT system (Perkin Elmer) or Newton 7.0 (Vilber).

Statistical analysis

All data were analysed and graphically represented using GraphPad Prism software (v.9.0.1, GraphPad Software Inc. CA). Means and standard errors of the means (SEM) were calculated and correspond to the indicated independent experiments. Student's t-test was used to analyse differences between two experimental groups. The differences between multiple groups were performed by one-way or two-way ANOVA, whenever appropriate, with Tukey's Multiple Comparison Test. The normality of the data was verified by the Shapiro-Wilk normality test. Survival curves were plotted using Kaplan-Meier plot, and statistical significance was determined using the log-rank (Mantel-Cox) test. CFU data were $\log_{10}$ transformed. Differences were considered significant for $P \leq 0.05$ and represented by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. The number of samples and independent experiments is expressed in the respective legend.

Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files). Further inquiries can be directed to the corresponding author, Elva Bonifácio Andrade.

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References

- Thigpen, M. C. et al. Bacterial meningitis in the United States, 1998–2007. *N Engl. J. Med.* **364**, 2016–2025 (2011).
- Tavares, T., Pinho, L. & Bonifácio Andrade, E. Group B Streptococcal neonatal meningitis. *Clin. Microbiol. Rev.* **35**, e00079–e00021 (2022).
- Wilson, C. B., Nizet, V., Maldonado, Y., Remington, J. S. & Klein, J. O. *Remington and Klein's Infectious Diseases of the Fetus and Newborn Infant* (Elsevier Health Sciences, 2015).
- Nanduri, S. A. et al. Epidemiology of Invasive Early-Onset and late-Onset Group B Streptococcal Disease in the United States, 2006 to 2015: Multistate Laboratory and Population-based surveillance. *JAMA Pediatr.* **173**, 224–233 (2019).
- Jamroz, D. et al. Increasing incidence of group B streptococcus neonatal infections in the Netherlands is associated with clonal expansion of CC17 and CC23. *Sci. Rep.* **10**, 9539 (2020).
- Plainvert, C. et al. Multidrug-resistant Hypervirulent Group B Streptococcus in neonatal invasive infections, France, 2007–2019. *Emerg. Infect. Dis.* **26**, 2721–2724 (2020).
- Verani, J. R., McGee, L. & Schrag, S. J. Division of Bacterial diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention (CDC). Prevention of perinatal group B streptococcal disease—revised guidelines from CDC, 2010. *MMWR Recomm. Rep. Morb. Mortal. Wkly. Rep. Recomm. Rep.* **59**, 1–36 (2010).
- Tazi, A. et al. The surface protein HvgA mediates group B streptococcus hypervirulence and meningeal tropism in neonates. *J. Exp. Med.* **207**, 2313–2322 (2010).
- Ferrieri, P., Burke, B. & Nelson, J. Production of bacteremia and meningitis in infant rats with group B streptococcal serotypes. *Infect. Immun.* **27**, 1023–1032 (1980).
- Mancuso, G. et al. Beneficial effects of interleukin-6 in neonatal mouse models of group B streptococcal disease. *Infect. Immun.* **62**, 4997–5002 (1994).
- Barichello, T. et al. Evaluation of the brain-derived neurotrophic factor, nerve growth factor and memory in adult rats survivors of the neonatal meningitis by *Streptococcus agalactiae*. *Brain Res. Bull.* **92**, 56–59 (2013).
- Andrade, E. B. et al. A mouse model reproducing the pathophysiology of neonatal group B streptococcal infection. *Nat. Commun.* **9**, 3138 (2018).
- Avci, P. et al. In-vivo monitoring of infectious diseases in living animals using bioluminescence imaging. *Virulence*. **9**, 28–63 (2018).
- Russel, W. M. S., Burch, R. L. & Hume, C. W. *The Principles of Humane Experimental Technique*, Vol. 238 (Methuen, London, 1992).
- Loh, J. M. S. & Proft, T. Stabilized plasmid-based system for bioluminescent labeling of multiple streptococcal species. *Biotechnol. Lett.* **38**, 139–143 (2016).
- Loh, J. M. S. & Proft, T. Comparison of firefly luciferase and NanoLuc luciferase for biophotonic labeling of group A Streptococcus. *Biotechnol. Lett.* **36**, 829–834 (2014).
- Sheel, M., Pandey, M., Good, M. F. & Batzloff, M. R. Correlation between Bioluminescence and bacterial burden in passively protected mice challenged with a recombinant bioluminescent M49 Group A Streptococcus strain. *Clin. Vaccine Immunol.* **17**, 127–133 (2010).
- Loh, J. M. S. & Proft, T. Toxin–antitoxin-stabilized reporter plasmids for biophotonic imaging of Group A streptococcus. *Appl. Microbiol. Biotechnol.* **97**, 9737–9745 (2013).
- Marques, S. M., Esteves Da Silva, J. C. G. Firefly bioluminescence: a mechanistic approach of luciferase catalyzed reactions. *IUBMB Life*. **61**, 6–17 (2009).
- Loimaranta, V., Tenovu, J., Koivisto, L. & Karp, M. Generation of bioluminescent Streptococcus mutans and its usage in Rapid Analysis of the efficacy of Antimicrobial compounds. *Antimicrob. Agents Chemother.* **42**, 1906–1910 (1998).
- Short, K. R. et al. Using bioluminescent imaging to Investigate Synergism between Streptococcus pneumoniae and Influenza A Virus in Infant mice. *JoVE J. Vis. Exp.* **e2357** <https://doi.org/10.3791/2357> (2011).
- Lamb, L. E. et al. Modelling invasive group A streptococcal disease using bioluminescence. *BMC Microbiol.* **18**, 60 (2018).
- Alam, F. M., Bateman, C., Turner, C. E., Wiles, S. & Sriskandan, S. Non-invasive monitoring of Streptococcus pyogenes Vaccine Efficacy using Biophotonic Imaging. *PLOS ONE*. **8**, e82123 (2013).
- Bellais, S. et al. Capsular switching in group B Streptococcus CC17 hypervirulent clone: a future challenge for polysaccharide vaccine development. *J. Infect. Dis.* **206**, 1745–1752 (2012).
- Landwehr-Kenzel, S. & Henneke, P. Interaction of Streptococcus agalactiae and Cellular Innate immunity in colonization and disease. *Front. Immunol.* **5**, 519 (2014).
- Baldwin, T. O. Firefly luciferase: the structure is known, but the mystery remains. *Structure*. **4**, 223–228 (1996).
- La Rosa, S. L., Diep, D. B., Nes, I. F. & Brede, D. A. Construction and application of a luxABCDE reporter system for real-time monitoring of Enterococcus faecalis gene expression and growth. *Appl. Environ. Microbiol.* **78**, 7003–7011 (2012).
- Gutekunst, H., Eikmanns, B. J. & Reinscheid, D. J. The Novel fibrinogen-binding protein FbsB promotes Streptococcus agalactiae Invasion into epithelial cells. *Infect. Immun.* **72**, 3495–3504 (2004).

29. Que, Y. A., Haefliger, J. A., Francioli, P. & Moreillon, P. Expression of *Staphylococcus aureus* clumping factor A in *Lactococcus lactis* subsp. *cremoris* using a new shuttle vector. *Infect. Immun.* **68**, 3516–3522 (2000).
30. Miller, R. J. et al. Development of a *Staphylococcus aureus* reporter strain with click beetle red luciferase for enhanced in vivo imaging of experimental bacteremia and mixed infections. *Sci. Rep.* **9**, 16663 (2019).
31. Simonyan, H., Hurr, C. & Young, C. N. A synthetic luciferin improves in vivo bioluminescence imaging of gene expression in cardiovascular brain regions. *Physiol. Genomics*. **48**, 762–770 (2016).
32. Evans, M. S. et al. A synthetic luciferin improves bioluminescence imaging in live mice. *Nat. Methods*. **11**, 393–395 (2014).
33. Archer, N. K., Francis, K. P. & Miller, L. S. Optical imaging. in *Imaging Infections: From Bench to Bedside* 43–76 Springer International Publishing, doi: (2017). https://doi.org/10.1007/978-3-319-54592-9_3
34. Close, D. M., Xu, T., Sayler, G. S. & Ripp, S. In vivo bioluminescent imaging (BLI): noninvasive visualization and interrogation of biological processes in living animals. *Sensors*. **11**, 180–206 (2011).
35. Zhao, H. et al. Emission Spectra of bioluminescent reporters and interaction with mammalian tissue determine the sensitivity of detection in vivo. *J. Biomed. Opt.* **10**, 41210 (2005).
36. Hutchens, M. & Luker, G. D. Applications of bioluminescence imaging to the study of infectious diseases. *Cell. Microbiol.* **9**, 2315–2322 (2007).
37. Petiet, A. E. et al. High-resolution magnetic resonance histology of the embryonic and neonatal mouse: A 4D atlas and morphologic database. *Proc. Natl. Acad. Sci.* **105**, 12331–12336 (2008).
38. Mofford, D. M. & Miller, S. C. Luciferins behave like drugs. *ACS Chem. Neurosci.* **6**, 1273–1275 (2015).
39. Fogh, J., Fogh, J. M. & Orfeo, T. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J. Natl. Cancer Inst.* **59**, 221–226 (1977).

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Author contributions

E.B.A. and I.L. conceived and designed the experiments. I.L., R.G., J.S. L.O., A.F. and E.B.A. performed the experiments. I.L., R.G., L.O. and E.B.A. analysed the data. L.O., A.F. and E.B.A. provided reagents and tools. E.B.A. supervised the study. I.L., A.F. and E.B.A. wrote the manuscript. All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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