

Glycans as dual orchestrators of microbiome and immune response dynamics in Inflammatory Bowel Disease

Cláudia Sousa Rodrigues

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2024

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D.ICBAS 2024

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Cláudia Manuela de Sousa Rodrigues



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**GLYCANS AS DUAL ORCHESTRATORS OF MICROBIOME AND
IMMUNE RESPONSE DYNAMICS IN INFLAMMATORY BOWEL
DISEASE**

Tese de Candidatura ao grau de Doutor em
Ciências Biomédicas;
Programa Doutoral da Universidade do Porto
(Instituto de Ciências Biomédicas de Abel
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O trabalho apresentado nesta tese foi apoiado financeiramente por:

Bolsa de doutoramento (2020.08422.BD) fornecida pela da Fundação Portuguesa para a Ciência e Tecnologia (FCT) do Ministério da Ciência, Tecnologia e Educação Superior.

The work presented in this thesis was financially supported by:

PhD Fellowship (2020.08422.BD) provided by the Portuguese Foundation for Science and Technology (FCT) of the Portuguese Ministry of Science, Technology and Higher Education.



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Cláudia Manuela Sousa Rodrigues

Cláudia Manuela de Sousa Rodrigues, MSc

“If you want to go fast go alone, if you want to go far go together.”

- African proverb

“True knowledge exists in knowing that you know nothing”

- Socrates

Acknowledgments

Desde cedo, a paixão pela ciência encontrou um lugar especial no meu coração, e desde então soube qual seria o meu caminho. Estava eu longe de imaginar o quão desafiadora, mas também quão gratificante esta viagem poderia ser. Uma tese de doutoramento nunca é um trabalho individual. Por isso, no final desta etapa, gostaria de agradecer a todas as pessoas que me acompanharam e que de alguma forma contribuíram para que todo este trabalho fosse realizado da melhor forma possível.

Em primeiro lugar, gostaria de agradecer à minha orientadora Salomé Pinho. Numa fase em que decidi sair da minha zona de conforto para realizar o doutoramento num novo grupo de investigação, num instituto e numa cidade diferente, foi a Salomé quem prontamente me abriu as portas do grupo Immunology, Cancer & Glycomedicine. Esta oportunidade permitiu-me continuar a perseguir o meu sonho, apoiando-me nesta minha procura constante por evolução e crescimento. E não imagina o quanto isso foi significativo para mim! Agradeço-lhe por me permitir desenvolver este projeto de doutoramento pela qual me apaixonei. Agradeço-lhe pela confiança, pelo apoio e pela orientação. Obrigada por ter apostado em mim, por todas as vezes que me colocou à prova e pelos incentivos ao longo deste percurso. Tudo isto foi sem dúvida determinante para o meu crescimento profissional e pessoal. Obrigada, também pela compreensão nos momentos necessários. Obrigada por todo o estímulo e transmissão de conhecimentos. Foi um gosto enorme aprender, trabalhar consigo e fazer parte deste grupo maravilhoso. A paixão e o entusiasmo que tem pela ciência é contagiante para quem a rodeia. É para mim um grande orgulho ter feito parte deste grupo e de ter contribuído em parte para o seu crescimento e sucesso. Vou guardar com muito carinho a sua orientação, as discussões científicas e todos os momentos de lazer nos quais sempre promoveu muito bem a união deste grupo. Obrigada por tudo.

Querida Joana, a minha “chefinha”, o meu braço direito. Já nos conhecíamos de outros tempos, mas foi ao longo destes 4 anos de doutoramento que construímos uma ligação que vai além da relação de aluna e supervisora. Juntas enfrentamos dois grandes projetos que nos desafiaram muito, que testou muitas vezes a nossa paciência e que nos fez suspirar vezes sem conta. Mas foram também estes desafios que tornaram estes quatro anos ainda mais especiais dos quais sinto que nos devemos sentir orgulhosas. Foram muitas as horas que passamos juntas, que nos rimos muito e de muitas conversas! Obrigada pelo teu sentido de humor que me fez chorar de rir tantas vezes! A ti agradeço todo o companheirismo, a amizade, os conselhos sábios e as palavras certas e reconfortantes. Obrigada por me teres puxado para cima quando precisei. Agradeço-te

muito por todo o carinho e apoio incondicional, que foram sem dúvida muito importantes para mim. Espero que, sempre que te lembrares de mim, possas sentir orgulho na tua primeira aluna de doutoramento.

I would like to express my gratitude to Professor Harry Sokol, my co-supervisor, for providing the necessary resources to enable me to complete my work. I am also grateful for the support throughout this process.

To all the co-authors of the publications for their important suggestions and contributions, namely, Jean-Frederic Colombel, Francesca Petralia, Joana Torres, Aonghus Lavelle, Ricardo Marcos-Pinto, Renee M. Laird, Mislav Novokmet, Jerko Štambuk, Gordan Lauc, Chad Porter, Nuno Padrão, Bruno Cavadas, Catarina Moreira-Barbosa and Ana M. Dias.

Ao Professor Doutor Eduardo Rocha agradeço a disponibilidade e todo o apoio enquanto diretor do Doutoramento em Ciências Biomédicas. À Helena Martins, como membro dos serviços académicos do ICBAS, agradeço a disponibilidade em esclarecer todas as dúvidas.

Quero agradecer a todo o grupo Immunology, Cancer & Glycomedicine! Um grupo maravilhoso, com um espírito de equipa fantástico, cooperativo e com mentes brilhantes. Inês Alves, obrigada por toda a ajuda científica e não só, pelas palavras sábias, pelos conselhos, pelas opiniões sinceras e pela amizade. Obrigada por tudo dentro e fora do laboratório. Admiro-te muito e levo-te como um exemplo e como uma pessoa que nasceu sem dúvida para a ciência! Minha querida Beatriz, a minha parceira do lado! Não tenho palavras para todo o carinho, preocupação, companheirismo e por estares sempre pronta a ajudar. Obrigada por tudo, por todas as vezes que perguntaste como eu estava e pelas palavras amigas! Guardo tudo no coração. A minha Du, a minha companheira de boas aventuras! Obrigada pelo carinho, pela amizade, pelos valores certos e pela leveza de encarar a vida! Ainda bem que regressaste ao grupo, fico muito contente por te ter conhecido e por todos os momentos que passamos juntas. A minha querida Ângela, obrigada de coração por todas as palavras meigas, de incentivo e pela empatia e preocupação. Não me vou esquecer das mensagens e perguntas sempre nos momentos certos. O teu carinho foi essencial ao longo deste percurso. Querida Leticia, foi sem dúvida uma surpresa muito boa conhecer-te e rapidamente ganhei um carinho muito grande por ti. Agradeço-te muito todos os conselhos, a empatia, o cuidado e as conversas que tanto reconfortaram. Rafaela, Carolina Dantas, Catarina, Guilherme, Pedro Almeida, Mariana, Pedro Lopes, Ariana e Raquel obrigada pela alegria, gargalhadas e companheirismo partilhados. Guardo todos com muito carinho. Vanda a melhor lab manager de sempre. Obrigada pela tua calma e paciência. Francisca, a minha pequena. Obrigada pelo teu

entusiasmo e pelo papel determinante que tiveste para o arranque do nosso projeto. Na verdade, começamos juntas nesta aventura entre microbioma e glicanos e não podia ter tipo melhor parceira para iniciar esta aventura. Márcia obrigada por toda a tua cooperação e entusiasmo. Mesmo longe, demonstraste sempre todo o interesse em ajudar! Manel agradeço-te por todos os conselhos científicos, pela simpatia, boa disposição e pela disponibilidade! À querida Mariana Silva por toda a simpatia e ajuda quando foi necessário. A todos vocês, muito obrigada pelos excelentes momentos tanto no trabalho como fora dele. Farão todos, para sempre, parte da minha história enquanto aluna de doutoramento. Viva aos nossos “sweet” glicanos!

Agradeço à minha querida Beatriz Ferreira, a minha amiga de sempre, obrigada por todo o carinho e compreensão. Obrigada pelas partilhas, conversas e desabafos. Ao meu querido Cláudio, por todos os conselhos e por toda a alegria e leveza que transmites quando estamos juntos.

Aos meus queridos Carolina Vasconcelos e Miguel Bica. Vocês são o exemplo da família que escolhemos. Uma amizade que tem praticamente tantos anos quanto tem esta tese, mas é uma amizade que parece que foi desde sempre. Obrigada por todas as gargalhadas, por todos os momentos absolutamente fantásticos que passamos juntos, pela preocupação e apoio. Obrigada pelo afilhado mais maravilhoso que aquece o meu coração. Não imaginam o quanto me faz bem ter-vos na minha vida. A vossa presença tornou este caminho muito mais doce.

Dona Teresa e Sr. Vítor não tenho palavras para agradecer a forma como receberam e me integraram na vossa família. Agradeço todo o apoio que me têm transmitido desde então. Um especial obrigado por toda a preocupação e cuidado, principalmente nesta última fase. O vosso carinho fez toda a diferença para aguentar este último esforço. Aos tios João e tia Nanda pela forma carinhosa como me tratam e pelo orgulho que transmitem. Marta e Pedro agradeço pelo apoio e pela alegria que trazem junto com os pequeninos, Miguel e Iris.

Quero agradecer à minha família, em especial ao meu padrinho, à minha madrinha, tia Tina, Sascha, tio Nel, tia Elisabete, aos meus primos Andreia, Pedro, Joel e Lia por todo o cuidado e apoio que sempre me fazem sentir. Ao meu avô João e avó São, pelo orgulho que expressam e por toda a preocupação sobre o meu bem-estar e felicidade. Todos contribuíram para uma estrutura de carinho e suporte muito importante para mim.

Por fim, quero agradecer às duas pessoas da minha vida.

Mãe, não há agradecimento suficiente para tudo o que fizeste e fazes por mim. Obrigada pelo orgulho que demonstras pelo meu trabalho e pela motivação incansável que me tens

dado. Obrigada pela força, amparo e amor incondicional. Obrigada por sempre me incentivares a fazer mais e melhor, mas sobretudo por acreditares sempre em mim. Agradeço por todos os valores que me transmites e que me seguem onde quer que esteja. Obrigada por nunca me fazeres sentir sozinha. És sem dúvida o grande pilar da minha vida e sem ti certamente nada disto seria possível. Espero que sempre te possas orgulhar de mim.

Sérgio, o meu porto de abrigo. As palavras não são suficientes para agradecer tudo o que fazes por mim. Obrigada por me apoiares sempre e por me fazeres acreditar que eu consigo atingir todos os meus objetivos e os meus sonhos. Obrigada por compreenderes todas as minhas ausências. Obrigada por ouvires os meus desaforos e dissabores. Obrigada por ouvires as minhas apresentações vezes sem conta. A tua paciência e preocupação ao longo destes anos foram incansáveis. Tiveste um papel tão, mas tão importante, que nem sei como expressar a minha gratidão. Sou imensamente grata por todo o teu amparo, dedicação, amor e altruísmo. Tornas tudo muito mais especial e nada disto teria o mesmo sentido se não fosse partilhado contigo. Não podia ter melhor companheiro ao meu lado.

A todos um sincero e grande obrigada.

Declaration

The author of this thesis declares that, in accordance with “no 2, alinea a) do artigo 31o do Decreto-Lei no 115/2013 de 7 de Agosto”, provided a major contribution to the design and technical execution of the work, interpretation of the results and manuscript preparation resulting in the following accepted articles and submitted:

Scientific publications:

Original articles

- **Rodrigues, C. S.***, Gaifem, J.*, Pereira, MS., Alves, M.F., Silva, M., Padrão, N., Cavadas, B., Moreira-Barbosa, C., Alves, I., Marcos-Pinto, R., Torres, J., Lavelle, A., Colombel, J. F., Sokol, H., Pinho, S. S. Alterations in mucosa branched *N*-glycans lead to dysbiosis and downregulation of ILC3: a key driver of intestinal inflammation. (*Under revision*); *These authors contributed equally to this work. (**Chapter 3**)
- Gaifem, J.*, **Rodrigues, C. S.***, Petralia, F., Alves, I., Leite-Gomes, E., Cavadas, B., Dias, A. M., Moreira-Barbosa, C., Revés, J., Laird, R. M., Novokmet, M., Štambuk, J., Habazin, S., Turhan, B., Gümüş, Z. H., Ungaro, R., Torres, J., Lauc, G., Colombel, J. F., Porter, C. K., ... Pinho, S. S. (2024). A unique serum IgG glycosylation signature predicts development of Crohn's disease and is associated with pathogenic antibodies to mannose glycan. *Nature immunology*, 25(9), 1692–1703. <https://doi.org/10.1038/s41590-024-01916-8>; *These authors contributed equally to this work. (**Chapter 4**)

Reviews & Book Chapters

- Crouch, L. I., **Rodrigues, C. S.**, Bakshani, C. R., Tavares-Gomes, L., Gaifem, J., & Pinho, S. S. (2024). The role of glycans in health and disease: Regulators of the interaction between gut microbiota and host immune system. *Seminars in immunology*, 73, 101891. Advance online publication. <https://doi.org/10.1016/j.smim.2024.101891> (**Chapter 5**)
- Catarina M. Azevedo*, Beatriz Santos-Pereira*, Eduarda Leite-Gomes*, **Cláudia S. Rodrigues***, Carolina S. Dantas, Guilherme Faria, Rafaela Nogueira, Pedro Almeida, Letícia Tavares-Gomes, Joana Gaifem, Inês Alves, Ângela Fernandes, Salomé S. Pinho. *N*-glycans as fundamental immune-checkpoints at the frontiers of inflammation, autoimmunity and cancer. (*Invited submission in The Royal Society of Chemistry*) *These authors contributed equally to this work (**Appendix I**)

- Alves, I., Vicente, M. M., Dias, A. M., Gaifem, J., **Rodrigues, C.**, Campar, A., & Pinho, S. S. (2021). The Role of Glycosylation in Inflammatory Diseases. *Advances in experimental medicine and biology*, 1325, 265–283. https://doi.org/10.1007/978-3-030-70115-4_13 (not included in the thesis)

Resumo

A doença inflamatória intestinal (DII) é um grupo de doenças crónicas debilitantes do trato gastrointestinal, que engloba a doença de Crohn e a colite ulcerosa. A DII está a emergir como uma doença global e incurável, caracterizada pela sua significativa complexidade e heterogeneidade. Embora a etiologia da DII permaneça ainda pouco clara, sabe-se que esta doença é mediada por uma resposta imunitária exacerbada associada a alterações da microbiota intestinal, impulsionada por vários fatores ambientais em indivíduos com uma forte predisposição genética. Para além disso, na DII, e predominantemente na doença de Crohn, várias evidências têm apontado para um período caracterizado por alterações imunológicas que precedem os sintomas e o dano intestinal, com início anos antes do diagnóstico da DII. Estes aspetos realçam a necessidade de maiores conhecimentos sobre a fase pré-clínica que possam permitir a previsão e a prevenção da doença. As causas que levam a uma composição desequilibrada da microbiota (disbiose) associada a uma inflamação intestinal aberrante não são ainda conhecidas. Desta forma, existe uma clara necessidade de uma melhor compreensão dos mecanismos subjacentes às interações entre o hospedeiro e o microbioma intestinal para uma melhor gestão clínica da DII. A este respeito, a glicosilação celular desempenha um papel fundamental na imunopatogénese da DII e o glicocálix, que é uma camada complexa de glicanos/cadeias de açúcar na superfície celular, constitui uma interface fundamental entre o hospedeiro e a microbiota intestinal. Se e como as alterações no glicoma da mucosa conduzem à disbiose associada à inflamação intestinal é uma questão fundamental que permanece sem resposta. Por conseguinte, determinar o papel da glicosilação do hospedeiro na modulação da microbiota e na resposta imunitária associada à DII, é um passo fundamental para melhorar o nosso conhecimento sobre a doença. Em particular, nesta tese pretendemos: 1) desvendar de que forma alterações no glicocálix intestinal têm impacto na composição da microbiota intestinal e na resposta imunitária associada à inflamação; 2) avaliar a assinatura do glicoma do hospedeiro na fase pré-clínica da DII.

Primeiramente, ao utilizar um modelo de ratinho geneticamente modificado, com ausência de expressão de *N*-glicanos complexos ramificados devido a ausência do gene *Mgat5*, verificamos que no seu estado basal existe um défice na resposta imunitária ILC3-IL22, concomitante com uma disfunção da barreira epitelial do intestino e uma microbiota disbiótica com níveis reduzidos de ácidos gordos de cadeia curta (AGCC). Avaliámos ainda se a suplementação profilática com glicanos era capaz de reverter a transição de uma condição saudável para a inflamação intestinal. De facto, a suplementação com *N*-acetilglucosamina (GlcNAc) foi capaz de preservar a homeostasia intestinal, promovendo,

por exemplo, a resposta imunitária ILC3-IL-22 e recuperando os níveis de AGCC. Para além disso, esta modulação induzida pela suplementação contribuiu para uma diminuição significativa da suscetibilidade à doença. Deste modo, demonstramos que a suplementação com GlcNAc é capaz de atuar como uma estratégia profilática eficiente em prevenir a transição de um ambiente homeostático para a inflamação intestinal.

Para compreender melhor o papel dos glicanos no período pré-clínico da DII e os mecanismos que ocorrem durante a transição de uma condição saudável para uma inflamação crónica que, consequentemente podem influenciar a suscetibilidade à DII, analisámos amostras de soro colhidas até seis anos antes do diagnóstico da doença. Identificámos um padrão de glicosilação distinto nos anticorpos IgG circulantes, marcado pela agalactosilação da IgG Fc. Verificamos que o padrão agalactosilado da região Fc das IgG2 permanece estável até ao diagnóstico e apresenta uma correlação significativa com os níveis elevados de anticorpos contra-*Saccharomyces cerevisiae* (ASCA). Demonstrámos que este eixo glicoma-ASCA prevê o desenvolvimento da doença de Crohn bem como a sua severidade. Através de estudos funcionais, descobrimos que as IgGs ASCA agalactosiladas desencadeiam respostas pró-inflamatórias através da ativação de células imunes inatas e da indução da via do inflamassoma. Por fim, validámos estes resultados em modelos animais, onde a transferência de IgGs ASCA resultou numa maior suscetibilidade ao desenvolvimento da inflamação intestinal.

Em conclusão, os resultados nesta tese apresentam novos conhecimentos sobre o papel fundamental do perfil de glicanos do hospedeiro na manutenção da imunidade intestinal protetora. Destacamos a função de uma assinatura de glicosilação alterada como um fator essencial nos processos inflamatórios iniciais ocorrentes na fase pré-clínica da DII. Deste modo, os nossos resultados sublinham a importância dos glicanos na manutenção de uma interação homeostática entre o microbioma e a imunidade do hospedeiro. A nossa investigação destaca o potencial das estratégias baseadas em glicanos como uma abordagem promissora para novas intervenções clínicas destinadas a prever e prevenir a DII.

Abstract

Inflammatory Bowel Disease (IBD) is a group of chronic debilitating disorders from the gastrointestinal tract, comprising Crohn's disease (CD) and ulcerative colitis (UC). IBD is emerging as a global and incurable disease, characterized by its significant complexity and heterogeneity. Although the precise aetiology of IBD is still elusive, it seems to result from the interplay of the host's genetic predisposition and abnormal immune response to an altered gut microbiota, driven by several environmental factors. Moreover, in IBD, and predominantly in CD, growing evidence has pinpointed towards the existence of a period characterized by immunological changes preceding symptoms and intestine injury, starting years before diagnosis. These aspects highlight the need to gain insight into the preclinical phase that could allow disease prediction and prevention. The causes leading to an imbalanced microbiota composition (dysbiosis) associated with a deleterious intestinal inflammation are still elusive. Thus, there is a clear demand from an improved understanding of the mechanisms underlying host and gut microbiome interactions for a better clinical management of IBD. Cellular glycosylation displays a key role in IBD immunopathogenesis and host glycocalyx, a complex layer of glycans at the surface of mucosa, is a major interface between intestinal mucosa and gut microbiota. Whether and how changes in mucosa glycocalyx leads to dysbiosis associated with intestinal inflammation is a key question that remains unanswered. Hence, determining the role of host glycosylation in the modulation of microbiota profile and immune response associated with IBD development is a fundamental step to fully understand this disease. In particular, in this thesis we aimed to 1) unveil how an altered host glycocalyx impacts gut microbiota and the initiation of intestinal inflammation; 2) assess the host glycome signature in the pre-clinical phase of IBD.

Using a glycoengineered mouse model (*Mgat5*^{-/-}) which lacks complex branched *N*-glycans, we observed that the increased susceptibility to severe colitis displayed by *Mgat5*^{-/-} mice coincides with a deficiency in ILC3-IL22 immune response, concomitant with intestinal epithelial dysfunction and a dysbiotic gut microbiome with reduced levels of short-chain fatty acids (SCFAs) at baseline. We further evaluated whether the prophylactic supplementation with glycans could revert the health to intestinal inflammation transition. Supplementation with *N*-acetylglucosamine (GlcNAc) was able to rescue intestinal homeostasis by enhancing ILC3-IL-22 immunity and recovering the levels of SCFAs. This was then translated to a decrease in disease susceptibility upon colitis induction. Therefore, supplementation with GlcNAc acted as an efficient prophylactic strategy to prevent health to intestinal inflammation transition.

To better understand the role of glycans in the preclinical period of IBD and to gain deeper insights about the mechanisms that take place during health-to-chronic inflammation transition that may influence IBD susceptibility we analysed serum samples taken up to six years before IBD diagnosis. We identified a distinct glycosylation pattern on circulating IgG antibodies marked by agalactosylation of IgG Fc. The agalactosylated IgG2 Fc pattern remains stable until diagnosis and correlates to higher levels of anti-*Saccharomyces cerevisiae* antibodies (ASCA). We demonstrated that this glycome-ASCA hub can predict CD development and a more severe disease course. We further performed mechanistic studies where we found that these agalactosylated ASCA IgGs trigger pro-inflammatory immune responses by activating innate immune cells and inflammasome pathways. Ultimately, we validated these findings in animal models, in which adoptive transfer of ASCA IgGs resulted in increased susceptibility to intestinal inflammation.

Collectively, the findings presented in this thesis provide novel insights regarding pivotal role of the host glycome in maintaining homeostatic gut immunity. We highlight the potential of an altered glycosylation signature to act as a pivotal factor in the early inflammatory processes observed in the preclinical phase of IBD. Likewise, our findings underscore the importance of glycans in maintaining a homeostatic crosstalk between the microbiome and host immunity. Our research ultimately sheds light on the potential of glycan-based strategies as a promising avenue for novel clinical interventions aimed at predicting and prevent IBD.

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List of abbreviations

ACCA – Anti-chitobioside carbohydrate antibodies
ADCC – Antibody-dependent cellular cytotoxicity
AIEC – Adherent invasive Escherichia coli
ALCA – Anti-laminaribioside carbohydrate antibodies
AMCA – Anti-mannobioside carbohydrate antibodies
AMPS – Antimicrobial peptides
Anti-OmpC – Anti-outer membrane protein C
APCs – Antigen-presenting cells
ASCA – Anti-Saccharomyces cerevisiae antibodies
ASIR – Age-standardized incidence
ASMR – Age-standardized mortality rate
ASPR – Age-standardized prevalence
BAs – Secondary bile acids
CDC – Complement-dependent cytotoxicity
CD – Crohn's disease
CRP – C-reactive protein
CTLs – C-type lectins
DAMPs – Damage-associated molecular patterns
DC-SIGN – Dendritic Cell-Specific ICAM-3 Grabbing Non-Integrin
DCs – Dendritic cells
DSS – Dextran sulfate sodium
EOMES – Eomesodermin
ER – Endoplasmic reticulum
Fc – Fragment crystallizable
FcγR – Fc gamma receptor
FDRs – First-degree relatives
FMT – Fecal microbiota transplantation
FOS – Fructooligosaccharides
Fuc – Fucose
FUT2 – α 1,2 fucosyltransferase
Gal – Galactose
GalNAc – *N*-acetylgalactosamine
GATA3 – GATA binding protein 3
Glc – Glucose
GlcA – Glucuronic acid

GlcNAc – *N*-acetylglucosamine
GOS – galactooligosaccharides
GPCRs – G-protein-coupled receptors
HDACs – Histone deacetylases
HMOs – Human milk oligosaccharides
IBD – Inflammatory Bowel Disease
IdoA – Iduronic acid
IECs – Intestinal epithelial cells
IFN γ – Interferon gamma
Ig – Immunoglobulin
ILCs – Innate lymphoid cells
IL – Interleukin
LacNAc – *N*-acetyllactosamine
LTi – Lymphoid tissue-inducer
Man – Mannose
MCT1 – Proton-coupled monocarboxylate transporter 1
MGAT – Mannosidase acetylglucosaminyltransferase
MUC – Mucin
NF κ B – Nuclear factor Kb
NK – Natural killer
NLRs – Leucine-rich repeat receptors
NOD2 – Nucleotide-binding oligomerization domain containing 2
PAMPs – Pathogen-associated molecular patterns
pANCA – Perinuclear anti-neutrophil cytoplasmic antibodies
PRRs – Pattern recognition receptors
RNA – Ribonucleic acid
ROR γ t – Retinoic acid-related orphan receptor gamma t
ROS – Reactive oxygen species
SCFAs – Short-chain fatty acids
SFB – Segmented filamentous bacteria
Sia – Sialic acids
SMCT1 – Sodium-coupled monocarboxylate transporter 1
SNPs – Single nucleotide polymorphisms
Tbet – T-box transcription factor Tbet
TCR – T cell receptor
Th – T helper cells
TLR – Toll-like receptor

TNF α – Tumor necrosis factor alpha

Treg – Regulatory T cells

UC – Ulcerative colitis

Xyl – Xylose

Chapter 1

General Introduction

1. Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) is a group of chronic inflammatory conditions affecting the gastrointestinal tract, being classified into two main different disease entities: Crohn's disease (CD) and ulcerative colitis (UC)[1-3]. There is the lack of efficient treatments for all patients, which makes IBD an incurable disorder. It is characterized by a progressive, debilitating and unpredictable disease course, with periods of relapsing and remission [3], causing significant morbidity and disability. This disease is also associated with several complications such as infections and cancer, which together with the absence of efficient therapies, leads to elevated healthcare costs and pressure on healthcare systems [4-6].

CD and UC display similar clinical symptoms, including faecal urgency, diarrhoea (often containing blood and mucus), abdominal pain, and weight loss. These conditions are often accompanied by systemic symptoms such as fatigue and heavy productivity losses [7]. Nevertheless, the disease processes in both conditions are heterogeneous, differing in the location and depth of inflammation [8]. In CD, inflammation is transmural and can occur in a discontinuous pattern throughout the gastrointestinal tract, most commonly affecting the ileum and colon [9, 10]. In turn, UC inflammation is confined to the mucosa and submucosa layers and extents continuously from the rectum to the colon [2].

1.1 Epidemiology: incidence and prevalence

The global burden of IBD has been progressively rising over the past few decades, placing it as a major public health challenge worldwide [11]. According to the Global Burden of Disease study from 2019 [4, 12], IBD accounted for 4.90 million cases globally, with an impressive 47.45% increase compared to 1990. The estimated number of new cases increased from 293,570 to 404,550 between 1990 and 2019. The incidence and prevalence of IBD vary across and within different geographic regions [13]. Traditionally, UC and CD have been most dominant in Western countries, in particular in North America and Europe, where incidence and prevalence rates are higher compared to developing countries [14]. Nevertheless, in newly industrialized regions such as Asia, the Middle East, Africa, and South America, the incidence of IBD has increased rapidly over the last decade [15]. The great acceleration is attributable to several factors, including the unmasking incidence. Advances in technology and the economy have been contributed to increased awareness and access to healthcare services, better diagnostic techniques, and an improved the ability to detect IBD, leading to the identification of previously undiagnosed cases. Simultaneously, the increased incidence of the disease is also a result of improvements in sanitary

conditions, advances in agriculture and diet, the progress of urbanisation, and the increasing interconnectivity of global economies and cultures (westernisation of society)[16].

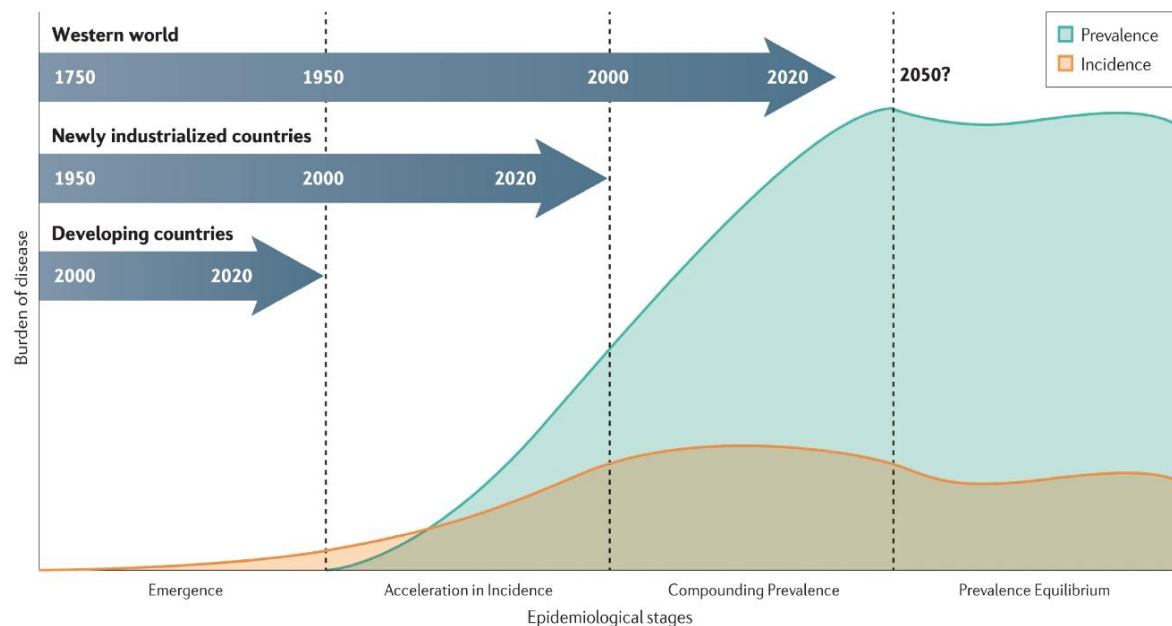


Figure 1. Different incidence and prevalence patterns of Inflammatory Bowel Disease across the four stages of disease evolution. Developing countries are currently in an emergent phase, while the new industrialized countries are undergoing an acceleration stage. Western countries are in the compounding prevalence stage, in which despite an increase in prevalence, incidence is reaching a point of stabilisation. Adapted from [16].

The study of IBD burden at regional level in 2019 indicated that the Western Europe still had the highest number of prevalent cases (1.03 million) [4]. Nevertheless, the largest change in age-standardized prevalence (ASPR) and age-standardized incidence (ASIR) rates occurred in Asia, with a substantial increase in ASPR (210.54 cases per 100,000 people) and with highest annual growth of ASIR [4]. Indeed, regions in Asia with higher population density exhibited a rise in CD and UC incidence [17, 18]. For instance, India has reported a considerable increase in IBD cases [19]. This pattern is consistent with increased urbanisation and industrialisation of certain areas, which are associated with a higher risk of developing IBD [20].

At the national level, China and the USA exhibited the highest number of prevalent IBD cases in 2019, with 911,405 and 762,890 cases, respectively, positioning themselves as prominent contributors to the global IBD burden. In Europe, the highest ASPR of IBD was observed in Norway, with 498.95 cases per 100,000 people [4]. Additionally, a Finnish study focusing on the IBD incidence between 2000 and 2020 revealed an increased IBD incidence

with 42,498 new cases. Moreover, the incidence rate ratio of UC raised from 20 to 35 cases per 100,000 person-years, while the incidence rate ratio in CD increased from 8 to 13 per 100,000 person-years [21]. Similarly, a study in Italy comparing the periods 2001–2006 and 2016–2021 found that IBD prevalence increased by 46%, from 200 to 321.2 cases per 100,000 individuals. The incidence also increased by 169%, from 6.7 to 18.0 per 100,000 person-years, with a greater increase in CD comparing to UC. During 2016–2021, the mean age at diagnosis was 42.0 ± 17.4 years, 30.9% of patients required at least one hospitalization, and 10.9% underwent at least one surgery [22]. In Portugal, the incidence rate is around 12 cases per 100,000 people, with the prevalence expected to rise from 161 cases per 100,000 in 2008 to approximately 390 cases per 100,000 in 2024. Notably, the forecasted prevalence is expected to be around 746 per 100,000 people in 2030 [23].

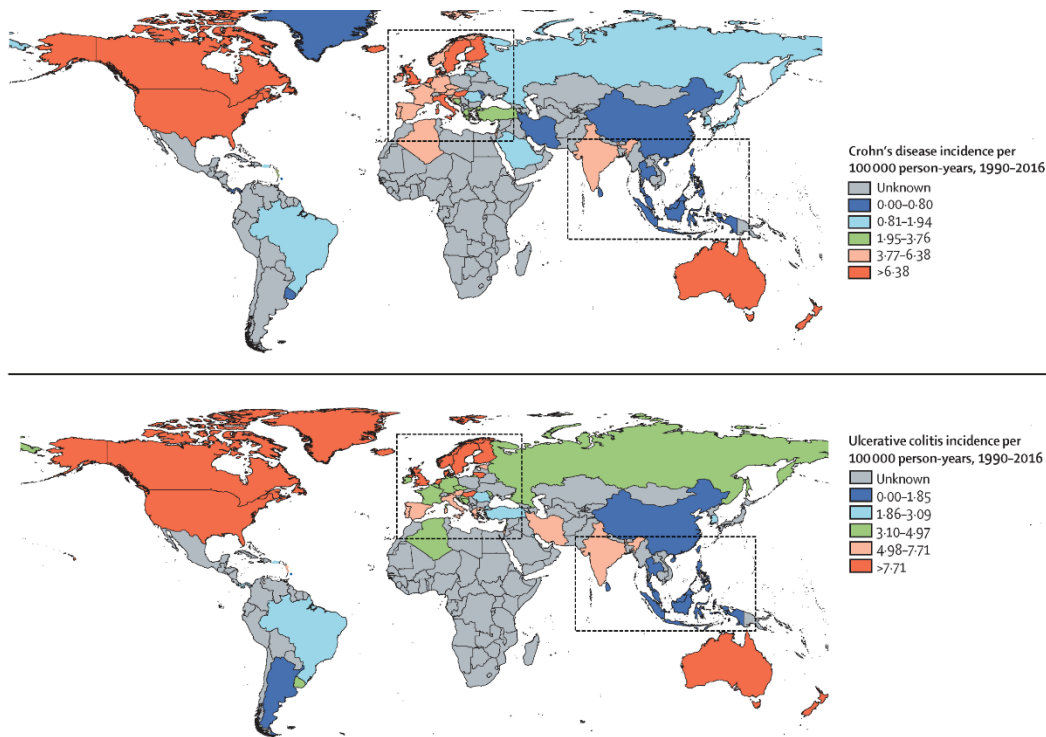


Figure 2. Worldwide incidence of Crohn's disease (CD) and ulcerative colitis (UC) between 1990 and 2016. Adapted from [24].

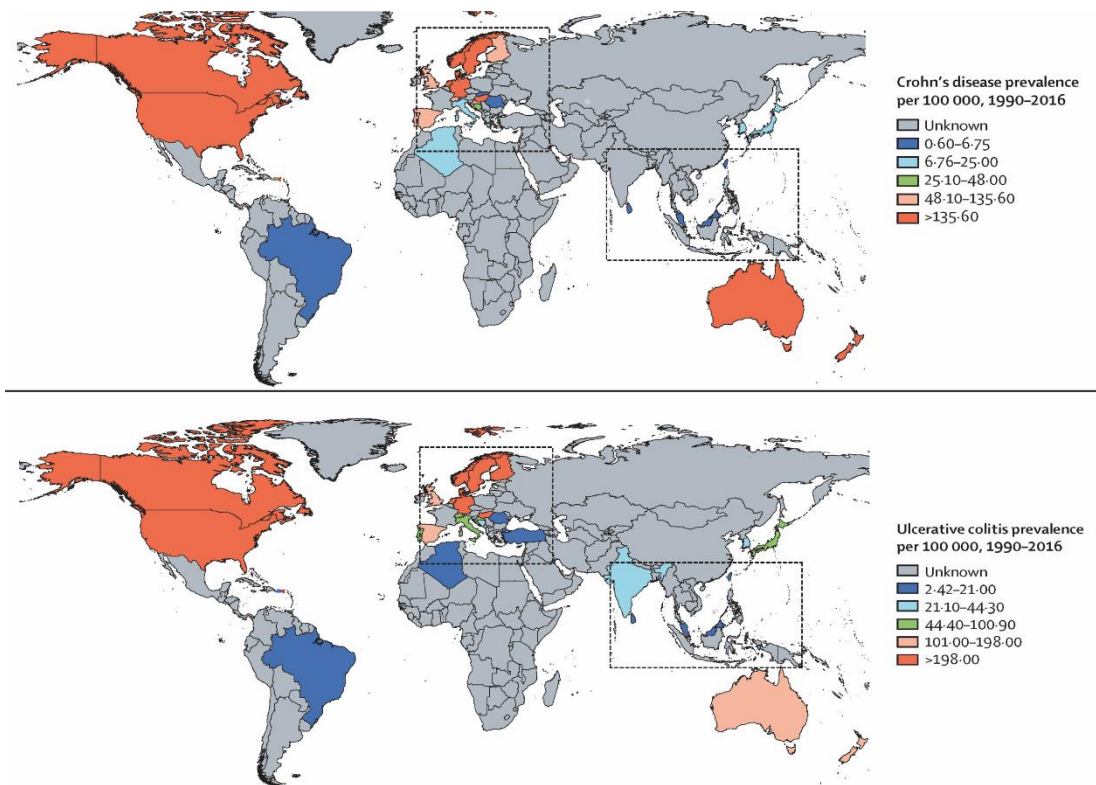


Figure 3. Worldwide prevalence of Crohn's disease (CD) and ulcerative colitis (UC) between 1990 and 2016. Adapted from [24].

The IBD burden also varies by age and gender. While older populations are seeing a decline in IBD incidence, young adults and adolescents in regions such as East Asia and high-income Asia-Pacific are experiencing an alarming increase. The peak age of onset of IBD is between 15 and 30 years of age, with rising incidence rates in those under 25. IBD is also rising in paediatric patients, with incidence rates of 5 to 11 cases per 100,000 children per year in Western countries. The prevalence among children and adolescents is estimated at 100 to 200 cases per 100,000, with approximately 20-25% of IBD cases being diagnosed before the age of 18 [25-27].

Data on the gender prevalence between UC and CD are still inconclusive. In UC, man and women seem to present similar incidence before the age of 45; after which men display a higher risk. For CD, women have a lower risk of CD until puberty, after which women present a higher risk than men [28, 29]. Overall, the number of prevalent cases of IBD was higher among women in 2019 [4].

As for mortality, the number of IBD-related deaths increased 68.75%, with 40,998 deaths in 2019 comparing to 1990 [30]. However, the global age-standardised mortality rate (ASMR) reduced from 0.67 to 0.54 per 100 000 people on the same period. Western Europe

showed the highest number of deaths (11,981) with the highest ASMR (1.11 per 100 000 people). Nationally, China and USA presented the highest number of deaths (4,676 and 5,910, respectively), although it was the Netherlands that revealed the highest ASMR in 2019. In India, between 1990 and 2019, the total number of deaths raised from 2770 to 4214, although the ASMR decreased. Globally, more IBD-related deaths were observed among females in 2019 [19].

1.2 Causes and risk factors in IBD: the power of genetics and environment

Although the aetiology of IBD is widely explored, the precise and definitive causes contributing to the disease's pathogenesis remains not well defined. Accumulating evidence suggests that CD and UC are multifactorial disorders [1, 2]. Nevertheless, a complex interplay of factors, including an abnormal immune response, altered intestinal microbiota, genetic predisposition, and environmental triggers, are known to play a pivotal role in the onset and progression of chronic intestinal inflammation [31, 32]. Indeed, the heterogenous incidence and prevalence across different populations worldwide, together with family aggregation (tendency of the disease to cluster in families), pinpoints the involvement of genetic and environmental factors in IBD [33, 34]. Remarkably, it was reported that first-degree relatives (FDRs) of affected individuals exhibit more than a five-fold risk to develop the disease [3]. Moreover, more than 15% of IBD patients have relatives which also develop the same condition [35]. In this regard, it is consensual that IBD has a strong genetic component, with inheritance playing a more prominent role in CD than in UC [36-38], with values of risk around 13.1% and 8.2%, respectively [39].

Genome-wide association studies (GWASs) in the last few years have identifying more than 240 allelic variants in genes, single nucleotide polymorphisms (SNPs), related to IBD susceptibility [34, 40]. Most of these genes are implicated in immunological regulatory pathways, such as: *NOD2*, *CLEC7A* and *CARD* family, known to be paramount in the recognition of microorganisms and activation of innate immune response [41-43]; *TNF*, *STAT3*, *STAT5B*, *SP110*, *JAK2*, *CLEC4K/CD207*, *IRF8*, *IL23R*, *IL10*, *IL10R*, and *IL1RA*, involved in lymphocyte regulation and pro- and anti-inflammatory responses [34]; *CDH1*, *HNF4- α* , that are linked to intestinal barrier integrity and function [34, 44].

Concerning to the IBD type, accumulative evidence demonstrates that there is a genetic overlapping between IBD forms. Indeed, at least one-third of genetic loci conferring susceptibility to the disease are shared both by CD and UC [1, 45]. Amongst the shared genes between CD and UC there are: *IL23R*, *IL12B*, *JAK2*, *TYK2*, *STAT3*, *IL10*, *IL1R2*, *REL*, *CARD*, *NKX2.3*, *CCNY*, *ICOSLG*, *PRDM1*, *SMAD3* and *ORMDL3* [1, 33]. Regarding

CD, *NOD2* is the most widely implicated gene related to increased disease susceptibility [46]. Besides conferring increased risk to CD, variants of the *NOD2* gene also impact the disease course, phenotype and prognosis, determining, for instance, the requirement of surgery [47]. Genetic variations in other genes, such as *SLC22A5*, *LACC1*, *FAMIN*, *IL23R*, *PTPN2*, *IRGM*, *GPX1* and *GPX3*, are typically more associated with CD pathogenesis [33, 40]. On the other hand, *ECM1*, *HERC2*, *STAT3*, *PTPN2*, *IL10*, *ARPC2*, *CDH1*, *LAMB*, *GNA12*, *CLEC7A*, *MUC5AC* and *HNF4A* are considered UC-susceptibility genes [1, 33, 48].

The presence of polymorphisms can vary across ethnic groups and may have either protective or detrimental effects on IBD depending on the specific gene variant involved [34]. For instance, the presence of *NOD2* polymorphisms is detected in European patients but not in Asiatic patients [32, 49]. In turn, while *CARD9* and *CARD10* gene expression seems to be significantly elevated in UC comparing to CD, *CARD11* and *CARD15* show higher expression in CD patients. Notably, decreased expression of *CARD14* in UC has been linked to a more favourable clinical course of the disease [1]. Numerous environmental factors have been highlighted as important triggers of IBD, including diet, smoking, exposure to antibiotics, alcohol consumption, breastfeeding, psychological stress, urban lifestyle, air pollution, hygiene in childhood (hygiene hypothesis), infections etc [13, 50, 51]. A report found that individuals raised in unhygienic rural conditions during their early years had a lower risk of developing IBD compared to those living in urban areas [52]. Similarly, a study showed that children of immigrants who arrived in Canada at a younger age had a higher susceptibility to IBD [53], emphasizing the significant role of environmental factors in the pathogenesis of the disease.

Diet has been also extensively studied. Several types of diet, including those high in animal fat, sugar, refined carbohydrates, emulsifiers, and chemical additives, are known to compromise the intestinal barrier and lead to pro-inflammatory reactions [54-56]. Notably, diet is one of the principal modulators of the composition and function of intestinal microbiota [57].

In the same line, the use of antibiotics and the lack of early childhood exposure to microorganisms are also closely related to the influence on the intestinal microbiome by reducing microbiota diversity and richness. This further alters the regulatory pathways between immune system and commensal microorganisms [40, 58]. Another interesting perspective concerns the role of environment, particularly diet, in epigenetic changes. Pro- or anti-inflammatory dietary patterns, alongside with microbiome, are recognized as strong inducers of epigenetic alterations that might play a key role in the IBD pathogenesis and treatment [59-61]. In fact, epigenetic modifications have been reported in both CD and UC [60, 62-65].

Hence, to sum up, all this evidence illustrates the complex relationship between genetic predisposition and environmental factors that disrupt the normal balance of intestinal microbiome and may contribute to an inappropriate mucosal immune response.

Notably, large part of the above-mentioned genetic alterations occurring in IBD leads to immunological dysfunctions and a deficient regulation to microbiota. As such, it is plausible that in genetically susceptible individuals, the modulation of the environmental exposure (e.g. through the diet) can potentially help to prevent IBD onset, as well as to mitigate disease outcome.

1.3 Clinical management of IBD

1.3.1 Diagnosis

The diagnostic of CD and UC mainly relies on patient's history and in clinical results obtained by colonoscopy, endoscopy, radiology, magnetic resonance imaging, histopathology, serologic and faecal analysis [9, 15, 66]. CD and UC are mainly distinct by the nature and location of inflammation. Characteristically, CD is characterized by a discontinuous and transmural inflammation affecting the whole digestive tract, mainly the terminal ileum, caecum, perianal area and colon [9, 67]. CD can be divided in five types: gastroduodenal (affecting the stomach and the duodenum), jejunoileitis (involving upper half of the small intestine), ileitis (only the ileum is affected), ileocolitis (inflammation involves the ileum and the colon) and Crohn's colitis (affects only the colon) [68]. Patients with CD have a wide spectrum of extra-intestinal manifestations, namely Crohn's disease-specific granulomatous skin lesions, characterized by tuberculoid granulomas like those observed in the gastrointestinal tract [69-71].

In UC, the inflammation is diffuse and continuous, affecting mainly the rectum but may extend throughout the colon, being confined to the mucosal layer. UC can be divided into four subtypes based on the affected area: proctitis (involvement limited to the rectum), proctosigmoiditis (affecting the rectum and sigmoid), distal/ left-sided colitis (involving descending colon, sigmoid and rectum), and extensive colitis/pancolitis (affecting the entire colon) [34, 48].

The symptomology associated to CD and UC are very similar depending on the extension of inflammation. Therefore, the endoscopic and pathological features are preponderant for the diagnosis [15]. Disease activity in CD can be assessed by two main scores: the simple Endoscopic Score (SES-CD), and Harvey-Bradshaw index (HBI). SES-CD is a simple, and easy-to-use endoscopic scoring system that has been widely used in

clinical practice. This score assesses the size of mucosal ulcers, the ulcerated surface, the endoscopic extension and the presence of stenosis [72, 73]. On the other hand, HBI focuses only on clinical features, allowing faster categorization of CD severity and detection of remission. HBI considers five parameters: patients' well-being, abdominal pain, stool frequency and complications such as ulcers, new fistulas and abscesses [74-76]. For UC diagnosis, the Mayo Score is the most used to assess UC activity and severity, including clinical and endoscopic domains. It is based on four parameters: stool frequency, rectal bleeding, endoscopic evaluation and physician's global valuation. Each parameter of the score ranges from zero to three, classifying the disease as normal or inactive (Mayo 0), mild (Mayo 1), moderate (Mayo 2) or severe (Mayo 3) [77].

Apart from radiology findings, endoscopic studies and histopathological examination, the integration of faecal and serologic biomarkers is essential for the distinction of UC from CD and for an accurate management of patients [78, 79]. Among blood biomarkers, C reactive protein (CRP) is a protein produced by hepatocytes stimulated by pro-inflammatory cytokines, mainly by IL-6, during the acute phase of inflammation. It is a serum biomarker widely used in clinical practice to determine acute inflammation, although it is not disease specific [80]. Nevertheless, the levels of CRP in IBD patients are a reliable indicator of disease activity (whether is active or inactive) as well as an indicator of response to treatment. For example, in UC, high CRP (modestly) correlates with endoscopic and clinical activity, with sensitivity of 67.1 and a specificity of 97% [81-83]. Perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) and anti-*Saccharomyces cerevisiae* antibodies (ASCA) have been widely used to discriminate UC and CD patients. Atypical pANCA is detected in 60–70% of sera from UC patients [80, 84, 85], discriminating UC from CD with 52% of sensitivity and 91% specificity. In turn, ASCA is a clinical diagnostic marker of CD with serological positivity values from 50 to 60%. Accumulative evidence has shown that utilization of pANCA and ASCA together improves the diagnostic power [86]. Alongside to ASCA, anti-carbohydrate antibodies including IgG anti-laminaribioside carbohydrate antibody (ALCA), IgA anti-chitobioside carbohydrate antibody (ACCA) and IgG antimannobioside carbohydrate antibody (AMCA) were also demonstrated to be specific to CD [87, 88]. ALCA is associated with ileocolonic CD and ACCA with penetrating and perianal disease [88]. IgA anti-outer membrane protein C (anti-OmpC), an antibody against a transport protein on the outer membrane of *E. coli* is mostly detected in the sera of CD patients with a prevalence value around 55% [87]. Faecal biomarkers like calprotectin, lactoferrin, Calgranulin C/S100A12, myeloperoxidase (MPO), and Matrix metalloproteinase 9 (MMP-9) are also valuable complementary tools for diagnosing and managing IBD [89, 90].

1.3.2 Treatments and therapeutic challenges

Currently, there is no curative treatment for IBD. Therapeutic goals have evolved to “treat-to-target” concept focused on mitigating inflammation and promoting endoscopic and histological healing. Consequently, the aim is to improve patient's quality of life by alleviating symptoms, achieving long-term remission, and preventing disease-related complications [91, 92].

The traditional therapeutic approach in IBD management has relied on progressive treatment intensification based on clinical symptoms, following a “step-up approach” [93]. In this method, treatment is prescribed in a stepwise manner, beginning with conventional first-line drugs, such as antibiotics and aminosalicylates, which carry limited toxicity. Depending on the response of patients, therapy progresses to more intense and aggressive treatments, including second-line drugs namely corticosteroids and immunosuppressants. Third-line therapies, such as biologics, are used in patients who fail to respond or are intolerant to previous therapies. Surgical resection is performed as final option when there is no control of inflammation. This may result in significant loss of the intestine and disability, potentially requiring permanent ostomies, which have a profound impact on the patient's life [94, 95].

Nevertheless, in some cases, according to disease severity, patients may undergo early administration of more potent drugs, defined as a “top-down approach”, which results in better outcomes [96]. Indeed, there is a critical early period following the onset of the disease denominated as “window of opportunity” in which the aggressive treatment seems to be more effective in hampering disease progression [97]. For example, initiating biologic therapies during this window may prevent disease progression and irreversible bowel damage, thus reducing the need for surgery in both CD and UC [98-101].

Treatment option includes antibiotics, aminosalicylates, corticosteroids, immunosuppressants and biologics [34]. Aminosalicylates are compounds that contain 5-aminosalicylic acid (5-ASA), such as mesalamine and sulfasalazine (SASP). These have multiple anti-inflammatory and immunomodulatory actions, such as scavenging reactive oxygen species (ROS); and inhibition of lipoxygenase and cyclooxygenase, interfering with pro-inflammatory cytokine secretion and induction of regulatory T cells (Treg). They are mainly used as primary treatment option to treat cases of mild-to-moderate and left-sided UC, being the second most prescribed treatment in IBD [102, 103]. Nausea, abdominal pain, diarrhoea, infertility, and haemolytic anaemia are some of the side effects of 5-ASA and SASP [104].

Corticosteroids, such as budesonide and prednisone, are commonly used for short-term treatment in moderate-to-severe CD and UC patients. They are highly effective in inducing

remission during flares, acting as broad-spectrum systemic anti-inflammatory agents, reducing the pro-inflammatory cytokine release [34]. Long-term treatment is not recommended due to the risk of adverse effects, such as increased risk of mortality, infection, osteoporosis, steroid-induced diabetes, insomnia and depression [34, 105, 106].

Immunosuppressors, like azathioprine (AZA), 6-mercaptopurine (6-MP), and methotrexate (MTX), control intestinal inflammation by restraining T lymphocyte proliferation and pro-inflammatory cytokines. They interfere with DNA synthesis, thus inhibiting the proliferation of these cells. Immunosuppressors have demonstrated efficacy in preventing recurrence and maintaining disease remission in both CD and UC [107-109]. Additionally, they are usually prescribed as adjuvant treatment to anti-TNF α biologics, to prevent the production of anti-drug antibodies [110].

In recent years, there was a significant progress in IBD therapeutic options, namely with the advent of biologics. These mainly comprise cytokine-targeted inhibitors such as anti-TNF α (infliximab, adalimumab, certolizumab, and golimumab), IL-12 (ustekinumab), IL-23 (ustekinumab, risankizumab, or mirikizumab), IL-6 (PF-04236921) and integrin antagonists (vedolizumab, natalizumab, etrolizumab or abrilumab) [98, 104]. Biological therapies are typically prescribed to patients who have moderate-to-severe active disease and act selectively on pro-inflammatory mediators relevant to IBD pathogenesis, being efficient in reducing symptoms, healing inflamed tissues, modifying IBD course and enabling long-standing remission. Others, such as the ones targeting integrins, are anti-adhesion molecules that prevents leukocytes infiltration contributing to the reduction of inflammatory response in the intestine. Despite their high efficacy and low toxicity, biological agents are extremely expensive [34, 98, 104]. Additional therapeutic strategies are also needed since there are still cases of primary no-response, secondary loss-of-response and adverse reactions in treatment with biologics [104].

Small-molecule drugs (SMDs) are emerging therapeutic agents in IBD [105, 111]. These include JAK inhibitors (e.g., upadacitinib, filgotinib, tofacitinib), which show efficacy in moderate-to-severe refractory UC and CD, and sphingosine-1-phosphate receptor modulators (e.g., etrasimod, ozanimod), which act as leucocyte trafficking/migrating inhibitors [34, 104, 112]. Other agents include toll-like receptor 9 (TLR9) agonists and human IL-22Fc Fusion Protein (UTTR1147A) a fusion protein that promotes IL-22 signalling pathway allowing tissue regeneration without immunosuppression [98].

Microbial-based therapies have also gained attention as IBD is closely linked to the imbalance of intestinal microbiota. Therapies such as probiotics, prebiotics, postbiotics, synbiotics, and faecal microbiota transplantation (FMT) have shown promise [113, 114]. Probiotics are live bacteria which can be obtained by non-digesting fermented foods [115]. For instance, *Bifidobacterium* given to UC patients significantly reduced clinical and

endoscopic disease activity [116]. Several studies have shown the potential effects of probiotics in UC, but this is less explored in CD [115, 117]. In turn, prebiotics are substrates utilized by host microbes and are easily found in fruits and vegetables. The three major classes of prebiotic are fructooligosaccharides (FOS), galactooligosaccharides (GOS) and human milk oligosaccharides (HMOs). They selectively stimulate the growth and/or activity of one or a limited number of bacteria in the colon, thereby improving intestinal environment [118]. Symbiotic are the combination of probiotics and prebiotics found in foods, drugs and supplements. They are often more effective in inducing or maintaining IBD remission since they combine the benefits of both probiotics and prebiotics [115, 119, 120].

Faecal microbiota transplantation (FMT) is a therapy that involves the transplantation of the “beneficial” microbiota from the faeces of healthy donors into the gastrointestinal tract of IBD patients, aiming to overcome of dysbiotic microbiota. The effectiveness of FMT in treating IBD is still not fully clear; besides, the choice of a suitable donor and patient is an issue under debate [121]. Nevertheless, successful FMT has been reported mainly in UC patients, demonstrating a positive impact in inducing remission in mild-to-moderate UC with rates of 33% [122, 123]. Moreover, a systematic analysis of several studies encompassing in total 122 patients have demonstrated that around 36–45% of IBD patients undergo clinical remission during follow-up [124]. Another systematic review focusing on the effectiveness of FMT in UC showed that approximately 42% of a total of 234 UC patients achieved clinical remission and 65% had a positive clinical response to FMT [125]. Considerably, FMT has been shown to be very promising in the treatment of recurrent and refractory *Clostridium difficile* infection, a major risk of IBD patients, with high success rate of 90% [126]. Likewise, there are positive findings for CD receiving FMT with clinical remission rates of 52% [123, 127]. Notwithstanding, there are several adverse events reported after FMT administration such as fever, abdominal pain, bloating, nausea, vomiting, diarrhoea, and constipation [124].

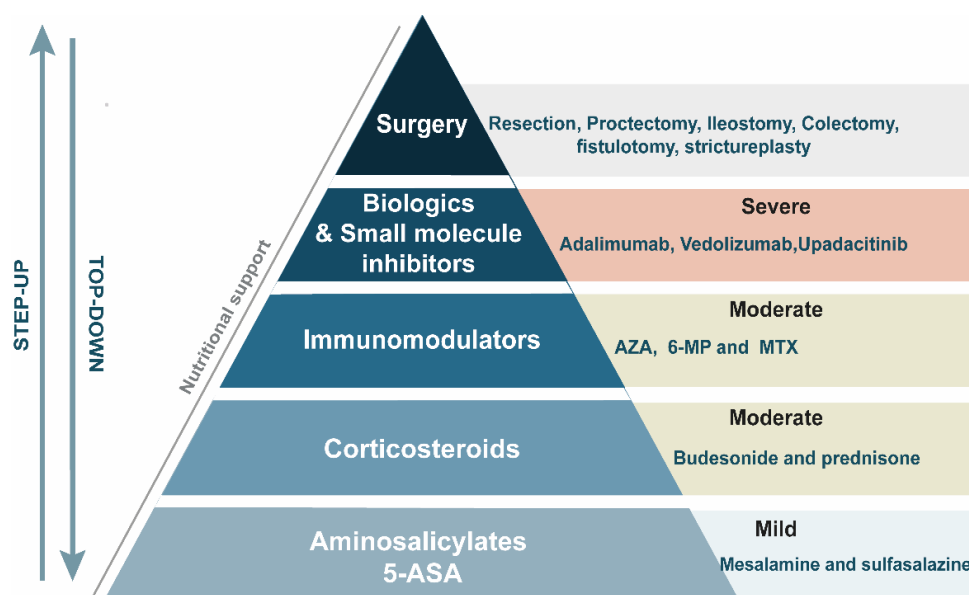


Figure 4. Therapeutic pyramid for IBD clinical management. Step-up approach: from mild to stronger and more toxic therapies. Top-down approach: early aggressive treatment with immunomodulators and biologic agents. ASA: Aminosaliclate; AZA: Azathioprine; MTX: Methotrexate; 6-MP: 6-mercaptopurine. Adapted from [114, 128].

1.3.3 Prognosis

Accurate and effective prognostics tools are extremely important to help to make informed choices regarding the possible disease course and the outcome of several available therapies. For instance, higher levels of faecal calprotectin were indicative of non-response to Infliximab (anti-TNF α) in severe UC patients [129, 130], while low values upon eight weeks of Vedolizumab treatment (anti- α 4 β 7 integrin) predicts a favourable response to therapy [131]. Furthermore, increased levels of ASCA were associated with an earlier onset of CD and increase disease severity [132, 133]. Higher values of CRP, faecal calprotectin, faecal lactoferrin and the presence of deep ulceration are indicative of increased risk to develop complicated CD (need for surgery, hospitalization, stricture formation, or combinations of these). Similar, the presence of anti-OmpC antibodies in CD patients indicate higher probability to require surgery by internal perforating inflammation. In turn, elevated pANCA seems to be associated with more severe UC and treatment resistance [34]. Moreover, it was reported a linked between the presence of pANCA and anti-OmpC antibodies and poor response to Infliximab therapy [134, 135].

In Europe and North America, the risk of colectomy varies between 1–7% after one year and 4–13% after five years after diagnosis. Similar tendencies are observed for Asian patients [136]. In CD, studies in patients from North America demonstrated that after five-

and ten- years of diagnosis the risk of undergoing abdominal surgery was 38.2% and 47.6%, respectively. In Europe, the risk ranges between 15–25%. Remarkably, a study performed in Epi-IBD cohort indicates that 13% and 22% of CD patients one- and five years, respectively, following diagnosis undergo intestinal resection [136]. This demonstrates the need of proper prognosis markers for IBD management, in order to adapt the therapy schemes to avoid severe complications.

Besides the complications inherent from the disease, it is known that patients with UC face an elevated risk of developing colorectal cancer. This risk is particularly pronounced in males and in those diagnosed with extensive colitis at a young age [15, 137, 138]. The main risk factors for colorectal cancer (CRC) in IBD are persistent active inflammation and immunosuppression. UC and CD patients display a 10 to 25 times higher risk of CRC compared with the general population [31].

A timely and appropriate treatment may prevent disease progression to a more severe, chronic, or refractory state. Hence, the early and precise diagnosis, combined with reliable prognosis, can help to discriminate patients and provide critical information to guide therapy decisions.

2. Intestinal microenvironment in homeostasis

The intestine is a complex ecosystem composed by a vast number of microorganisms collectively termed the microbiota, along with the epithelium barrier and mucosal immune system. The symbiotic host-gut microbiota interaction plays a vital role in the individual health status with a soft equilibrium between immune tolerance and host defence. Many benefits in nutrition, metabolism and immune maturation are provided by the microbiota, while immune and mucosal epithelial cells constitute a protective barrier against harmful microorganisms, preserving the integrity of the intestinal tract.

2.1 Gut microbiome in health

The human gastrointestinal tract harbours an abundant microbial community, consisting of approximately 100 trillion microorganisms namely bacteria, fungi (gut mycobiota) and viruses[139-141]. In the healthy human intestine, fungal diversity is generally lower than bacterial diversity, and is dominated by the phyla Ascomycota and Basidiomycota, making up 70% and 30% of the gut mycobiota, respectively. In descending order of abundance, the

core of fungal species include *Candida* (in particular *Candida albicans*), *Saccharomyces* (especially *Saccharomyces cerevisiae*), *Penicillium*, *Aspergillus*, *Cryptococcus*, *Malassezia* (mainly *Malassezia restricta*), *Cladosporium*, *Galactomyces*, *Debaryomyces*, and *Trichosporon* [142].

Bacteria, in turn, are the dominant microbial component of gastrointestinal tract, encompassing approximately 1,000 to 1,500 different bacterial species [143]. Phylum Bacteroidota (Bacteroidetes) and Bacillota (Firmicutes), which together make up over 90% of the healthy human microbiota, followed by Pseudomonadota (Proteobacteria), Actinomycetota (Actinobacteria), Fusobacteria, and other phyla [141, 144, 145]. The composition and distribution of microorganisms across the gastrointestinal tract is variable, with certain Proteobacteria components, such as Enterobacteriaceae, found only in the small intestine, while Bacteroidota, including *Bacteroidaceae*, *Prevotellaceae* and *Rikenellaceae*, are frequently observed in the colon [141]. However, the relative composition and distribution of microbiome is unique between individuals mostly due to age, genetics and environmental context [139, 143]. Although a clear definition of a “normal” healthy gut microbiome remains undetermined, it is generally accepted that the commensal microbiota is characterised by higher diversity and richness of beneficial bacteria and a lower presence of pathogenic bacteria or pathobionts [139, 143].

Host-gut microbiota relationship is mutually beneficial through a range of mechanisms. While the intestine provides an distinct place for microorganisms’ colonization, with higher abundance of nutrients, commensal gut microbiota contributes to the education of host mucosal immune system without producing any pathological symptoms. Indeed, the microbial colonization of neonatal gastrointestinal tract is a critical event for the initiation of host immunity development and maturation [146]. Additionally, gut microbiota influences different biological processes, providing metabolites that stimulate signalling pathways and promote gut homeostasis [145, 147]. In this line, gut microbiota holds numerous enzymes that ferment complex host and diet carbohydrates [140, 148]. As a result of microbial fermentation, several metabolites are produced including short chain fatty acids (SCFAs), secondary bile acids (BAs), tryptophan metabolites and vitamins [149].

SCFAs are organic products mainly composed of acetate, propionate, and butyrate, and are key products of microbial fermentation [150]. *Faecalibacterium prausnitzii*, *Clostridium leptum* (*Ruminococcaceae* family), *Eubacterium rectale* and *Roseburia spp* (*Lachnospiraceae* family) are pointed out as main butyrate-producing bacteria, followed by members of the phyla Fusobacteria, Proteobacteria, Actinobacteria, Spirochaetes and Thermotogae [145]. Members of Bacteroidota and Verrucomicrobia phyla, for instance *Akkermansia muciniphila*, are associated with secretion of acetate and propionate [145, 151].

SCFAs play a critical role in maintaining host homeostasis. They provide an energy source for colonocytes and regulate important physiological functions of intestinal epithelium barrier and immune system [149, 150]. SCFAs enter the epithelial and immune cells via proton-coupled monocarboxylate transporter 1 (MCT1) and sodium-coupled monocarboxylate transporter 1 (SMCT1) [145, 152]. Furthermore, they can bind G-protein-coupled receptors (GPCRs) with variable affinities, activating anti-inflammatory signalling pathways. Some examples are GPR41, GPR43 and GPR109A, expressed in adipocytes, colon epithelial and immune cells [145].

Among SCFAs, butyrate is particularly important. Balanced levels of butyrate facilitate the proliferation [149] of intestinal epithelial cells (IECs) and increase the expression of tight junctions, contributing to strengthen epithelial barrier [145]. Also, butyrate enhances mucus production and modulates the immunological function of IECs by regulating gene expression of cytokines [149]. Specifically, activation of GPR41 and GPR43 drives the defence against pathogenic bacteria, leading to the production of cytokines and chemokines by colonic epithelium. In turn, butyrate elicits the expression of IL-18 via GPR109a activation, protecting towards intestinal inflammation. Importantly, butyrate released in gut environment leads to HIF stabilization, contributing for tight junction regulation, MUC2 expression, and production of host defence antimicrobial peptides (AMPs), such β -defensin 1 [153, 154].

Both innate and adaptive immune responses are also critically regulated by gut microbiota and their metabolites. For instance, SCFAs exert anti-inflammatory effects in intestinal mucosa by histone deacetylases (HDACs) inhibition and GPRs signalling, mediating further cell migration and activation. Specifically, butyrate regulates intestinal immune cells including macrophage and dendritic cells (DCs), either suppressing proinflammatory cytokine production (IL-8, IL-6, TNF- α , IFN- γ , and IL-1 β) or upregulating IL-10 secretion [155, 156]. Notably, butyrate regulates IgA secretion, controlling gut microbial community and limiting the hyperresponsiveness of macrophages towards intestinal commensals [157]. In turn, acetate controls immune cell recruitment via GPR43, inhibiting neutrophil migration [155]. SCFAs also play an important role in helping to prevent the colonization of opportunistic pathogens, such as *Salmonella enterica*, *Citrobacter rodentium* and *E. coli*. As example, butyrate boosts gut defence of macrophages against invasive pathogens without causing tissue-damaging inflammation, while acetate elicits pathogen clearance through ROS activation by neutrophils [155].

Additionally, gut microbiota has an active role in the education of adaptive immunity, mediating adequate gut B cell responses and T cell differentiation towards Th1 and Th17 subtypes [145, 158, 159]. An early B cell lineage is strongly regulated by signals from commensal microbiota, influencing the repertoire of intestinal immunoglobulins [160]. Treg

cells are also affected by intestinal microbiota; for instance, *Clostridium* clusters XIVa and IV and *Bacteroides* species play a crucial role in boost gut homeostasis by inducing the accumulation of Treg cells [161, 162]. Moreover, butyrate enhances Foxp3⁺ expression, thereby facilitating the differentiation and expansion of intestinal Treg cells [145, 159]. In the same line, *Lactobacillus rhamnosus* and *Lactobacillus reuteri* foster IL-10-producing Tregs [163]. Several members of gut microbiota, including *Enterobacter aerogenes*, *Clostridium limosum*, *Clostridium tetani*, *Clostridium lentoputrescens*, *Clostridium bifermentans*, *Clostridium melanomenatum* (Bacillota phylum members), as well as Fusobacteria, Proteobacteria and some members of Bacteroidota phylum, promote IL-22-derived homeostatic immune response as a result of tryptophan fermentation [145]. The commensal bacterial tryptophan fermentation also can produce indole, which is known to enhance the expression of tight junctions such as occludins and claudins in IECs [164, 165].

Importantly, alongside its synergy with the immune system, the commensal microbiota selectively modulates the gut bacterial community, preventing the invasion of external pathogens and limiting the growth of resident pathobionts via direct commensal-pathogen interaction (a protective mechanism known as colonisation resistance) [160, 166]. Despite the precise mechanisms by which commensal gut microbiota confer colonization resistance are not yet fully understood, many of these mechanisms comprise direct interactions, including nutrient competition, niche exclusion, production of toxic substances and metabolite production (e.g bacteriocin, secondary bile acids and SCFAs) [167].

2.2 Gatekeepers of the Gut: immune-epithelial dynamics in microbiota regulation

The intestinal mucosal barrier is traditionally recognised as the first line of defence, equipped with a multi-layered system of chemical and physical components that are essential to maintain the intestinal microbiota compartmentalized from the mucosa [140]. Markedly, there is a gradual increase in the complexity of mucosal barrier composition along the intestine, possibly fostered by the higher number of microorganisms [168]. In the small intestine, the mucosal barrier is essentially formed by a chemical layer and by IECs accompanied by a very thin and discontinuous layer of mucus. On the other hand, closer to and within the large intestine, there is a solid physical barrier consisting of an outer loose mucus layer, an inner firm mucus layer, the intestinal epithelium, which are further reinforced by the chemical layer [140].

The chemical layer contains a pool of AMPs involved in the control of gut microbiota. Most intestinal AMPs, including α -defensins, lysozyme C, C-type lectins (CTLs), such as Reg3 family proteins, and secretory phospholipase 2 (sPLA2), are constitutively shed by Paneth cells in the small intestine (cells usually absent in the large intestine), while β -defensins and cathelicidins are more commonly expressed in the colon [139, 149, 169, 170]. The multiple layers of mucus observed in the large intestine are supported by the higher number of goblet cells [149] which synthesize and secrete gel-forming mucin, namely hyperglycosylated MUC2 - the main component of the intestinal mucus [149]. The mucins of the inner mucus layer are insoluble, firmly attached to the epithelium and are progressively converted to the soluble and expanded outer mucus layer via proteolytic cleavage by the host or gut bacteria [171]. Conversely the outer mucus layer, which is in constant contact with the commensal intestinal microbiota, the inner mucus layer is devoid of bacteria. This is mainly due to the dense network of mucins, and due to the continuous secretion of several antimicrobial molecules (chemical barrier), by epithelial and immune cells, which further shields against the invasion and overgrowth of symbiotic microbiota [149].

In addition to the double mucus barrier, IECs display on the uppermost surface a network of carbohydrate moieties of glycolipids and glycoproteins, including transmembrane mucins, called the glycocalyx (the role of glycocalyx will be further discussed in detail in the following sections). This act as an additional physical wall that hampers microbial invasion of the mucosa [140]. Importantly, the expression of different tight (e.g. claudins, occludins) and adherents (e.g. E-cadherin) junctions constitutes a fundamental feature that governs the connection of IECs and the paracellular permeability, thereby controlling the influx of luminal elements [149]. IECs are also equipped with several pattern recognition receptors

(PRRs), such as Toll-like receptors (TLRs) and NOD1 and NOD2. In particular, the production of antimicrobial molecules by IECs driven by gut microbes is regulated by the TLR4/MyD88 and NOD2 signalling pathways [140]. Moreover, the expression of these receptors allows IECs to recognise bacterial components and respond by producing cytokines and chemokines that recruit immune cells, actively participating in immune surveillance, beyond their direct defence against microorganisms [139].

Therefore, in parallel with the epithelial barrier, several immune cell populations resident in lamina propria are key sentinels programmed to hinder harmful antigens. Immune cells continuously communicate with luminal microbes or their products through diverse receptors (e.g TLRs, NOD, leucine rich repeat receptors (NLRs), C-type lectin and Fcγ receptors) [140, 149]. In specific, bacterial sensor NOD2 plays an essential role in limiting the growth of commensal bacteria, namely *Bacteroides vulgatus* [160].

Among the subsets of antigen-presenting cells (APCs), DCs are one of the dominant subtypes in the intestinal lamina propria being able to recognize, process, and present antigens. They can extend through the interstices of epithelium (extension of transepithelial dendrites) and access the lumen, where they directly phagocytose harmful microbes, such as *Salmonella* and *E. coli* [172]. Likewise, DCs can continuously sample luminal antigens and transport them to gut-associated lymphoid tissues, such as mesenteric lymph nodes (MLNs) and Peyer's patches. There, DCs present antigens to T cells, regulating adaptive immune responses and preventing unnecessary inflammatory responses to commensal bacteria [139]. In this line, under non-inflammatory conditions, DCs play an important role in regulating intestinal immune tolerance. DCs facilitate the differentiation of naïve CD4⁺ T cells into Tregs and various T helper (Th) cells, including Th1, Th2, and Th17 [149]. In homeostasis, Th17 cells and Tregs are in dynamic balance. Commensal bacterial products activate TLR signalling, particularly TLR2/TLR1, which fosters tolerance to luminal pathogens by downregulating PRRs, by inhibiting Th17 differentiation, and enhancing Treg activity along to IL-10 secretion. This ultimately allows the immune tolerance and mucosal wound healing [161]. IL-10 downregulates antigen presentation and acts on APCs to prevent their activation and release of proinflammatory molecules, thus hindering T cell recruitment into the intestine [173]. Furthermore, through the production of large amounts of cytokines and chemokines, such as IL-23, IL-6 and IL-1β, DCs induce the activation of homeostatic innate lymphoid cells (ILCs) [149].

ILCs are emerging family of innate immune cells enriched at barrier surfaces, that has been recognized as essential in initiating, regulating, and resolving inflammation [174]. As tissue-resident lymphocytes they mediate an essential part of innate mucosal immunity, specifically the maintenance of intestinal homeostasis [175, 176]. ILCs encompass several immune cell subsets, such as type 1 ILCs (ILC1) constitutively express T-box transcription

factor Tbet; type 2 ILCs (ILC2) expressing GATA binding protein 3 (GATA3) and type 3 ILCs (ILC3) which express retinoic acid-related orphan receptor gamma t (ROR γ t).

ILC1 population is heterogeneous comprising distinct subsets, namely the intraepithelial CD127⁻ILC1, CD127⁺ ILC1 and Natural killer (NK) cells. ILC1 are particularly important in immunity to intracellular pathogens [177]. While CD127⁻ ILC1 is linked to a more cytotoxic effect similar to CD8⁺ T cells phenotype, CD127⁺ ILC1 are broadly noncytotoxic, being comparable to Th1 cells, producing IFN- γ in response to IL-12, IL-18 and TNF- α [175, 178]. Conventional NK cells express Eomesodermin (EOMES) but not Tbet [174]. They have a cytotoxic profile due to their capacity to produce IFN- γ , TNF- α , perforin and granzymes. In the intestine, NK cells activity is strongly relied on priming by commensal activated-DCs, further using different TLRs to interact with bacteria [179]. It is noteworthy that NK cell subsets can be defined based on the expression of CD56 and CD16 (FCR γ III) on the cell surface. The latter enables antibody-dependent cellular cytotoxicity [180]. ILC3 constitute the majority of ILCs present in the intestine and exert homeostatic control of intestinal immunity, mainly due to the production of IL-22, as well as IL-17 and GM-CSF [178, 181]. ILC3 embrace different cellular subsets such as CCR6⁺ lymphoid tissue-inducer (LTi) cells, natural cytotoxicity receptor (NCR)⁺ILC3s and NCR⁻ILC3 [182, 183]. LTi-like ILC3s are enriched in the intestine and lymphoid tissues [174]. ILC3 are usually involved in the immune response to extracellular pathogens or fungi and the regulation of adaptive T cell responses through the expression of class of major histocompatibility complex (MHC) II molecules in response to commensal microbiota [178]. ILCs are highly plastic and can undergo different functional phenotypes according to the environmental cues [182, 184]. For instance, ILC3 can acquire an ILC1-like phenotype by increased Tbet expression and downregulation of ROR γ t, being able to produce IFN γ [181].

Hence, the ability of ILCs to polarize into the different phenotypes place them as key dynamic players either in homeostatic/protective responses or in the development of inflammation. In the intestinal mucosa, IL-22 produced by ILC3s limits the growth of segmented filamentous bacteria (SFB), which are also known to stimulate Th17 cell response [139, 149]. The gut harbours distinct populations of Th17 cells whose inflammatory potential is largely influenced by the bacteria responsible for their differentiation. For instance, Th17 cells induced by SFB are non-inflammatory, whereas those induced by *Citrobacter* are a potent source of pro-inflammatory cytokines in mice [185]. Importantly, IL-22 and IL-17 produced by Th17 cells or ILC3 act in an alliance to limit microbial invasion [179]. Nevertheless, ILC3 are considered the main intestinal IL22-producing cells and thus a key element supporting to the resolution of inflammation in the gastrointestinal tract [174]. Notably, in the gut the receptor for IL-22, the IL22R1, is mainly expressed by IECs making them a relevant target of IL-22 produced by the immune cells

[179]. Pleiotropic effects of IL-22 help strengthen the epithelium, promotes the expression of AMPs such as Reg3 family proteins, and increases mucus secretion from goblet cells. This reduces bacterial translocation across the epithelial barrier, protecting against infection by *C. rodentium*, ultimately promoting the balance of the commensal microbiota [149, 179]. Likewise, NCR⁺ ILC3 cells were demonstrated to be essential for maintaining cecal homeostasis in mice during *C. rodentium* infection [160].

The impact of several immune mediators can be defined by the context in which it occurs. As an example, excessive IL-22 secretion can be also detrimental, since IL-22 can boost the pathogenic *Salmonella Typhimurium* colonization in the inflamed intestine, by suppressing commensal *Enterobacteriaceae* via AMPs induction [186]. In parallel, while an excess of IL-17 contributes to inflammation and tissue damage, its presence is fundamental to control fungal and bacterial pathogenic species (e.g. *Candida*, *Cryptococcus*, *Klebsiella* and *Staphylococcus*), thereby maintaining a healthy balance of gut microbiota [187]. IL-17 is produced at low levels to help maintain a balanced microbiota. It enhances the epithelial barrier defences in response to resident microbes by stimulating the production of AMPs, helps to reinforce the epithelial barrier by inducing tight junction formation and promoting intestinal immunoglobulin A (IgA) secretion [179, 188]. In addition, IL-17 aids in the recruitment of neutrophils to control pathogens [189].

In addition, B cells within the intestinal mucosa are committed in maintaining the intestinal barrier homeostasis by producing a wide range of secretory IgA in response to commensals. The IgA repertoire is a mainstay of protective humoral mucosal immunity and, together with AMPs, aids to protect the intestinal epithelium from bacterial overgrowth, contributing to the balanced microbiome [149]. Secretory IgA can be produced in either a T cell-independent or in a dependent manner. As example, Treg cells stimulated by CBir1, a microbiota flagellin, induce the proliferation of IgA-producing B cells, which in turn leads to a reduction in the pathogenic burden and downregulation of systemic T-cell activation [160, 161].

In summary, the gut microbiota and the immune response are in constant interaction, with each influencing the other. The balance of intestinal immunity, involving DCs, ILCs, Th17 and Tregs, is largely governed by signals from gut bacteria, which are closely linked to the composition of the microbiota (as partially discussed in the previous section). In turn, gut immunity plays a pivotal role in shaping the microbial configuration. While the gut microbiota can enhance mucosal barrier function, it does so directly or indirectly by triggering immune responses activated by both commensal and pathogenic bacteria.

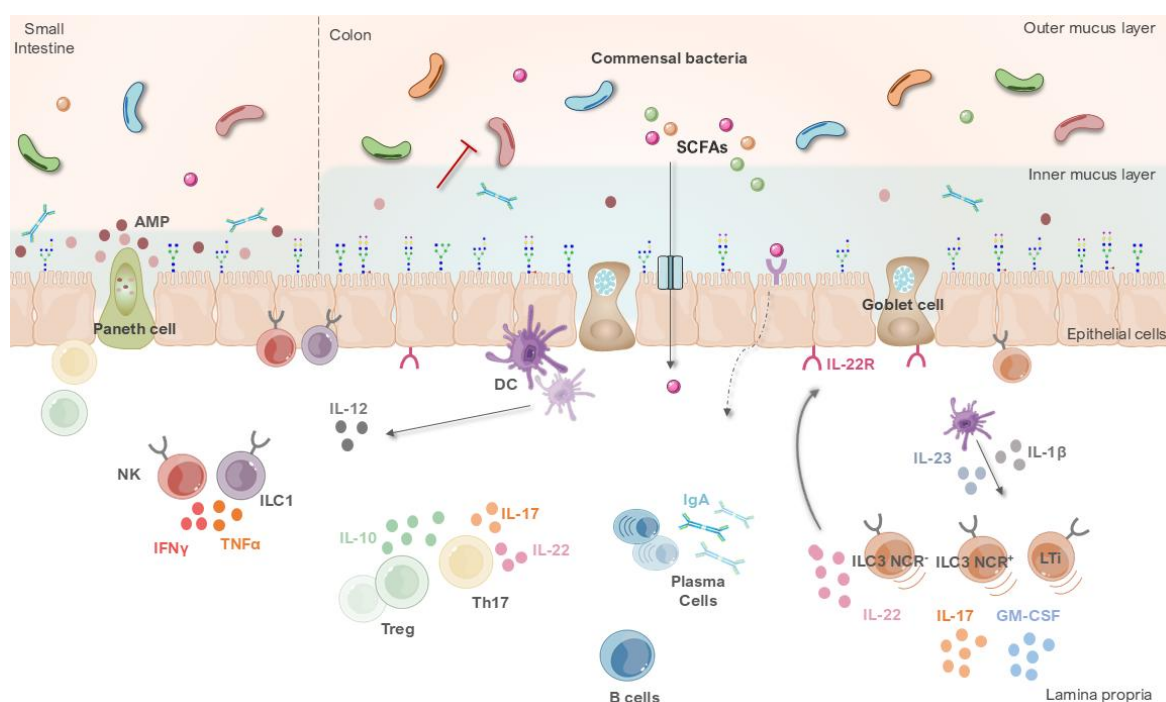


Figure 5. Dynamic interactions between the gut microbiota and the immune system. AMPs: antimicrobial peptides; SCFAs: short-chain fatty acids.

3. Gut microbiome & intestinal inflammation in IBD pathogenesis

Aberrant interactions between the microbiome and the host immune system have been implicated as one of the major mechanisms contributing to the development of complex immune-mediated diseases such as IBD.

3.1 Gut microbiota in IBD

Several lines of evidence point to a central role of gut microbiome imbalance in the pathogenesis of IBD, mainly due to its impact on the inflammatory response and the integrity of the intestinal barrier. The gut microbiota of patients with IBD is characterized by a marked shift in the abundance of certain bacterial taxa, accompanied by reduced bacterial α -diversity and richness – a condition termed dysbiosis [145]. The decline in beneficial, anti-inflammatory bacteria and an overgrowth of opportunistic or pathogenic microorganisms are often characteristics of gut dysbiosis [190].

The analysis of the gut microbiome signature of healthy individuals and of IBD, has revealed clear differences. The patterns of dysbiosis in CD and UC vary between studies, possible due to the significant inter-individual variability in microbiota composition [191]. Nevertheless, IBD-related dysbiosis is typically associated to a decreased abundance of

bacterial taxa from the Firmicutes and Bacteroides phyla, and increased abundance of Proteobacteria [40].

Compared to healthy individuals, IBD patients often display increased levels of potentially pathogenic members of Proteobacteria (e.g *Escherichia*, *Salmonella*, *Yersinia*, *Desulfovibrio*, *Helicobacter*), as well as species from the families *Pasteurellaceae*, *Veillonellaceae*, *Fusobacterium* species, and mucolytic bacteria *Ruminococcus gnavus* [40, 121, 190]. Notably, *Fusobacterium* species, alongside with *Enterococcus faecalis*, are common residents of the human GI tract and have been recognized as opportunistic pathogens implicated in IBD [192, 193]. The invasive ability of *Fusobacterium* and *E. faecalis* was shown to be positively correlated with the severity of IBD. Indeed, increased levels of mucosa-associated *Fusobacterium* and *E. faecalis* are observed in UC patients, correlating with disease activity, along with elevated *E. faecalis*-specific IgG reactivity [194-196]. Similarly, adherent-invasive *E. coli* (AIEC) strains have been isolated from ileal CD and UC biopsies. The presence of higher abundance of AIEC and *E. faecalis* in IBD patients have been associated with an impact in intestinal permeability, alter gut microbiota composition, and contribute to intestinal inflammation [197, 198].

In contrast, IBD patients tend to show reduced representation of bacteria belonging to the genera *Bacteroides*, *Eubacterium*, and *Lactobacillus*, as well as members of the *Clostridiales* order. Key species from the Firmicutes phylum, including *Faecalibacterium*, *Ruminococcus*, and members of the *Lachnospiraceae* family (such as *Roseburia* and *Blautia*), are typically underrepresented [121, 141, 145, 193]. Notably, many of these bacteria are SCFAs producers, namely *F. prausnitzii*, *Ruminococcus torques*, *Roseburia inulinivorans*, *Roseburia hominis*, *Blautia faecis*, and *Clostridium* cluster IV/XIV species [145, 193, 199, 200]. Other SCFA-producing bacteria, such as *Phascolarctobacterium* and *Leuconostocaceae*, are reduced in CD and UC patients, respectively [193]. This imbalance is reflected by lower SCFAs concentrations, specifically butyrate, in tissue and faecal samples of patients with IBD, and significantly correlates with higher levels of inflammatory markers, reflecting increased inflammation [190, 201].

In addition to bacterial dysbiosis, recent evidence suggests that the fungal community (mycobiota) is also skewed in IBD [202, 203]. The detection of high levels of ASCA in the blood of CD patients is one of the most powerful pieces of evidence pointing to the role of mycobiota in the development and progression of intestinal inflammation [204]. Fungal dysbiosis in IBD is mainly characterized by an increased Basidiomycota/Ascomycota ratio [203]. Studies identifying disease-specific patterns of fungal diversity have suggested that the gut environment in CD may favour fungal overgrowth at the expense of bacteria, in contrast to UC and CD patients without ileal involvement, which tend to show reduced fungal diversity [202]. Accordingly, in both stool and biopsy samples, decreased levels of *S.*

cerevisiae have been reported in patients with CD, whereas *Candida* spp, *specifically Candida tropicalis*, *C. albicans*, *Candida glabrata*, as well as *Clavispora lusitanae*, *Cyberlindnera jadinii*, and *Kluyveromyces marxianus*, showed to be overrepresented compared with healthy individuals [142, 205-207]. Furthermore, the presence of *S. cerevisiae* and *Filobasidium uniguttulatum* species were associated with non-inflamed mucosa [202].

The use of microbial markers has been demonstrated to facilitate the prediction of disease progression and the assessment of treatment outcomes. Increased ileal abundance of *F. prausnitzii* predicts increased risk of post-operative recurrence in CD patients [200]. Besides that, certain dysbiotic-microbial signatures aids in predict response to therapy being, for instance, associated with relapse in patients who ceased infliximab treatment [193].

3.2 The inflammatory immune response in IBD

Intestinal barrier dysfunction is strongly associated with intestinal inflammation and is proposed as a risk factor for the development of IBD [208]. A defective barrier is characterized by a thinner mucus layer, epithelial injury, abnormal expression of tight and adhesion molecules and impaired AMPs [193, 208, 209]. Analysis of UC and CD inflamed tissue have revealed significant alterations in the levels of claudins, specifically an increase in pore-forming claudins, and a reduction in those that perform a sealing function [210]. Indeed, overexpression of claudin-1 (sealing tight junction) and claudin-2 (pore-forming tight junction) has been detected in patients with active IBD and positively correlated with inflammatory activity [211].

The immune response in UC and CD is characterized by an imbalance between anti-inflammatory and pro-inflammatory signals, with a significant shift of leukocytes towards the intestinal epithelium. Nonetheless, there are some differences regarding the mechanisms involved in the inflammatory response between the IBD subtypes.

Innate immune response in IBD is characterized by a marked presence of DCs, macrophages and neutrophils [48]. In IBD patients, colonic DCs display an abnormal immature phenotype showing enhanced expression of CCR9 and integrin β 7, which are associated with leukocytes adhesion and recruitment [212]. Moreover, colonocytes and mucosal DCs show abnormal increase in TLR2 and TLR4 expression, which has been associated with improper activation of innate and adaptive immune responses [48, 212]. Upon activation of PRRs, several signalling pathways, including NF- κ B, are triggered, leading to the persistent release of inflammatory cytokines, such as IL-23, IL-12, IL-1 β , IL-

6, IL-18, and TNF- α . This inappropriate and sustained cytokine production promotes and exacerbates local intestinal inflammation [48, 212]. Consistently, elevated levels of IL-6 and TNF- α are frequently observed in both UC and CD patients. Importantly, serum levels of TNF- α strongly correlates with the clinical disease severity [213, 214]. Mucosal DCs from CD patients exhibit higher CD40 expression and increased IL-6 and IL-12 secretion comparing to cells from healthy individuals [212]. DCs, in combination with other myeloid cells as well as IECs, play a pivotal role in the activation of ILCs in the context of inflammation [215, 216]. IECs contribute to ILC activation through the secretion of IL-18, IL-33, and IL-37. It is noteworthy that IL-33 levels are elevated in the intestinal mucosa of IBD patients, thus facilitating the exacerbation of inflammation associated with colitis [48, 217].

Considerably, intestinal inflammation in IBD is densely associated with substantial alterations in the composition of ILC pools. Indeed, activated ILCs contribute to the inflammatory burden through the enhanced secretion of IFN- γ , IL-17 and IL-22 [48]. Several studies have pointed out the increased accumulation of CD127⁺ ILC1 subset and respective higher IFN- γ secretion (drive by IL-12 and IL-18 signalling), in the inflamed tissues of CD patients [218, 219]. Accordingly, an increased local secretion of IL-12 was reported in CD [220]. The raised levels of ILC1 are accompanied by a decline in ILC3 populations, particularly NCR⁺ ILC3s, along with a reduction in IL-22 production. This was demonstrated to correlates with increased disease severity [218, 221-223]. Moreover, contrary to CD patients, UC patients have been associated with higher accumulation of NKp44⁻ ILC3s [223]. Notably, in paediatric IBD patients, MHCII expression on colonic ILC3 was found to be reduced along with increased Th17 frequency. This suggests that MHCII-expressing ILC3 may exert a protective regulatory influence over Th17 activity in IBD [178]. It is important to highlight that some contradictory evidence exists, indicating that both UC and CD exhibit enhanced capacity for IL-22 production by ILC3. This suggests the possibility of the potentially pathogenic effect of IL-22, at the expense of a protective and regenerative role [178], which again demonstrates the relevance of context for the impact of immune mediators in the gut. A greater number of ILC2s were also observed in the inflamed tissue of UC patients in comparison with non-inflamed regions or healthy individuals [223]. This finding is consistent with the elevated levels of IL-33 observed in UC, as previously mentioned. Although IL-33 have been indicated as a stronger inducer of proliferation and activation of ILC2s, the effective role of IL-33/ILC2 axis in IBD is not consensual, since some evidence point towards either a pathogenic or protective effect [224, 225].

Alterations in the frequency of NK cells have been also reported in intestinal inflammation. Increased numbers of CD16⁺ NK cells were detected in the colonic lamina propria of IBD patients compared to controls [226]. CD56⁺ HLA-DR⁺ NK cell subset was associated with intestinal inflammation of UC patients, while NKp46⁺ NK cells might mediate

the pathogenesis of CD by producing IFN- γ and facilitating Th17 differentiation [175, 221]. Also, the production of IFN- γ is known to promote gut inflammation by reducing tight junction integrity, enhancing leukocyte recruitment, and stimulating phagocytes [175].

Components of the innate immune response not only contribute to inflammatory overload, but also play a crucial role in triggering adaptive immunity essential for the progression of inflammation at both local and systemic levels. One of the hallmark features of IBD is the infiltration of inflammatory CD4⁺ T cells in intestinal tissue. UC is thought to be driven by higher activity of Th2 intestinal immunity, with increased levels of IL-13 and IL-5. On the other hand, CD is often associated to a Th1-driven inflammation with high IFN- γ levels in response to IL-12 and IL-18 [48]. Despite this, Th17 cells are considered a key pathogenic factor in both UC and CD [227]. Although Th17 cells and related cytokines play a critical role in fighting infection and ensuring intestinal homeostasis, when dysregulated, the hyperproliferation and activation of these cells induce abnormal immune responses that can exacerbate intestinal inflammation [227]. IL-23, IL-12 and IL-1 β are pivotal in driving the transition of Th17 cells to a pro-inflammatory state, promoting the proliferation and accumulation of Th17 cells in the gut. At the same time, they inhibit the differentiation of induced Tregs and suppress IL-10 production in the colon [228]. Multiple evidence shows a massive infiltration of Th17 cells alongside with elevated Th17-related cytokines, particularly IL-17, in the inflamed intestinal mucosa and serum of patients with active IBD when compared to those patients in remission or healthy individuals [214, 229, 230]. Accordingly, RNA transcripts for IL-17A and IL-17F are significantly upregulated in the inflamed mucosa of IBD individuals. Besides this, elevated levels of Th17 cells and their associated cytokines in IBD patients correlate with disease activity, endoscopic and histological severity, as well as with markers like CRP. In line with this, patients with quiescent UC, display increased levels of IL-17A, IL-17F, and IL-21 in the colonic mucosa, which have been linked to higher rates of relapse [228]. Th17 can transdifferentiate to a Th1-like phenotype (IFN- γ ⁺ IL-17A⁺ Th cells) under the influence of IL-23 and IL-12 [228]. Indeed, patients with IBD display an increased presence of IFN- γ ⁺ IL-17A⁺ Th cells in inflamed mucosal tissues [231]. Interestingly, correlation analysis between IL-23 expression and the transcription factors ROR γ t, T-bet and Gata3 in biopsies from UC patients revealed that besides IL-23 showing lower correlation with ROR γ t in UC inflamed tissue, IL23 strongly associated with T-bet and GATA3 expression. These observations suggest that IL-23 is potentially associated with transdifferentiating Th17 cells towards a more pathogenic/inflammatory phenotype [228].

In the context of inflammation, the balance between Th17 cells and Tregs shifts to favour Th17. IL-10-producing Tregs regulate Th17 cell proliferation by inhibiting ROR γ t via FOXP3 signalling. Additionally, Tregs suppress the proliferation and activity of Th1 and Th2 subsets

by secreting IL-10 and TGF- β [232]. In fact, in CD, the number of Tregs and expression of TGF- β were found to be downregulated [227]. In agreement, it has been detected a specific subset of IL17-producing FoxP3⁺ T cells in the inflamed mucosa of CD patients, supporting the Treg/Th17 axis [233, 234]. The origin and the specific mechanisms of action of this distinct cell population is not completely clear; however, growing evidence have pinpointed a potential involvement of gut microbiota in shaping these cells [235].

The dysregulation of humoral immunity has been widely reported to be a key feature of IBD. A significant genetic alteration detected in UC is on *FCGR2A*, a gene encoding an activating IgG Fc γ receptor (Fc γ R) [236, 237]. Fc γ R signalling is linked to several pro-inflammatory effector functions in myeloid cells, which are known to play a role in the pathogenesis of IBD, as illustrated earlier. The associated-Fc γ R signalling outcomes comprise Th17 cell polarisation, pro-inflammatory cytokine production, antigen presentation, activation of the complement system and ROS production. In line with this, a considerable increase in the number of neutrophils expressing Fc γ Rs has been found in the inflamed mucosa of IBD patients [238, 239].

Additionally, increased infiltration of IgG⁺ B cells, plasma cells, as well as enriched microbiota-reactive IgG levels, in the inflamed tissue are also important pathological hallmarks of IBD [240, 241]. Increased expansion of B cells alongside with higher expression of BAFF, a B cell activation factor, have been observed in UC patients [242]. Furthermore, in contrast to CD patients, which display a smaller pool of mucosal IgG plasma cells mainly composed by IgG2, UC patients show an higher accumulation of IgG⁺ plasma cells, specifically, IgG1⁺ [243].

The humoral response in IBD is characterized by the presence of two main groups of antibodies: some targeting microbial antigens, and also autoantibodies which recognize intestinal and non-intestinal self-antigens [244]. As previously outlined, UC individuals have higher anti-tropomyosin 5 (TM5) IgG1 autoantibodies, ANCA and antibodies against goblet cells (GAB) [242, 245]. On the other side, CD patients have increased levels of IgG and IgA against *S. cerevisiae* (ASCA), flagellin (the anti-Cbir1), and against *E. coli* (anti-OmpC) [242]. Remarkably, it was demonstrated that commensal-targeting IgG, which are present in higher abundance in the colonic mucosa of UC patients, play a pivotal role in the pathogenesis of intestinal inflammation and Th17 immunity. Specifically, this commensal-targeting IgG is described to activate colonic macrophages through Fc γ R signalling, thereby inducing NLRP3 and ROS pathways. This activation results in the production of IL-1 β , and neutrophil-recruiting chemokines and the enhancement of intestinal inflammation associated with IL-1 β -driven type 17 immune responses [246]. In accordance with these observations, it was demonstrated that IgA reactive to bacteria, such as IgA-coated *E. coli*

detected in IBD patients, are involved in the inflammatory processes characterized by mucosal and systemic Th17-dependent inflammation [247, 248].

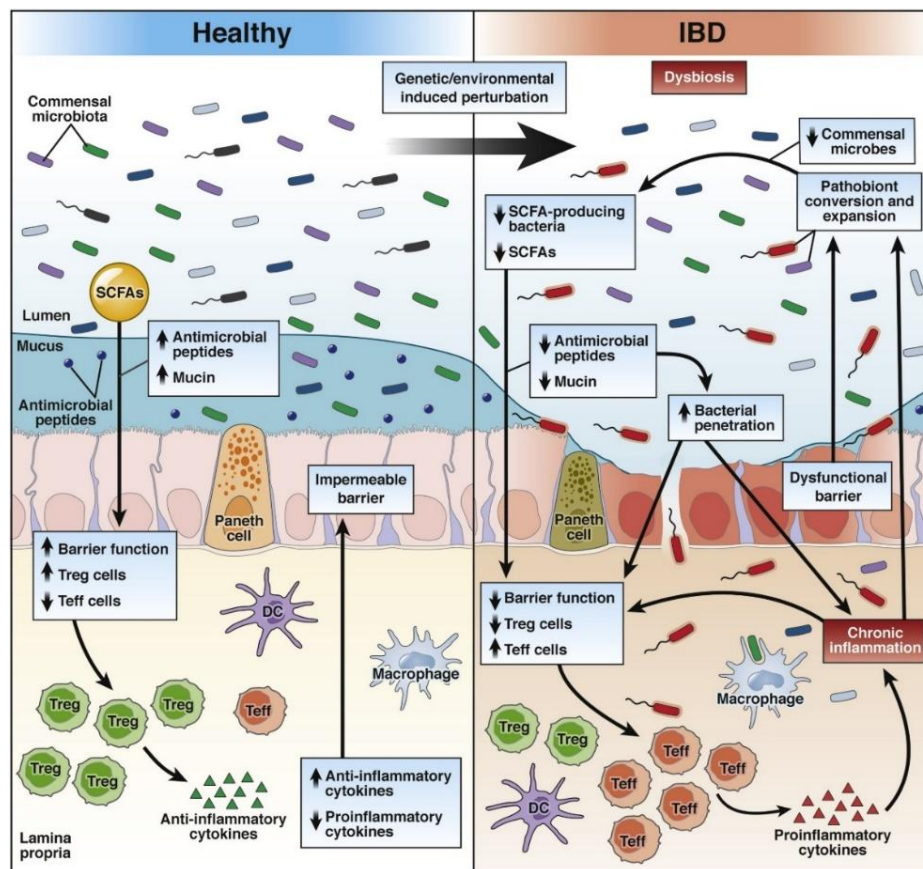


Figure 6. Defective mucosal barrier in IBD. The gut microbiota composition is altered (dysbiosis) characterized by the proliferation of opportunistic pathogenic microorganisms and impaired SCFAs secretion. The integrity of the gut barrier is compromised with a thinner mucus layer and reduced expression of tight junctions, leading to increased paracellular permeability. Consequently, a decreased production of antimicrobial peptides occurs, favouring the inefficient control of pathogenic bacteria invasion. The unbalanced intestinal immune system leads to an over-secretion of proinflammatory mediators and exacerbated inflammatory response. Chronic inflammation further aggravates gut barrier dysfunction leading to a vicious cycle. SCFAs: short-chain fatty acids; Treg: regulatory T cells; Teff: effector T cells; DC: dendritic cells [249].

4. Cellular glycosylation

The surfaces of all living organisms, cells, and most secreted proteins share a common feature: they are glycosylated [250]. Glycans—saccharides or sugar chains—are highly diverse and can exist either freely or covalently attached to proteins or lipids, forming simple or complex glycoconjugates such as glycoproteins, proteoglycans, and glycolipids. The

biosynthesis of glycoconjugates occurs through glycosylation, a major post-translational modification that takes place in the endoplasmic reticulum (ER) and in the *cis*-, *medial*- and *trans*- compartments of the Golgi complex. This process is mediated by specific enzymes, namely glycosidases and glycosyltransferases, which generate different families of glycoconjugates [251]. Common monosaccharides found in these glycoconjugates include galactose (Gal), glucose (Glc), mannose (Man), fucose (Fuc), xylose (Xyl), N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), glucuronic acid (GlcA), iduronic acid (IdoA), and sialic acids (Sia), among others [251, 252].

Concerning protein glycosylation, there are two main types of glycoproteins according to the glycan-protein linkage: *N*-linked glycans (GlcNAc is attached to the nitrogen atom of an asparagine (Asn) side chain by a β -1N linkage); and *O*-glycans (GlcNAc or GalNAc are linked to an oxygen atom of Ser or Thr residues) [251, 252].

N-linked glycosylated proteins can vary in their glycan sequences and chain lengths, resulting in three main structures with different complexities: high-mannose *N*-glycans, hybrid *N*-glycans or complex *N*-glycans (see Figure 7) [251, 252]. The development of *N*-glycans begins with synthesis of a lipid-glycan precursor: the Glc3Man9GlcNAc2 structure attached to a dolichol polypeptide chain (Dol-P) in the cytoplasmic side of ER. After being transferred by oligosaccharyltransferase (OST) to an Asn on a Asn-X-Ser/Thr (where X can be any amino acid except proline), this carbohydrate-protein conjugate undergoes further quality-control processes in the ER, involving the sequential removal of glucose residues by α -glucosidases (α Glc-I and α Glc-II) and protein folding. Once the proper folding is assured, the last glucose is removed as part of a quality-control process, and following endo-mannosidase activity, the high-mannose structure (Man8GlcNAc2) is formed [253]. The glycoprotein is then transferred to the *cis*-Golgi apparatus, where three mannoses are removed by α 1,2-mannosidase I (α Man-I) forming the Man5GlcNAc2 structure. Still within the *cis*-Golgi, *N*-acetylglucosaminyltransferase I (GnT-I) adds GlcNAc in a β 1,2 linkage, producing the GlcNAc1Man5GlcNAc2, a primary hybrid *N*-glycan structure, thus initiating the development of GlcNAc-carrying branches (called antennae) [253, 254]. Afterward, in the *medial*-Golgi, GlcNAc1Man5GlcNAc2 structure is processed by α 1,2-mannosidase II (α Man-II) which trims the terminal α 1,3-Man and α 1,6-Man residues to form the core GlcNAc1Man3GlcNAc2. This hybrid *N*-glycan then serves as a substrate to GnT-II, that catalyses the conversion of hybrid to complex *N*-glycans, by adding one GlcNAc in a β 1,2-linkage (GlcNAc2Man3GlcNAc2). The branching can be continued by a series of GnT enzymes (GnT-IV, V) which add GlcNAc to structures in diverse linkages. In detail, GnT-IV forms a β 1-4 GlcNAc branch on the α 1-3 mannose arm; and GnT-V (encoded by *MGAT5*), catalyses the formation of β 1,6-GlcNAc branched *N*-glycans (addition of β 1-6-linked *N*-

acetylglucosamine branch to α 1-6-linked mannose). Therefore, complex *N*-glycans can exist in bi-, tri- and tetraantennary forms. In addition, it is important to note that both hybrid and complex *N*-glycans can also display a bisecting GlcNAc residue at the mannosyl core, as a result of the activity of GnT-III. When GnT-III act on the hybrid *N*-glycan GlcNAc₁Man₅GlcNAc₂ (after GnT-I action), it inhibits α -mannosidase II [254]. Furthermore, the GlcNAc residue added by GnT-III is the only one that cannot be extended by additional monosaccharide linkages [254]. GlcNAcT-III action can also inhibit branching led by GlcNAcT-IV and GlcNAcT-V [254].

Finally, hybrid and the different forms of complex *N*-glycans are transferred to the trans-Golgi for maturation. Here, occurs the elongation and capping of all branching GlcNAc residues with other sugars (except for bisecting GlcNAc). Usually, the elongation of the antennae of complex and hybrid *N*-glycans involves the attachment of a β -Galactose to the β 1-2 linked GlcNAc (catalyzed by β 1,4 galactosyltransferase - β 4Gal-T), thus creating *N*-acetyllactosamine (LacNAc) units. These structures can be further elongated through the sequential repetition of GlcNAc and LacNAc forming poly-*N*-acetyllactosamine (polyLacNAc). Additionally, *N*-glycans can also be capped/decorated by the sequential addition of galactose, fucose and sialic acids sugars by several transferases, such as galactosyltransferases (Gal-T), fucosyltransferase (Fuc-T) and sialyltransferase (Sialyl-T) [251, 255]. The GlcNAc core modification of both hybrid and complex *N*-linked glycans can occur as well by the addition of fucose via α 1-6-fucosyltransferase (FucT-VIII) activity.

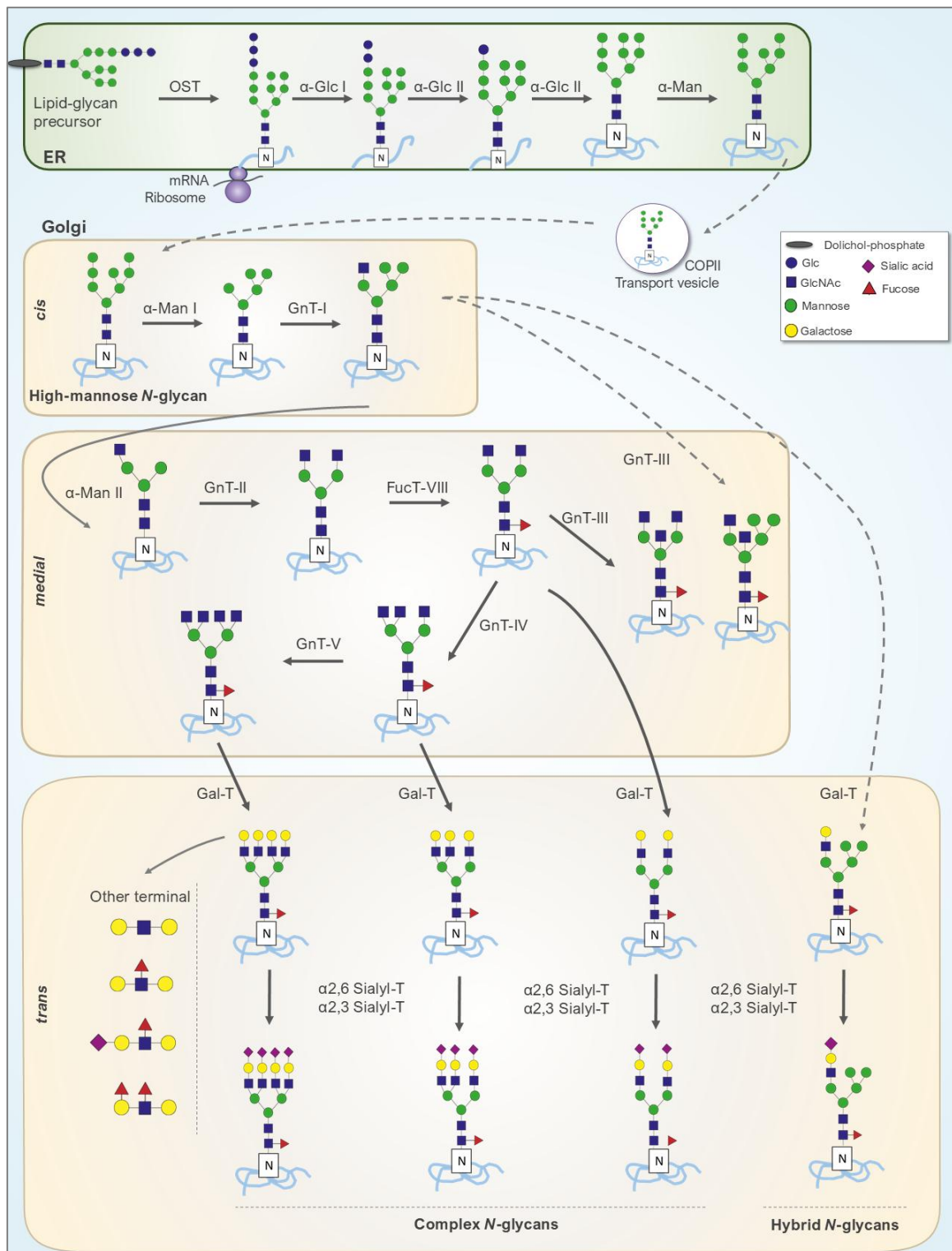


Figure 7. N-glycosylation biosynthetic pathway. The synthesis of N-glycans starts with the OST-mediated addition of a glycan precursor to an Asn, in the ER. After protein folding, the glycoprotein is transferred to Golgi, and the synthesis of N-glycans is mediated by the sequential action of action of glycosidases and glycosyltransferases.

5. Glycans: key mediators of host immunity and gut microbiome interactions

5.1 An overview of biological function of glycans

Free glycans, along with O- and N-glycoconjugates, form a highly diverse and complex layer of sugar chains on cell surfaces known as the glycocalyx. This structure encodes a broad spectrum of biological functions, including cell interaction and adhesion, migration, protein folding and stability, cell fate determination, regulation of receptor activation, and signal transduction [256, 257]. In the immune system, N-glycans and O-glycans play vital roles in the early development and differentiation of T and B cells, as well as in the regulation of immune cell trafficking and recruitment [258]. Furthermore, glycans can serve as key recognition molecules, as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), mediating host immune signalling. These “glyco-signals”, which usually comprise mannose-, sialic acid-, and N-acetylglucosamine-rich glycoconjugates, are commonly detected by innate immune cells via carbohydrate-recognizing receptors such as C-type lectins, Siglecs, and galectins, respectively [258, 259]. In turn, glycan-microbial immune reactions mediated by lectins trigger proinflammatory responses, involving downstream adaptive immune engagement and pathogen clearance [260]. Furthermore, glycans constitute a crucial element in self- *versus* non-self-recognition, functioning as a cellular fingerprint that enables the immune system to distinguish between its own components and foreign entities [261, 262]. Consequently, the host glycocalyx also exerts a modulatory influence on the functions of both innate and adaptive immune cells. For example, the transition from immature (iDCs) to mature DCs is accompanied by changes in the surface glycosylation patterns [263]. Loss of complex N-glycans expression on APCs were shown impact MHC-II binding, resulting in impaired antigen presentation to T cells [264, 265]. Moreover, N-glycosylation patterns govern the proliferation and activity of both T and B cells [266].

5.2 Mucosal glycocalyx at the interface of Host-gut microbiota crosstalk

The intestinal mucosal glycocalyx plays a vital role in sustaining gut homeostasis and maintaining the equilibrium of the gut microbiota. It establishes a bidirectional crosstalk between the host and commensal microbiota, serving as a biological and physical shield while simultaneously providing a central niche for microbial colonization and attachment [267].

As mentioned previously, gut microbiota possesses an extensive glycan-utilization system, comprising glycan-binding proteins, transporters, and glycan-degrading enzymes (CAZymes), that enables the recognition, import and breakdown of glycocalyx elements [268, 269]. The range and type of glycans that microorganisms can use is determined by the repertoire of glycan-utilization systems that they hold [270]. Species from the Bacteroidetes/Bacteroidota phylum, particularly *Bacteroides*, *Prevotella*, *Parabacteroides*, and *Alistipes* genera, display a vast arsenal of CAZymes, which allow them to degrade several types of glycans, being therefore considered as primary and glycan utilization-generalists. In contrast, members of Firmicutes/Bacillota phylum, such as *F. prausnitzii*, *E. rectale*, *Eubacterium hallii*, and Actinobacteria phylum are classified as "specialist" degraders, due to their ability to metabolize only a limited range of glycans [270-272].

It has been demonstrated that certain commensal bacteria belonging to the *Porphyromonadaceae*, *Ruminococcaceae*, and *Bacteroides* species (e.g. *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*), exploit intestinal α 1,2-fucose residues. The utilisation of epithelial fucosylation by these commensals enables the restriction of harmful microorganisms via mechanisms of colonization resistance [273, 274]. Briefly, the presence of intestinal α 1,2-fucose yield the growth of beneficial commensal microbiota, which ensures the occupation of pathogenic niches, interfering as well with pathogen attachment [275, 276]. Consistently, the presence of α 1,2-fucose in IEC has been described as an important protective platform against pathogenic and opportunistic microorganisms, such as *C. rodentium*, *S. typhimurium*, *E. faecalis*, *Salmonella*, *Campylobacter jejuni*, and *C. albicans* [275, 276]. This was supported by Fut2-deficient mice, which revealed an increased susceptibility to infection posed by *S. typhimurium* and *C. albicans* [277, 278].

Alongside to fucose residues, host-derived GlcNAc was identified as a crucial requirement for the growth of certain commensal bacteria, particularly, *Phascolarctobacterium* spp. [279]. The presence of optimal levels of *Phascolarctobacterium* spp. within the intestinal tract holds particular relevance, as it serves to inhibit the opportunistic proliferation of *C. difficile* [280]. Moreover, *B. thetaiotaomicron* utilizes mammalian high-mannose *N*-glycans (HMNG), yeast α -mannan and complex *N*-glycans [281-283]. In addition, under carbon starvation, this gut symbiont boosts the utilization of mucin-derived O-glycans [269]. Similar, *R. gnavu* and *A. muciniphila* are also mucus-layer-degrading bacteria. It is noteworthy that the presence of mucin-degrading gut commensals, specifically *Parabacteroides distasonis*, *B. thetaiotaomicron* and *A. muciniphila*, has been demonstrated to suppress intestinal epithelial inflammation induced by *E. coli* through the downregulation of the NF- κ B pathway and the enhancement of tight junction barrier function [284]. Nevertheless, excess mucin degradation may lead to the disruption and erosion of colonic mucous layer, culminating in increased susceptibility to enteric pathogens [284].

Besides this, many microorganisms exploit host glycans for its pathogenesis, virulence and immune invasion. For instance, the breakdown of GlcNAc impacts the virulence mechanism of several human pathogens, including *C. albicans*, *E. coli*, *Vibrio cholerae*, *Leishmania donovani* and *Mycobacterium* [285]. Several bacterial pathogens and respective toxins present lectin-like properties that recognize specific host glycans [286]. As example, *V. cholerae* expresses adhesin targeting complex *N*-glycans on IECs supporting colonization [287]. Similar, host complex-type *N*-glycans and core 1 *O*-glycans can be also targeted by toxins released e.g. by *S. Typhi*, *Salmonella*, *V. cholerae* and *C. difficile*, contributing to the dynamic invasion of the bacteria [286]. In turn, *Bacillus subtilis* utilize host fucosylated glycans to adhere and colonize gastrointestinal tract [288].

Additionally, some luminal microbes incorporate host glycans into their own surface glycoconjugates. For instance, upon incorporation and metabolization of intestinal epithelial fucoses, *B. fragilis* and *B. thetaiotaomicron* recycle fucosylated capsular polysaccharides, which are components of the bacterial cell wall [283]. This phenomenon, described as molecular mimicry, is a crucial event that encompasses either colonization/mutualistic interactions or pathogenic invasion strategies [289, 290]. The expose of host glycan structures on the surface of microorganisms enables them to camouflage themselves from host immune surveillance, thereby evading recognition of their PAMPs and hampering the host immune response [291].

Notably, relevant studies have indicated the contribution of microbial stimuli in reprogramming intestinal epithelial glycosylation, in particular α 1,2-fucosylation [292]. Supporting this evidence, germ-free mice showed reduced expression of α 1,2-fucose on IECs, that was restored following colonization with commensal *B. thetaiotaomicron* [273, 274]. Symbionts like *B. fragilis* and SFB also induce intestinal epithelial α 1,2-fucosylation [293]. In fact, SFB-deficient mice displayed a defective ileal epithelial fucosylation, placing SFB as an important player of epithelial α 1,2-fucosylation at homeostasis [294]. Nevertheless, also pathogenic species, in particular, *S. typhimurium* and *C. rodentium*, have been shown to trigger epithelial *Fut2* expression and consequently α 2-fucosylation expression in the cecum [295].

As an example of the microbiota-immune response-glycosylation axis, mucosal immune cells also play a critical role in the mechanisms of microbiota-induced epithelial α 1,2-fucosylation [275, 293]. Markedly, commensal gut microbiota fosters ILC3 to produce IL-22, which in turn induces *Fut2* expression on IECs. As a feedback loop, this process enhances protection against pathogenic and opportunistic bacteria by stabilizing commensal gut microbiota composition [275, 293]. Also relevant, IL-22 acts almost exclusively on non-haematopoietic cells like IECs, and thus ILC3 are extremely important for improving the intestinal epithelial barrier via IL-22 by stimulating AMPs (RegIII γ and RegIII β) and mucus

production [174]. Conversely to ILC3, adaptive immune cells, namely IL-10-producing T cells, seem to suppress α 1,2-fucose and Fut2 expression in the epithelium [296]. Therefore, intestinal innate and acquired immune system seem to cooperatively orchestrate epithelial α 1,2-fucosylation, controlling the homeostatic microenvironment in the intestine.

The fermentation of glycans by gut microbiota generates several metabolites that provide a myriad of benefits to the host (as aforementioned). As such, these microbial metabolites, including SCFAs, have been demonstrated to be key mediators in mucosal host-gut microbiota interplay. In addition to its ability to strengthen the epithelial barrier and regulate intestinal immunity, butyrate also exerts a cytotoxic effect on specific microbial species, thereby limiting the growth and activity of gut pathogens [149, 150, 297]. While *B. vulgatus* was found to be butyrate-sensitive, *B. thetaiotaomicron* can thrive in butyrate-rich environments [297]. Markedly, the inhibitory effect of butyrate on specific *Bacteroides* strains is modulated by the availability of distinct glycans. Indeed, the availability of different sugars influences the fitness of strains, activating context-dependent inhibitory capacities in response to butyrate [298].

Certain butyrogenic bacteria can rely on other microbes' fermentation by-products to produce butyrate or propionate. Specifically, to produce butyrate, *C. difficile* metabolises succinate, a fermentation end-product of commensal *B. thetaiotaomicron* [299]. In turn, *Anaerobutyricum soehngenii* secretes butyrate and propionate using lactate generated by *A. rectalis* and *Bacteroides ovatus* [300]. *Roseburia intestinalis* and *F. prausnitzii* are acetate-consuming gut microbes and are cross-fed by members of *Coprococcus* sp, *Bifidobacterium* sp, (e.g. *B. infantis* and *B. adolescentis*) and Bacteroidetes [301-303]

Collectively, a substantial body of evidence shows that mucosal glycocalyx participate in many biological processes integrating host, microbial and environmental cues. While mucosal glycans provide ligands and nutrients to commensal microbiota, which confers significant competitive advantages among the microbial community, they concomitantly shape gut immunity inducing bidirectional signalling between intestinal epithelial and immune cells which, in turn, fine tune the gut microbiota. This complex and delicate interplay operates as a synergy to establish symbiotic conditions with gut microbiota. Hence, modifications in mucosal glycosylation can disrupt the inter-microbial interactions, lead to gut dysbiosis and aberrant immune responses, potentially promoting an intestinal environment more prone to disease.

6. Unravelling the glycome shift in IBD: how altered glycosylation drives the disease

A substantial body of evidence has illustrated the significant role of glycans in the disruption of intestinal homeostasis and in the regulation of immune circuits involved in the development and progression of IBD [259, 304]. Remarkably, among the numerous genes involved in glycan biosynthesis, approximately 20 have been implicated as IBD-associated risk genes [257]. Intestinal inflammation is markedly fostered by changes in the glycosylation patterns of the gut mucosal barrier. A prominent example of these alterations in IECs is the increased expression of truncated or shortened O-glycans [305, 306]. These alterations are frequently observed in patients with active UC, being associated with increased mucosal thickness, which may facilitate greater bacterial contact with the colonic epithelium, ultimately triggering inflammation [307]. Furthermore, an altered O-glycosylation profile of MUC2 (specifically a decrease in sulfation of O-glycans) has been described in patients with active UC compared to healthy individuals or patients in remission [306, 308]. Likewise, the role of modified mucin glycosylation was demonstrated by the specific deletion of core 1–derived O-glycans on gut epithelial cells, achieved using a conditional mouse model lacking *C1galt1* specifically in IECs (C1GALT1 IEC knockout mice). This resulted in the development of spontaneous colitis, characterized by exacerbated immune infiltration, including TNF-producing myeloid cells and neutrophils, by crypt abscesses, and histopathology that closely resembles UC [305]. Similarly, mice deficient in core-3 derived glycosyltransferases exhibit diminished levels of total intestinal O-glycans, reflecting an enhanced susceptibility to DSS-induced colitis [309].

Additionally, SNPs in intestinal α 1–2 fucosyltransferases, namely FUT1 and FUT2, have been considered IBD-associated genetic risk factors. In particular, loss-of-function mutations in *FUT2* have been shown to promote disease-like intestinal inflammation associated with microbial dysbiosis [310, 311]. Supporting these observations, oral fucose supplementation alleviated both chronic and acute DSS-induced colitis in mice. This was achieved by the modulation of bile acid profile and by promoting beneficial gut microbiota. Moreover, fucose supplementation was shown to hinder macrophage M1 polarization, NLRP3 inflammasome activation, and NF- κ B signalling, thereby attenuating inflammatory responses [312–314]. Given that intestinal epithelial α 1-2-fucosylation profoundly influences gut microbial composition, as early described, the altered epithelial fucosylation in IBD patients may serve as a driving force related to the dysbiotic-predisposing microbiota that underlies IBD pathogenesis.

Evidence have demonstrated that the uncontrolled activation of adaptive immune mediators, particularly T cells, is a critical factor in the pathogenesis of IBD. Notably, altered

T-cell glycosylation is directly implicated in the pathology of IBD [266, 315]. In patients with UC, lamina propria T cells exhibited deficient levels of surface β 1,6-GlcNAc–branched *N*-glycans, namely on the T cell receptor (TCR). This defect results from the reduced GnT-V activity due to lower *MGAT5* expression. The lower levels of complex branched *N*-glycans on T cells were also associated with increased Th1 and Th17 differentiation, T cell hyperactivation, and higher production of TNF- α , IFN- γ , and IL17A [315, 316]. Mechanistically, defective expression of complex branched *N*-glycans fosters TCR clustering, lowering the threshold for T cell activation and improving susceptibility to experimental colitis [266].

Importantly, reduced levels of β 1,6-GlcNAc–branched *N*-glycans on T cells at the time of UC diagnosis are not only linked to increased disease severity [315, 316], but also serve as predictors of poor response to standard therapies, suggesting more aggressive disease progression [317]. Concomitantly, genetic variants downregulating the *MGAT5* expression were identified in T cells of UC patients. These SNPs hold clinical relevance in UC management, as they can determine disease outcomes and enable stratification of patients based on disease severity. Additionally, they are associated with an increased need for biologic therapies [318].

Remarkably, GlcNAc supplementation of mucosal T cells from patients with active UC significantly increased the levels of complex branched *N*-glycans levels on the TCR. These led to significant suppression of T-cell activity and Th1/Th17 immune responses. The promising effect of glycan supplementation was further confirmed in mice lacking branched *N*-glycosylation, where GlcNAc treatment effectively mitigated colitis severity and progression [315, 316]. These findings underscore the potential role of GlcNAc as an immunomodulatory therapy for patients with UC.

Similar to changes in branching *N*-glycosylation, enhanced levels of core fucosylation due to the increased expression of fucosyltransferase 8 (FUT8) were found in intestinal T cells from patients with severe IBD [319]. Conversely, the absence of Fut8 in mouse models was described to confer protection against the development of colitis, potentially attributable to diminished pro-inflammatory cytokine synthesis by T cells. This supports the central role of core fucosylation of TCRs for T-cell signalling and the induction of intestinal inflammation [319].

Changes in glycosylation profile of circulating IgG have also been reported in patients with IBD [320, 321]. Indeed, 5 of the 16 genes involved in regulating of IgG glycosylation have been identified as containing SNPs related to increased risk for IBD [322]. IgG is a glycoprotein in which both Fab and Fc regions are glycosylated [323]. The IgG glycome elicits different effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), through interactions between their

fragment crystallizable (Fc) region and several Fc receptors [324]. Therefore, alterations in glycan composition modulate IgG effector function, activating immune response towards either pro- or anti-inflammatory pathways [257]. Patients with UC and CD exhibit smaller levels of serum IgG sialylation [320, 325]. Terminal sialic acids exert an anti-inflammatory effect on IgG by binding to the inhibitory receptor FcγRIIB and the Dendritic Cell-Specific ICAM-3 Grabbing Non-Integrin (DC-SIGN) [326, 327]. On the other hand, increased levels of afucosylated IgGs have been detected in UC patients [320, 325]. More than 90% of IgG glycans in human serum are usually core fucosylated, which hampers IgG binding to the activating receptor FcγRIIIa [324]. Thus, the loss of core fucose and sialic acid residues in IgGs underlies the inflammatory mechanisms contributing to the development of UC. In CD patients, an elevated proportion of fucosylated IgGs has been identified, concomitantly with higher prevalence of agalactosylated IgG [320, 325]. This pattern is correlated with elevated levels of the inflammatory biomarker CRP, suggesting that changes in IgG galactosylation are linked to disease severity in IBD patients [328]. The levels of Fc IgG galactosylation in the serum of a healthy individual are heterogeneous, with a distribution among galactosylated (G0F), mono-galactosylated (G1F), or di-galactosylated, representing approximately 35%, 35%, and 15% of total IgG glycoforms, respectively [323, 329]. The role of terminal galactose in Fc of IgG is not consensual, with reports indicating a pro-inflammatory role through enhanced binding to FcγRIIIa and classical complement activation [330-332], while agalactosyl IgG was also demonstrated to exhibit increased affinity to FcγRIIb and C-type lectin-like receptor dectin-1 exerting an anti-inflammatory effect [333]. Interestingly, specific genetic variations on *MGAT5* gene occurring in UC patients have been associated with agalactosylation of IgGs [318].

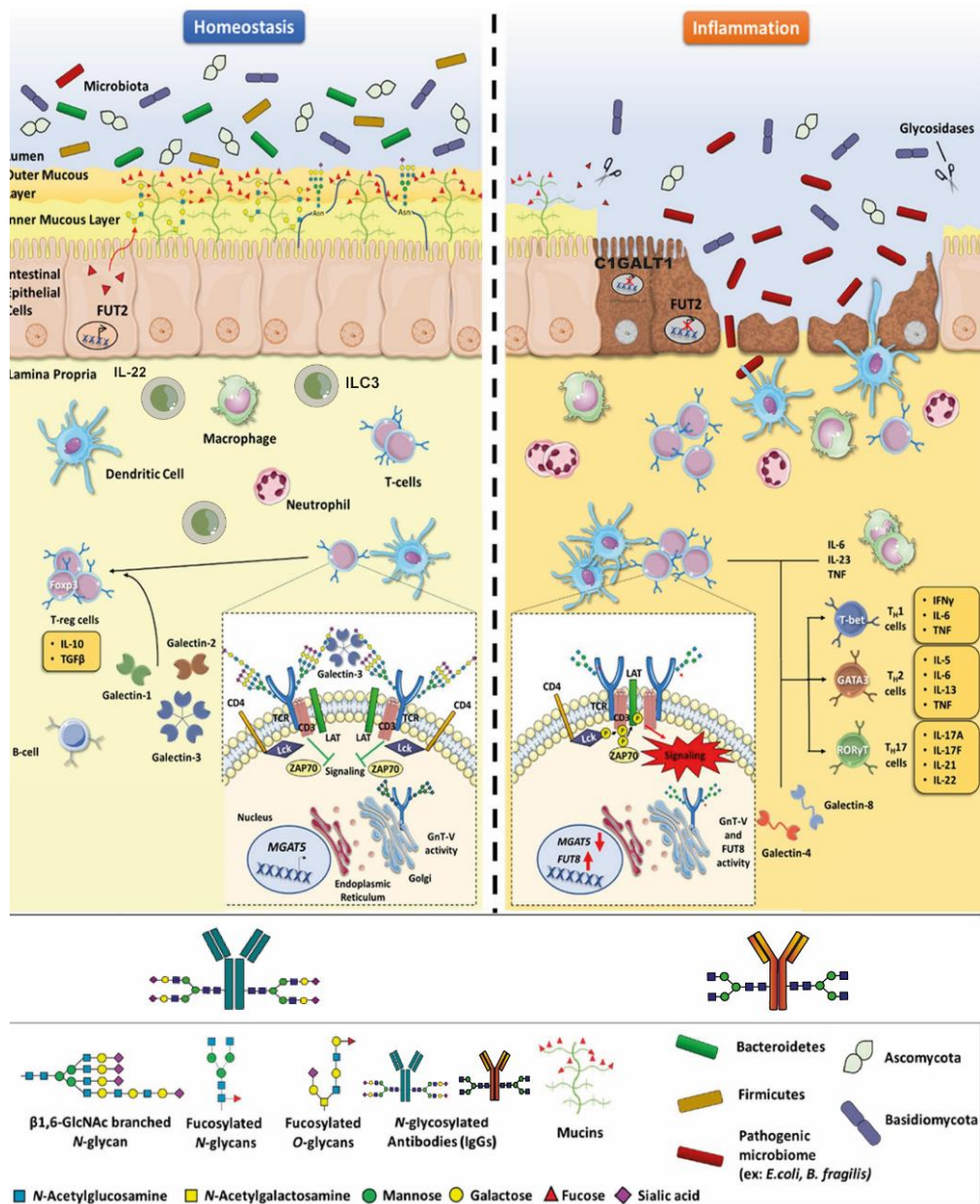


Figure 8. Impact of the glycosylation changes in gut homeostasis and inflammation. During the transition from normal to inflamed gut mucosa changes in the glycosylation signature occur. Abnormal expression of glycans contribute to abnormal gut immunity. Glycans composition namely in mucins are crucial to maintain the protective function of the intestinal barrier against pathogenic microbiota. Lack of intestinal C1GALT1 activity contributes to the aberrant intestinal O-glycosylation contributing to a defective inner colonic mucus layer and increased contact between epithelial cells and bacteria. In parallel, the expression of α-1,2 fucosylation at the surface of epithelial cells is catalysed by FUT2 and is crucial in the regulation of commensal microbiota. Loss of function mutations in *FUT2* contribute to microbial dysbiosis. Glycosylation profile regulate T cell-mediated immune response. Deficiency of β1–6 branching on N-glycans by impaired *MGAT5* expression and the overexpression of core fucose by the upregulation α1-6 fucosyltransferase (FUT8) promote TCR clustering, decrease TCR activation thresholds, and increase T-cell activation leading aberrant cell

response. The profile of IgG glycosylation released in the serum exhibit predominantly galactosylated and terminal sialylated *N*-glycans in the Fc portion. In IBD, the expression profile of serum IgG is predominantly characterized by agalatosylation. C1GALT1: Core 1 synthase, glycoprotein-*N*-acetylgalactosamine 3-beta-galactosyltransferase; FUT2: Galactoside 2-alpha-L-fucosyltransferase 2; MGAT5: Alpha-1,6-Mannosylglycoprotein 6-Beta-*N*-Acetylglucosaminyltransferas; TCR: T-cell receptor. Adapted from [304].

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Chapter 2

Aims of the thesis

Context and specific aims

The complete understanding of IBD aetiology remains elusive. Despite significant advances in the clinical and therapeutic management, the proportion of patients achieving long-term remission and the reduction in severe disease complications remains disappointingly low. Additionally, current treatments rely on mitigating the consequences of inflammation rather than targeting the initial triggers that drive disease onset and progression.

Hence, there is an unmet need for a deeper comprehension of the pathogenesis of IBD, envisioning the identification of novel mechanisms that drive the disease. Particularly, it remains to be defined the host-microbial interactions and the metabolic causes of dysbiosis associated with the initiation of intestinal inflammation. Moreover, the existence of a preclinical phase in IBD, which precedes the formal clinical diagnosis and symptoms, is also poorly understood. In this line, an increasing body of evidence has supported the relevance of glycans in the immunopathogenesis of immune-mediated diseases, particularly in IBD.

Thus, the main GOAL of this thesis was to dissect the mechanisms by which alterations in host glycosylation increase IBD susceptibility, envisioning the identification of glycans as central modulators of microbiome and gut immunity, further investigating the glycosylation profile in the pre-clinical phase of IBD. This new knowledge is expected to facilitate the discovery of innovative targets and biomarkers for the development of personalized medical interventions.

To address this goal, two outlined aims were defined:

1: Unveil the role of host *N*-glycans as pivotal regulators of the gut microbiota and the immune response associated with intestinal inflammation.

(Chapter 3, *Rodrigues & Gaifem et al.* work under revision)

2: Explore the host glycome signature in the pre-clinical phase of IBD and its potential association with disease development.

(Chapter 4, *Gaifem & Rodrigues et al.* Nature Immunology 2024)

Chapter 3

Alterations in mucosa branched *N*-glycans
lead to dysbiosis and downregulation of
ILC3: a key driver of intestinal inflammation

Alterations in mucosa branched *N*-glycans lead to dysbiosis and downregulation of ILC3: a key driver of intestinal inflammation

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Abstract

The perturbation of the symbiotic relationship between microbes and intestinal immune system contributes to gut inflammation and Inflammatory Bowel Disease (IBD) development. The host mucosa glycans (glycocalyx) creates a major biological interface between gut microorganisms and host immunity, that remains ill-defined. Glycans are essential players in IBD immunopathogenesis, even years before disease onset. However, how changes in mucosa glycosylation shape microbiome and how this impact gut immune response and inflammation remain to be clarified. Here, we revealed that alterations in the expression of complex branched *N*-glycans at gut mucosa surface, modelled in glycoengineered mice, resulted in dysbiosis, with a deficiency in Firmicutes bacteria. Concomitantly, this mucosa *N*-glycan switch was associated with a downregulation of type 3 innate lymphoid cells (ILC3)-mediated immune response, leading to the transition of ILC3 towards an ILC1 proinflammatory phenotype and increased TNF α production. In addition, we demonstrated that the mucosa glycosylation remodeling through prophylactic supplementation with glycans at steady state was able to restore microbial-derived short-chain fatty acids and microbial sensing (by *NOD2* expression) alongside the rescue of the expression of ILC3 module, suppressing intestinal inflammation and controlling disease onset. In a complementary approach, we further showed that IBD patients, often displaying dysbiosis, exhibited a tendency of decreased *MGAT5* expression at epithelial cells that was accompanied by reduced ILC3 expression in gut mucosa. Altogether, these results unlock the effects of alterations in mucosa glycome composition in the regulation of the bidirectional crosstalk between microbiota and gut immune response, revealing host branched *N*-glycans/microbiota/ILC3 axis as an essential pathway in gut homeostasis and in preventing health to intestinal inflammation transition.

Keywords: *N*-glycans; Intestinal inflammation; microbiome; ILC3; Prophylaxis.

Introduction

The human gut is home to a diverse collection of microorganisms – the intestinal microbiota – that has co-evolved with the immune system, maintaining a symbiotic relationship essential for homeostasis [1]. The perturbation of the cooperative relationship between microbes and the intestinal immune system is shown to contribute to the onset of many inflammatory diseases, such as Inflammatory Bowel Disease (IBD) [2, 3], an event that can occur years before diagnosis [4]. Despite advances in therapeutic resources, IBD remains incurable [5]. Therapies modulating microbiota are an emerging field of research [6, 7], with potential to revolutionize IBD therapy in a near future. Thus, it is essential to understand the causes underlying the metabolic changes associated with loss of gut microbial equilibrium (dysbiosis) and with gut inflammation, envisioning novel disease biomarkers and new therapeutic targets.

Host glycocalyx, which is the repertoire of glycans/sugar chains expressed at the surface of all cells, is a major biological and physical interface between intestinal mucosa, microorganisms and host immune response, essential to guarantee homeostasis [8-10]. Hence, host glycocalyx is a key target in a disease context that remains largely unexplored. In fact, the dense and complex coat of sugar-chains that cover our gut mucosa is an essential niche for microbiota colonization [11]. Moreover, the human gut microbiota is able to degrade and use host glycans as a major source of nutrients and energy [12, 13], producing short-chain fatty acids (SCFAs) that are fundamental in maintaining intestinal homeostasis through the regulation of epithelial and immune cells [14-16].

Mucins are the typical example of mucosa glycoproteins, heavily decorated with O-glycans, which are essential for building barriers that protect our inner surfaces from bacteria [17, 18]. Changes in the expression of truncated mucins O-glycans or the disruption of epithelial fucosylation were associated with increased susceptibility to bacterial infection [19]. However, the diversity of glycans presentation at the gut mucosa is enormous, going far beyond mucin O-glycans. The prominent expression of *N*-glycan structures in the human intestine places them as ideal, hitherto poorly defined, molecular interfaces between microorganisms and the immune microenvironment [20]. However, whether and how *N*-glycans at the surface of gut mucosa act as shapers of the gut microbiota composition and function and how this impact in the breakdown of gut homeostasis associated with IBD immunopathogenesis is a fundamental question that remains unanswered.

Previous evidence from our group have been demonstrating the key role of protein glycosylation in IBD immunopathogenesis, revealing the power of glycome alterations even years before disease diagnosis [8, 21-23]. We showed that ulcerative colitis patients exhibit a deficiency in the expression of complex branched *N*-glycans structures, associated with

T cell hyperactivity and disease severity [22]. We also demonstrated that mice deficient in *Mgat5* glycoenzyme, thus absent of *N*-acetylglucosaminyltransferase-V (GnT-V)-mediated complex branched *N*-glycans structures, have an increased susceptibility to severe forms of colitis and to early onset disease [22]. Importantly, changes in glycome were found to occur years before Crohn's disease (CD) diagnosis, supporting the predominant biological relevance of glycans in health to intestinal inflammation transition and IBD onset [23]. In addition, alterations in mucosa glycosylation have been also correlated with risk for colitis-associated cancer development [21, 24-26]. Altogether, these previous evidence highlight the prominent role of *N*-glycans in modulating gut immunity, with promising translational clinical applications in IBD prognosis and treatment [21, 27, 28]. However, when, why and how an altered host mucosa glycome shapes microbiome composition associated with immune activation and IBD development is a key question that remains ill-defined. In this study, we created a glycoengineered mice deficient in *Mgat5* gene to investigate whether and how an altered host glycocalyx, translated into fluctuations in the abundance and spatial distribution of mucosa *N*-glycans, is a fundamental factor that mediates loss of immune-tolerance, by leading to the selective overgrowth of pathobionts, triggering dysbiosis and consequently the activation of intestinal immune response. Our results reveal host glycocalyx and specifically mucosa branched *N*-glycans as master regulators at the frontier of microbiome and ILC3/ILC1-mediated immune response, bringing to light the power of glycosylation reprogramming of gut mucosa as a promising intervention strategy to prevent IBD onset with clinical applications.

Materials and Methods

Human cohort

To characterize the immune populations, fresh intestinal biopsies from 5 patients diagnosed with IBD (n=2 UC; n=3 CD) and 3 healthy controls were retrieved at Centro Hospitalar Universitário Santo António, Porto, Hospital Beatriz Ângelo, Loures, and Hospital da Luz, Lisbon. Healthy individuals without significant pathological findings. The samples were taken from the terminal ileum or the sigmoidal colon. In the disease samples, both inflamed and non-inflamed material were collected (See Supplementary Table 1). Patient identity was taken properly into account to allow for dependent samples in the statistical analyses. Ethical approval was obtained at the Ethical Committees of all Hospitals. All study participants gave written informed consent prior to sampling and data collection.

Animals

C57BL/6 wild-type (*Mgat5*^{WT}) and *Mgat5*-deficient mice (*Mgat5*^{-/-}, kindly provided by Michael Pierce, University of Georgia, Athens, GA) were bred and maintained in accredited animal facilities at the Institute for Research and Innovation in Health (i3S). Mice were housed in groups of 5-10, in HEPA filter-bearing cages, under 12-hour light/dark cycles. Autoclaved chow and water were provided *ad libitum*. Cages were enriched with nesting materials. Females between 9-14 weeks were used. Experiments were conducted with the approval of the Animal Ethics Committee and the Animal Welfare Body of the i3S. The protocols are in agreement with in-house standards and rules for animal experimentation, comply with national legislation and are integrated within a project, which is licensed by the competent authority, DGAV (license number 009268/2022-06-02).

Co-housing

4-week-old *Mgat5*^{WT} and *Mgat5*^{-/-} mice were weaned and moved to the same cage, in order to share the same environment. Mice were housed in the same cage at a maximum amount of 10 mice per cage. Co-housing was performed for 5 weeks. Stool samples were collected before and after co-housing for both *Mgat5*^{WT} and *Mgat5*^{-/-} mice.

GlcNAc supplementation

To assess the prophylactic properties of glycan supplementation, 4-week-old *Mgat5*^{WT} and *Mgat5*^{-/-} mice were treated with 800 mg/Kg/day of GlcNAc (Wellesley Therapeutics, Inc.) in the drinking water, *ad libitum*, for 8 weeks. No alterations in the regular behaviour and water consumption were observed. Stool samples were collected before and after GlcNAc

supplementation. The histological profile, as well as the immune response, were analysed upon GlcNAc treatment.

DSS-induced colitis

Mice with 9 to 11 weeks of age were given dextran sulfate sodium (DSS; 2% (w/v), molecular weight approximately 36000-50000 Da; MP Biomedicals) in the drinking water *ad libitum* for 7 days. Clinical signs of colitis, such as weight loss, stool consistency, and presence of blood were monitored daily and measured by the disease activity index (DAI) (See Supplementary Table 2). Mice were euthanized at the end of each experiment or earlier, if the symptoms of clinical disease reached one of these endpoints: more than 20% weight loss, diarrhea, or gross bleeding. The histological profile, as well as the immune response, were analyzed after colitis induction.

Isolation of lamina propria leukocytes (LPL)

To isolate lamina propria leukocytes (LPL), colons were flushed with Ca- and Mg-free PBS. Fragments of 0.5-1 cm were incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1mM CaCl₂, 1mM MgCl₂, and 1 mg/mL of collagenase IV (Sigma), under 100 rpm agitation at 37°C for 40 minutes. Tissues were dissociated and filtered through a 70 µm cell strainer (BD Biosciences). Cell suspension was centrifuged, the pellet was resuspended in RPMI 1640 medium supplemented with 10% FBS, and 1% penicillin/streptomycin, and layered upon Lymphoprep solution in a proportion of 1:2 (Lymphoprep:cell suspension). After gradient centrifugation at 800g for 20 minutes at 20°C (without acceleration or break), cells retained in the interface were collected for the staining of immune cells, whereas cells retained in the bottom of the tube were collected for staining of non-immune cells. Both fractions were washed in RPMI and kept in ice before analysis.

To isolate lamina propria leukocytes from human fresh colonic biopsies it was performed a mechanical dissociation in HBSS with 1% Pen/Strep and 0.1% Gentamycin. Then human fresh colonic biopsies were digested with 0.9 mg/mL of collagenase IV in RPMI supplemented with 10% FBS, 100 U/mL Pen/Strep, 1 mM CaCl₂ and 1 mM MgCl₂, for 45 min with agitation, at 37 °C. Cell suspensions were then filtered in 70 µm cell strainers and washed with PBS. Following, cells were centrifuged for 10 min at 300 g and 4 °C, and the pellet was resuspended in FACS buffer and ready to proceed for flow cytometry staining.

Flow cytometry analysis

All cells analyzed by flow cytometry were stained with Fixable Viability Dye (FVD) eFluor™ 780 for viability analysis and exclusion of dead cells. For lectin staining, cells were incubated

for 15 minutes, at 4°C protected from light, with conjugated lectins: L-PHA-fluorescein, L-PHA-biotin, GNA-fluorescein, Sambucus Nigra Lectin (SNA)-fluorescein, Maackia Amurensis Lectin II (MAL-II)-biotin and Ulex Europaeus Agglutinin I (UEA-I)-biotin, for evaluation of complex branched *N*-glycans (L-PHA), high-mannose residues (GNA), α -2,6 sialic acid (SNA), α -2,3 sialic acid (MAL-II), and α -linked fucose residues (UEA-I). Cells stained with biotinylated lectins were then incubated with streptavidin-PE or streptavidin-SparkBlue 550 for 30 minutes.

For staining of lamina propria lymphocytes, cells were stimulated with 20 ng/mL of phorbol myristate acetate (PMA), 200 ng/mL ionomycin calcium salt, and 10 μ g/mL of brefeldin A for 4 hours at 37°C. After staining with FVD, surface staining was performed by incubation for 30 minutes at 4°C with the surface antibodies shown in S. Table 3. Intracellular staining was performed using the eBioscience Foxp3 / Transcription Factor Staining Buffer set and the intracellular antibodies displayed in Supplementary Table 3. Gating strategy is presented in Figure S2A. Cells analysis was performed on a BD FACSCanto II (Becton Dickinson) or Cytex Aurora (Cytex). Data was analyzed using FlowJo software (Tree Star).

Tissue histochemistry and histological characterization

Samples from colons were fixed in 4% paraformaldehyde and 5 μ m paraffin-embedded sections were stained with hematoxylin and eosin (H&E). Inflammation was assessed blindly using a graduated semi quantitative system as described below. Besides H&E staining, lectin histochemistry was performed to assess the glycan profile of the colonic tissue. Paraffin-embedded sections were incubated with lectins Phaseolus Vulgaris Leucoagglutinin (L-PHA) and Galanthus Nivalis Lectin (GNA) for evaluation of complex branched *N*-glycans (L-PHA) and high-mannose residues (GNA). Staining with Alcian Blue/Periodic Acid-Schiff (AB/PAS) of colon section was performed to evaluate polysaccharide structures. Goblet cell number was assessed for each experimental condition in a blinded fashion. Only intact crypts, cut longitudinally from crypt opening to bottom, were quantified. Images were captured using an Brightfield Microscope – Leica DM2000 LED microscope and recorded with a digital camera Flexacam C3 using Leica Application Suite X (LAS X) software. Image analysis was performed using Fiji (ImageJ) software.

Intestinal permeability assay

In vivo intestinal permeability was assessed by administration of fluorescein isothiocyanate (FITC) labelled dextran. Food and water were withdrawn for 8 hours. Mice were administered 44 mg/100 g body weight of FITC-labelled dextran (TdB Consultancy; 4 kDa)

by oral gavage. Serum was collected four hours later, and fluorescence intensity was measured by spectrophotofluorimetry (excitation: 485 nm; emission: 528 nm).

RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA from mice was extracted using RNAqueous-Micro Kit (Invitrogen) according to the manufacturer's protocol. Total RNA was quantified using the Nanodrop 1000 system and RNA was transcribed into single-stranded cDNA using Superscript IV Reverse Transcriptase (Invitrogen) and Random Hexamer Primers (Invitrogen, Oregon, USA) according to the manufacturer's recommendations. The quantitative real-time PCR (qRT-PCR) was performed in 96-well reaction plates. cDNA was amplified using NZYSupreme qPCR Green Master Mix, ROX (NZYtech) using specific primers stated on Supplementary Table 4. Amplification data were acquired with 7500 Fast Real-Time PCR System (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) were used as housekeeping gene. Relative quantification values for gene expression were calculated based on the ΔC_t method, as follows: $2^{-(\text{Target gene mRNA expression} - \text{housekeeping gene mRNA expression})}$.

Cytokine quantification

For the colon explants, cytokine concentrations were analyzed by flow cytometry using cytometric bead arrays: the LEGENDplex™ MU Th Cytokine Panel (13-plex) (Biolegend), according to the manufacturer's instructions. Samples were measured on the BD Accuri C6 instrument (BD Biosciences, US) using a specific template provided by BD Biosciences

SCFAs quantification

Colonic tissue was minced to small pieces and homogenized in 500 μ l of cold PBS with a glass homogenizer on ice. The suspension was ultrasonicated followed by a centrifugation for 15 minutes at 5000 rpm. The quantitative determination of SCFAs in colonic tissue was determined using the Mouse Short-Chain Fatty Acids (SCFAs) Elisa kit (Amsbio), according to the manufacturer's instructions. Optical Density (O.D.) was measured at 450 nm using a microplate reader (Biotek Instruments).

Bioinformatic analysis

16S rRNA sequencing, bioinformatic and statistical analysis of gut microbiota composition

Stools were collected before DSS treatment and snap frozen in liquid nitrogen. Microbiome composition was determined by 16S rRNA gene sequencing and analyzed as previously

described[29]. Sequencing was performed on an Illumina MiSeq platform (Illumina) at GenoScreen with a 250-bp paired-end sequencing protocol. Raw paired-end reads were subjected to the following process: (1) quality filtering using the PRINSEQ-lite PERL script [30] by truncating the bases from the 3' end that did not exhibit a quality < 30 based on the Phred algorithm; (2) paired-end read assembly using FLASH (fast length adjustment of short reads to improve genome assemblies) with a minimum overlap of 30 bases and a 97% overlap identity; and (3) searching and removing both forward and reverse primer sequences using CutAdapt, with no mismatches allowed in the primers sequences. Assembled sequences without forward and reverse primers were removed.

Joined sequences were imported into the amplicon version of Qiime2 (version 2023.9) [31]. Sequence inference was performed using Deblur [32]. Taxonomy was assigned using the SILVA database (v138.1) [33]. Functional inference was performed using Picrust2 (v2.4.1) [34]. All downstream analysis was performed in R and RStudio. The phyloseq package (v1.40.0) was used to handle and analyze microbiome data[35]. The vegan package (v2.6.2) was used to perform diversity analyses. Samples were rarefied to an even depth of the sample with the lowest counts prior to performing diversity analyses. Alpha diversity was calculated using the Chao1 and Shannon indices and beta diversity was calculated using the unweighted unifracs distance [36]. Pairwise differential abundance testing was performed between single-housed wild type vs single-housed *Mgat5*^{-/-}, as well as *Mgat5*^{-/-} single-housed vs co-housed animals with taxa agglomerated at the genus level and rarefied, as recently suggested [37], using MaAsLin 2 [38], with the variable of interest a fixed-effect and the experiment as a random effect. "TSS" normalization and "LOG" transformation was used with prevalence filtering at 0.1. A q-value of 0.2 was applied to filter taxa. A similar analysis was performed using the unstratified pathway output of Picrust2 (*Mgat5*^{-/-} single-housed vs co-housed animals), without prior rarefaction and using a q-value of 0.05 and prevalence filtering of pathways in at least 50% of samples. For correlation of disease activity and differentially abundant taxa between *Mgat5*^{-/-} single-housed vs co-housed animals, the area-under-the-curve (calculated using the AUC function in the DescTools package (v0.99.54) with the "trapezoid" method) was calculated between day 0 and day 10 (with day 7 removed as some measurements were missing). Taxa were proportion-normalized, and the spearman correlation coefficient was used. Samples with a correlation p-value of <0.05 were manually plotted with the 'stat_smooth' function in ggplot2 using a "gam" method. All plotting was performed using ggplot2 (v3.4.1) and ggpubr (v0.4.0). Statistical tests of pairwise comparisons between independent groups were performed using the Wilcoxon rank sum test.

Human Single-cell and Bulk RNA sequencing analysis

Single cell RNA-seq data from Martin et al. (2019) with the accession number GSE134809 was downloaded from NCBI's GEO database. Cell type annotations provided by the authors were used for subsequent analysis. For the quantification of *MGAT5* in the epithelial component, the mean of all cells with above zero expression, per patient, was calculated. Epithelial cells were considered all those annotated as "Activated fibroblasts", "ACKR1+ endothelial cells", "Pericytes", "Lymphatics", "Fibroblasts", and "CD36⁺ endothelial cells". Significance was calculated using the Wilcoxon test. Percentage of ILC1 and ILC3 was calculated based on the total number of cells within each patient.

Bulk RNA sequencing from 12 healthy controls and 9 Crohn disease with inflamed epithelial were retrieved from dataset published by Häsler et al. (2017). Violin plots using Deseq2 normalized counts of *MGAT5* and *RORC* were generated to assess differences in expressions amongst groups.

Statistical analysis

All statistical analyses were executed using GraphPad Prism 8.0 Software. Data from flow cytometry was analysed with Flow Cytometry Analysis Software (FlowJo). The presence of outliers was evaluated using ROUT method (Q=1%). Normality was assessed by performing D'Agostino-Pearson or Shapiro-Wilk normality tests. For statistical analyses unpaired *t*-test, or Mann-Whitney test, according to normality. For DAI analysis, two-way ANOVA was used. Data obtained were expressed as mean $\pm$ standard deviation (SD). Statistical significance was considered at $p \leq 0.05$ and statistically significant values were represented as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

Results

Changes in mucosa branched *N*-glycosylation promote gut dysbiosis and increase intestinal permeability associated with intestinal inflammation.

Our previous evidence showed that the deficiency in the expression of complex branched *N*-glycans in *Mgat5* knockout mice (*Mgat5*^{-/-}) resulted in an early onset colitis in dextran sulfate sodium (DSS)-induced model, with mice developing severe forms of intestinal inflammation (Supplementary Figure 1A) [22]. However, whether this deficiency on mucosa glycome may impact microbiota composition associated with colitis susceptibility remains unknown. An extensive characterization of the mucosa glycosylation profile was performed in *Mgat5*^{-/-} mice using lectins that specifically recognize different glycan structures (Supplementary Figure 1B). The results showed the clear deficiency in β 1,6-GlcNAc complex branched *N*-glycans (detected by L-PHA) in the intestinal epithelial cell surface (CD45⁻ cells) of colitis-susceptible *Mgat5*^{-/-} mice compared with *Mgat5*^{WT} (Figure 1A and B). In contrast, higher levels of less complex, mannose-enriched *N*-glycans (GNA binding) at the gut mucosa surface were detected in *Mgat5*^{-/-} mice (Figure 1C and D) in comparison with *Mgat5*^{WT}. These observations are supported by the lower ratio of LPHA/GNA exhibited by *Mgat5*^{-/-} mice (Supplementary Figure 1C), which points towards an overexposure of mannose-enriched epitopes at the gut mucosa surface. A slight decrease in α (1,2)-fucosylation (UEA-I binding) (Supplementary Figure 1D) was observed and no alterations in terminal α 2,6 sialylation (SNA binding) (Supplementary Figure 1E) or α (2,3) linked sialic acid (MAL II binding) (Supplementary Figure 1F) in epithelia were detected. Despite the fact that mucus layer is mainly O-glycosylated, the presence of *N*-glycans may also affect mucins folding and dimerization [39]. Thus, the impact of the deficiency in β 1,6-GlcNAc complex branched *N*-glycans in mucus layer of *Mgat5*^{-/-} mice was analyzed, showing that the number of goblet cells seems to be reduced in *Mgat5*^{-/-} mice relative to *Mgat5*^{WT} (Supplementary Figure 1G)

Then, we evaluated how the mucosa glycan switch imposed by the deficiency complex branched *N*-glycans and overexposure of mannose-enriched glycans affects the gut microbiota composition, by performing 16S rRNA sequencing of stool content from *Mgat5*^{-/-} mice and *Mgat5*^{WT} mice in steady state. The results showed that mice lacking complex branched *N*-glycans display a clear dysbiotic microbiota composition at steady state when compared with *Mgat5*^{WT} mice (Figure 1E). Indeed, *Mgat5*^{-/-} mice showed an imbalance in the composition of different phyla with significantly decreased abundance of specific taxa belonging to Firmicutes phylum, specifically *Blautia*, *NK4A214 group* and *Lachnoclostridium* genera. In contrast, the increased abundance of bacterial species such

as *Marvinbryantia*, *Ruminococcaceae*, *Clostridia_UCG_014*, *Anaeroplasma*, and *Lachnospiraceae_FCS020_group*, were detected in *Mgat5^{-/-}* mice. Additionally, *Muribaculaceae* family from Bacteroidota phylum, *Coriobacteriaceae_UCG_002*, *Eggerthellaceae* from Actinobacteriota phylum and *Paracoccus* genus belonging to Proteobacteria phyla, also exhibited increased abundance in *Mgat5^{-/-}* mice (Figure 1E). Accordingly, we also found that the colonic microenvironment of *Mgat5^{-/-}* mice exhibited significant decreased levels of SCFAs, that are considered major players in intestinal homeostasis [40] (Figure 1F). In line with this, we also demonstrated a concomitant reduced expression of receptors and transporters of SCFAs[41], namely *Gpr43*, *Gpr109a*, *Mct1* and *Smct1* (Figure 1G-J).

Given the biological relevance of intestinal glycocalyx in maintaining the integrity of gut barrier, we then evaluated the impact of the deficiency of complex branched *N*-glycans in intestinal permeability. By performing FITC-dextran assay, we showed that *Mgat5^{-/-}* mice exhibited an increased permeability of intestinal epithelial barrier as illustrated by the increased FITC-dextran levels detected in *Mgat5^{-/-}* mice serum (Figure 1K). In addition, and since the regulation of tissue integrity and gut permeability also depends on the expression and interaction of proteins in cell–cell junctional complexes, such as the tight-junctions [42], we also evaluated the expression of claudin-1, 2, 3, 4 and occludin. The results showed an increased expression of claudin 1 and 2 in *Mgat5^{-/-}* mice (Figure 1L and M), as described to occur in IBD [43, 44], alongside a marked loss of sealing claudins 3 and 4 in comparison with *Mgat5^{WT}* mice (Figure 1N and O). No significant differences were observed in the expression of occludin between *Mgat5^{WT}* and *Mgat5^{-/-}* mice (Supplementary Figure 1H). Altogether, these results demonstrate the functional impact of changes in the gut mucosa *N*-glycome composition in triggering alterations of gut microbiome alongside with perturbations of the intestinal barrier integrity.

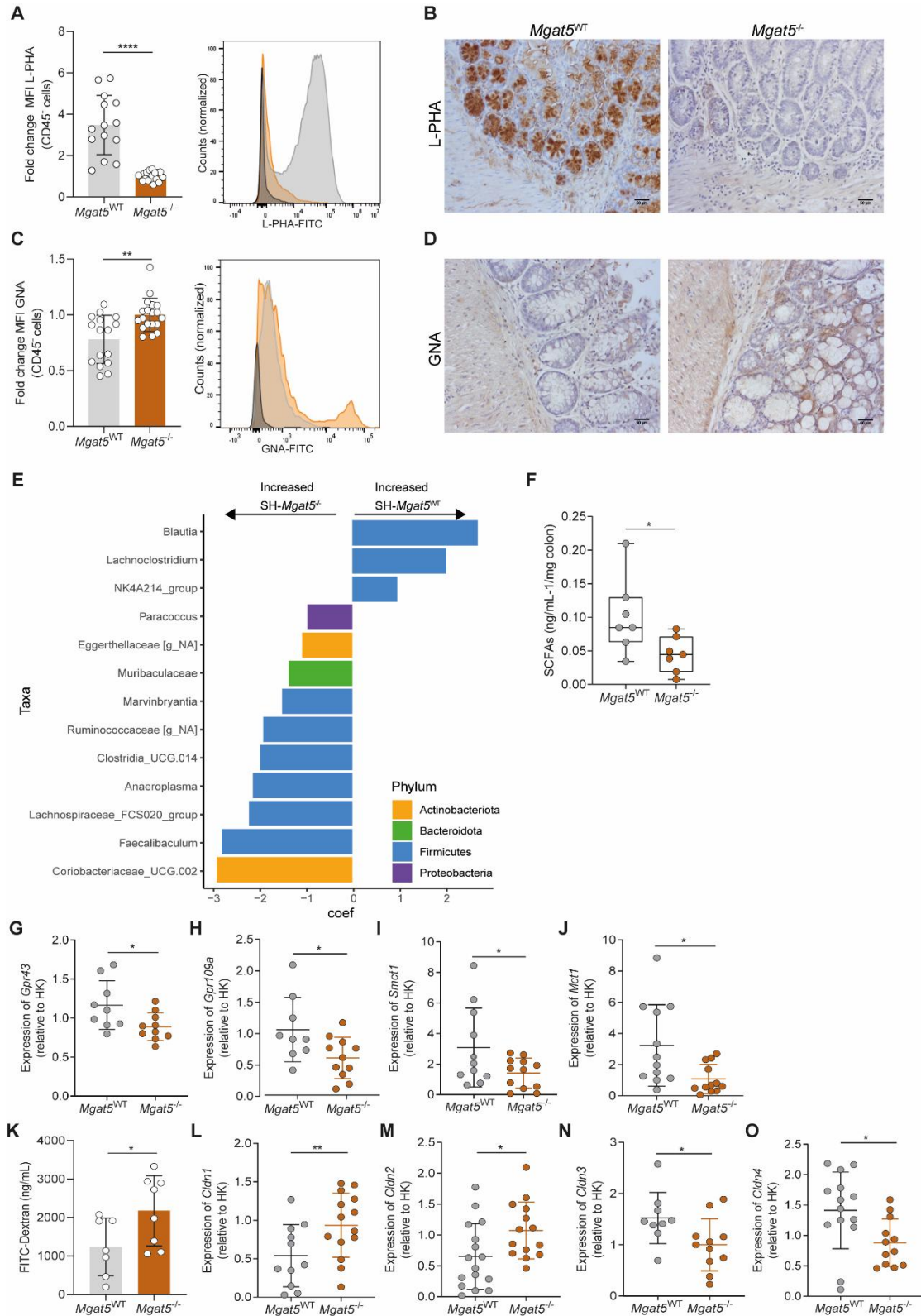


Figure 1. Reduction of branched *N*-glycans in mice promotes intestinal permeability and gut dysbiosis. (A and B) Levels of β 1,6-branching *N*-glycans at steady state in epithelial cells (CD45⁺ cells) from *Mgat5*^{-/-} mice and *Mgat5*^{WT} controls. (A) L-PHA lectin was used to detect branched *N*-glycans and the median fluorescence intensity (MFI) was determined by flow cytometry. MFI was normalized for the average of *Mgat5*^{-/-} mice MFI; in the representative histogram, dark grey, light grey

and orange depicts unstained control, *Mgat5*^{WT} and *Mgat5*^{-/-}, respectively. (B) Lectin histochemistry staining with L-PHA in mouse colonic samples. Scale bar = 50 μ m. (C and D) Levels of high-mannose *N*-glycans at steady state in epithelial cells (CD45⁺ cells) from *Mgat5*^{-/-} mice and *Mgat5*^{WT} controls. (C) GNA lectin was used to detect mannose *N*-glycans and the median fluorescence intensity (MFI) was determined by flow cytometry. MFI was normalized for the average of *Mgat5*^{-/-} mice MFI; in the representative histogram, dark grey, light grey and orange depicts unstained control, *Mgat5*^{WT} and *Mgat5*^{-/-}, respectively. (D) Lectin histochemistry staining with GNA in mouse colonic samples. Scale bar = 50 μ m. (E) Linear discriminant analysis (LDA) of the gut microbiota composition based on 16S rRNA sequencing in the faecal samples from *Mgat5*^{-/-} and *Mgat5*^{WT} mice at steady state. (F) Concentration of short-chain fatty acids (SCFAs) measured by ELISA in the colon of *Mgat5*^{-/-} and *Mgat5*^{WT} mice at steady-state. (G-J) The mRNA expression levels at steady state of genes encoding SCFAs receptors Gpr43 (G), Gpr109a (H) and SCFAs transporters Smct1 (I) and Mct1 (J) in the colonic tissue from *Mgat5*^{-/-} and *Mgat5*^{WT} mice measured by RT-qPCR. Expression of target gene mRNAs was calculated based on housekeeping gene (*Gapdh*). mRNA expression levels were normalized for the average of mRNA levels of *Mgat5*^{-/-} mice. (K) Intestinal permeability measured by FITC-labelled dextran in *Mgat5*^{-/-} and *Mgat5*^{WT} mice at steady state. (L-O) The mRNA expression levels at steady state of genes encoding for claudin-1 (L), claudin-2 (M), claudin-3 (N) and claudin-4 (O) in the colonic tissue from *Mgat5*^{-/-} mice and *Mgat5*^{WT} controls measured by RT-qPCR. Expression of target gene mRNAs was calculated based on housekeeping gene (*Gapdh*). mRNA expression levels were normalized for the average of mRNA levels of *Mgat5*^{-/-} mice. (A and C) n = 14–19 per group. (F) n = 7 per group. (G-H) n = 9–12 per group. (K) n = 7–8 per group (L-O) n = 9–16 per group. Each datapoint represents an individual animal. Data is represented as mean $\pm$ SD. *p < 0.05; **p < 0.01; ****p < 0.0001 using an unpaired two-tailed Student's *t*-test or Mann-Whitney test.

Sharing of the intestinal microbiota between *Mgat5*^{-/-} and *Mgat5*^{WT} mice impact on colitis susceptibility.

We then sought to explore the role of the dysbiotic microbiota imposed by changes in the host's glycocalyx, in defining susceptibility to intestinal inflammation. To explore this, *Mgat5*^{-/-} and *Mgat5*^{WT} mice were co-housed in the same cage, aiming to promote the sharing of their microbiota in a glycoengineered-dependent environment. Upon co-housing, colitis was induced with DSS (Figure 2A). Interestingly, cohoused *Mgat5*^{-/-} mice (CH-*Mgat5*^{-/-}) developed a less severe disease, as indicated by lower disease activity index (DAI) and area under the curve (AUC) when compared with single-housing *Mgat5*^{-/-} (SH-*Mgat5*^{-/-}) (Figure 2B and C). Remarkably, co-housed *Mgat5*^{WT} (CH-*Mgat5*^{WT}) exhibited a worse disease course (Figure 2B and C), which supports the relevance of host glycosylation in dictating the susceptibility to intestinal inflammation in a process mediated by microbiota alterations.

Gut microbiota composition analysis upon co-housing further revealed a clear gain in terms of increased abundance and diversity in CH-*Mgat5*^{-/-} comparing with SH- *Mgat5*^{-/-} (Figure 2D and E), which is in line with the milder disease symptoms. Indeed, the microbiota of CH colitis-susceptible *Mgat5*^{-/-} mice was mainly enriched by Firmicutes bacteria upon cohousing (Figure 2F).

These results demonstrate the clear impact of the intestinal microbiota profile induced by mucosa *N*-glycosylation alterations (modeled in *Mgat5*^{-/-} mice), in defining susceptibility to intestinal inflammation, putting glycans at the frontiers of host-microbiota interactions.

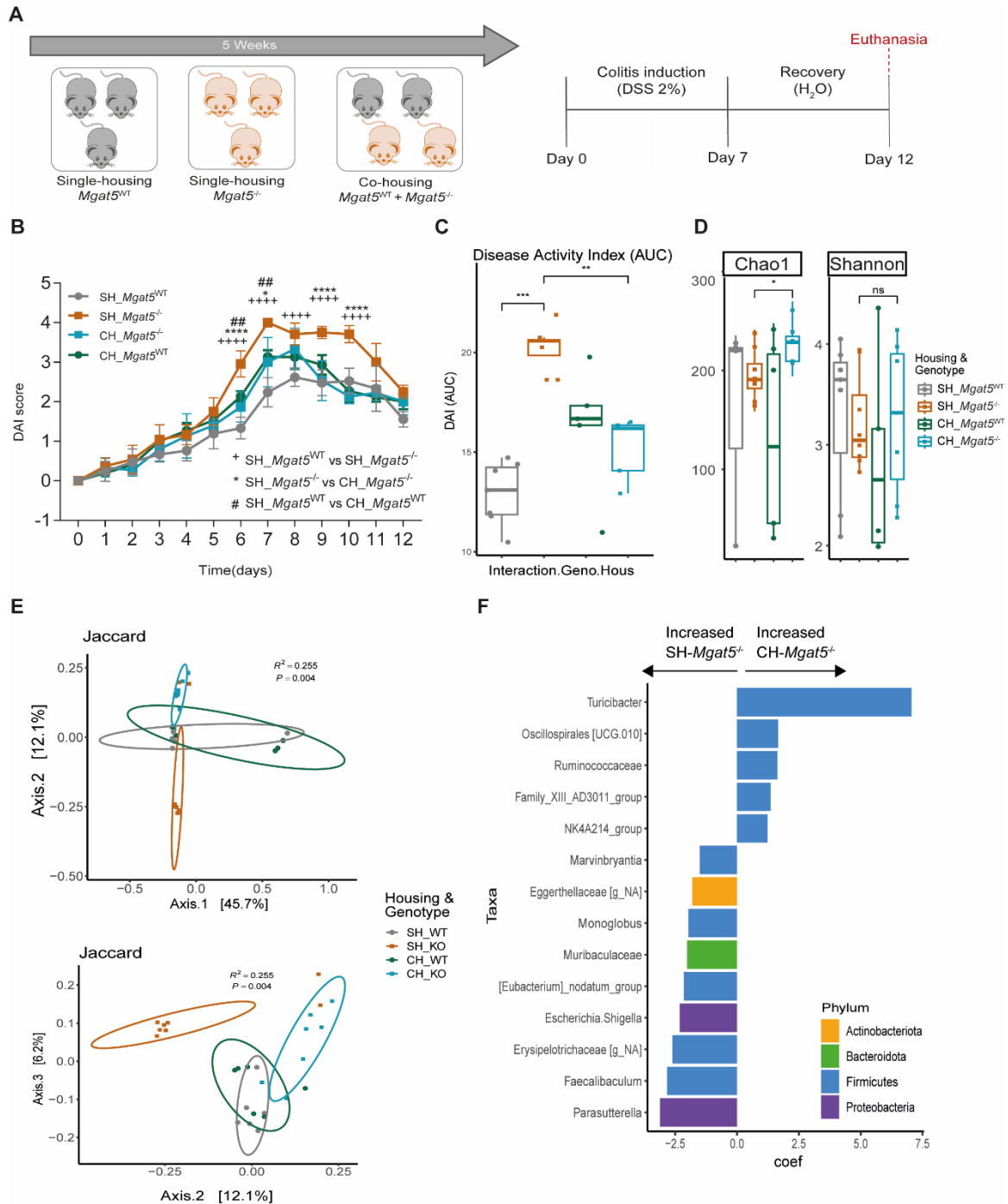


Figure 2. Co-housing of *Mgat5*^{-/-} mice with *Mgat5*^{WT} mice impacts gut dysbiosis and colitis susceptibility. (A) Schematic representation of co-housing experiment. *Mgat5*^{-/-} and *Mgat5*^{WT} mice (controls) were cohoused for 5 weeks. Then, colitis was induced by giving *ad libitum* 2% DSS, in the drinking-water, for 7 days followed by normal water for 5 additional days. (B) Disease activity score (DAI) of single-housed (SH) and co-housed (CH) *Mgat5*^{-/-} or *Mgat5*^{WT} mice upon DSS-induced colitis. (C) The area-under-the-curve (AUC) of DAI between single-housed (SH) and co-housed (CH) *Mgat5*^{-/-} or *Mgat5*^{WT} mice upon DSS-induced colitis. (D) Richness and evenness of faecal microbiota composition from mice before and after co-housing analysed by 16S rRNA sequencing. (E) Principal component analysis (PCoA) of gut microbiota composition generated on Jaccard based on 16S rRNA sequencing. (F) Linear discriminant analysis (LDA) of the gut microbiota composition based on 16S rRNA sequencing in the faecal samples SH and CH *Mgat5*^{-/-} at steady state (B-F) n = 5–8 per group. Each datapoint represents an individual animal. Data is represented as mean ± SD. *p < 0.05; **p < 0.01; ****p < 0.0001 using Two-way ANOVA.

Impaired β 1,6-GlcNAc complex branched *N*-glycosylation leads to a downregulation of ILC3- IL22 mediated immune response in the gut.

Taking into consideration the impact of gut mucosa *N*-glycome in shaping microbiota composition associated with severity to colitis, we then investigated their mechanistic effect in the regulation of intestinal inflammatory pathways. An extensive characterization of the different immune subsets in intestinal mucosa was performed at baseline (Supplementary Figure 2 and Figure 3) and after colitis induction (Supplementary Figure 3), comparing *Mgat5*^{WT} and *Mgat5*^{-/-} mice. No significant alterations were observed in the frequency of total T cells (CD3⁺), Th17 cells, regulatory T cells (Treg), B cells or macrophages at baseline (Supplementary Figures 2B-F). However, a remarkable downregulation in type 3 innate lymphoid cells (ILC3) was found in *Mgat5*^{-/-} mice, at baseline, in comparison with *Mgat5*^{WT} mice (Figure 3A). This significantly decreased expression of ILC3 was maintained upon disease induction (Supplementary Figure 3A). Specifically, and among the sub-populations of ILC3, we observed that the deficiency in mucosa complex branched *N*-glycans appears to selectively impact the downregulation of the NCR⁺CCR6⁺RORγt⁺ and CCR6⁺ILC3 (LTi-like cells) sub-populations at baseline (Figure 3B and C), contrary to NCR⁺RORγt⁺ ILC3 (Supplementary Figure 2G). This impact in innate immune response was also observed through the upregulation of dendritic cells in *Mgat5*^{-/-} mice at baseline in comparison with *Mgat5*^{WT} (Supplementary Figure 2H).

After disease induction, as expected, alongside a decrease in ILC3 expression (as well as CCR6⁺ILC3 (LTi-like cells) sub-population), (Supplementary Figures 3A and B), we also observed an increased Th17 response (Supplementary Figure 3C), with no alterations found in other immune sub-populations (Supplementary Figures 3D-G). Furthermore, a decrease in the production of IL-22 by ILC3 cells in *Mgat5*^{-/-} mice (Figure 3D and E) was

observed and maintained upon colitis induction (Supplementary Figure 3H and I). This impact in IL-22 production in *Mgat5*^{-/-} mice were found to be specific for the ILC3 population since no differences were observed in IL-22 produced by Th17 cells (Supplementary Figure 2I and J) both at baseline and upon disease induction (Supplementary Figure 3J and K). Additionally, after DSS-induced colitis, but not at baseline (Supplementary Figure 2K and L), *Mgat5*^{-/-} mice displayed reduced levels of IL-22 production by ILC3 NCR⁺RORγt⁺ (Supplementary Figure 3L and M).

In fact, ILCs are highly plastic immune cells, that are able to adapt their phenotype and function depending on the environmental cues, such as dietary signals and microbial counterparts[45]. The balance between ILC3 and ILC1 immune populations is known to play a key role in gut homeostasis, whereas disruption of this balance has been described as contributing to intestinal inflammation, such as IBD[46]. However, whether changes in host glycocalyx may affect this ILC3/ILC1 immune balance remain unknown. Our results pioneer the direct interplay between mucosa *N*-glycosylation and ILCs activity and function (Figure 3A). In addition, we also showed that *Mgat5*^{-/-} mice displayed a clear trans-differentiation of ILCs population towards an ILC1 phenotype (Figure 3F), as observed by the increase in ex-ILC3/ILC1-like cells (Figure 3G and Supplementary Figure 2O-P). This is also demonstrated by the reduced ILC3/ILC1 ratio in *Mgat5*^{-/-} mice (Figure 3H). A similar profile was also found in *Mgat5*^{-/-} mice after DSS-induced colitis (Supplementary Figure 3N and O). These results demonstrate, for the first time the direct impact of changes in mucosa *N*-glycosylation in the regulation of ILC3 module in the trans-differentiation towards an ILC1-mediated proinflammatory phenotype. This result is in line with previous observations reporting ILC3-to-ILC1 transition in inflamed mucosal biopsies from CD patients associated with anti-GM-CSF autoantibodies against glycosylation [47]. This proinflammatory environment imposed by the ILC3/ILC1 plasticity, triggered upon alterations in gut mucosa glycome, was further supported by the increased production of TNFα and IL-6 proinflammatory cytokines in colonic explants from *Mgat5*^{-/-} mice at baseline (Figure 3I and J) and upon colitis induction (Supplementary Figure 3P-Q), and by the decreased levels of anti-inflammatory IL-10 cytokine at baseline (Figure 3K). However, after DSS-induced colitis, between *Mgat5*^{-/-} and *Mgat5*^{WT} mice did not exhibit significant differences in terms of IL-10 secretion (Supplementary Figure 3R). In the same line, no alterations were found to IL-22 production in colonic explants at baseline and upon colitis induction (Figure 3L and Supplementary Figure 3S). Nevertheless, the impaired IL-22 signaling observed in *Mgat5*^{-/-} mice was also supported by the significant increased expression of *Il22ra2*, the gene encoding to IL22 binding protein (IL-22BP) (Figure 3M), a cytosolic protein involved in IL-22 blockade [48, 49].

IL-22 signaling is known to induce the production of nucleotide oligomerization domain-containing protein 2 (NOD2) which is related to the regulation of innate immune response and microbial recognition [50]. Furthermore, *NOD2* is one of the most widely implicated genes in the etiology of Crohn's disease [51]. We here demonstrated that *Mgat5*^{-/-} mice also exhibited a significant downregulation of *Nod2* (Figure 3N) reinforcing the negative impact of the impaired IL-22 signaling (either by decreased IL22-derived ILC3 or increased *Il22ra2*) in the homeostatic gut environment. In addition, and in line with previous evidence suggesting that IL-22 produced by ILC3 impacts epithelial cell glycosylation by regulating the expression of fucosyltransferase 2 (*Fut2*) [52], we also observed that *Fut2* expression was decreased in *Mgat5*^{-/-} mice (Figure 3O).

Taking together, these results demonstrate the clear impact of the deficiency in the β 1,6-GlcNAc complex branched *N*-glycans (associated with dysbiosis) in the impairment of ILC3-IL22 immune response that culminate in *Nod2* downregulation that together converge in the activation of inflammatory cues promoting health to intestinal inflammation transition.

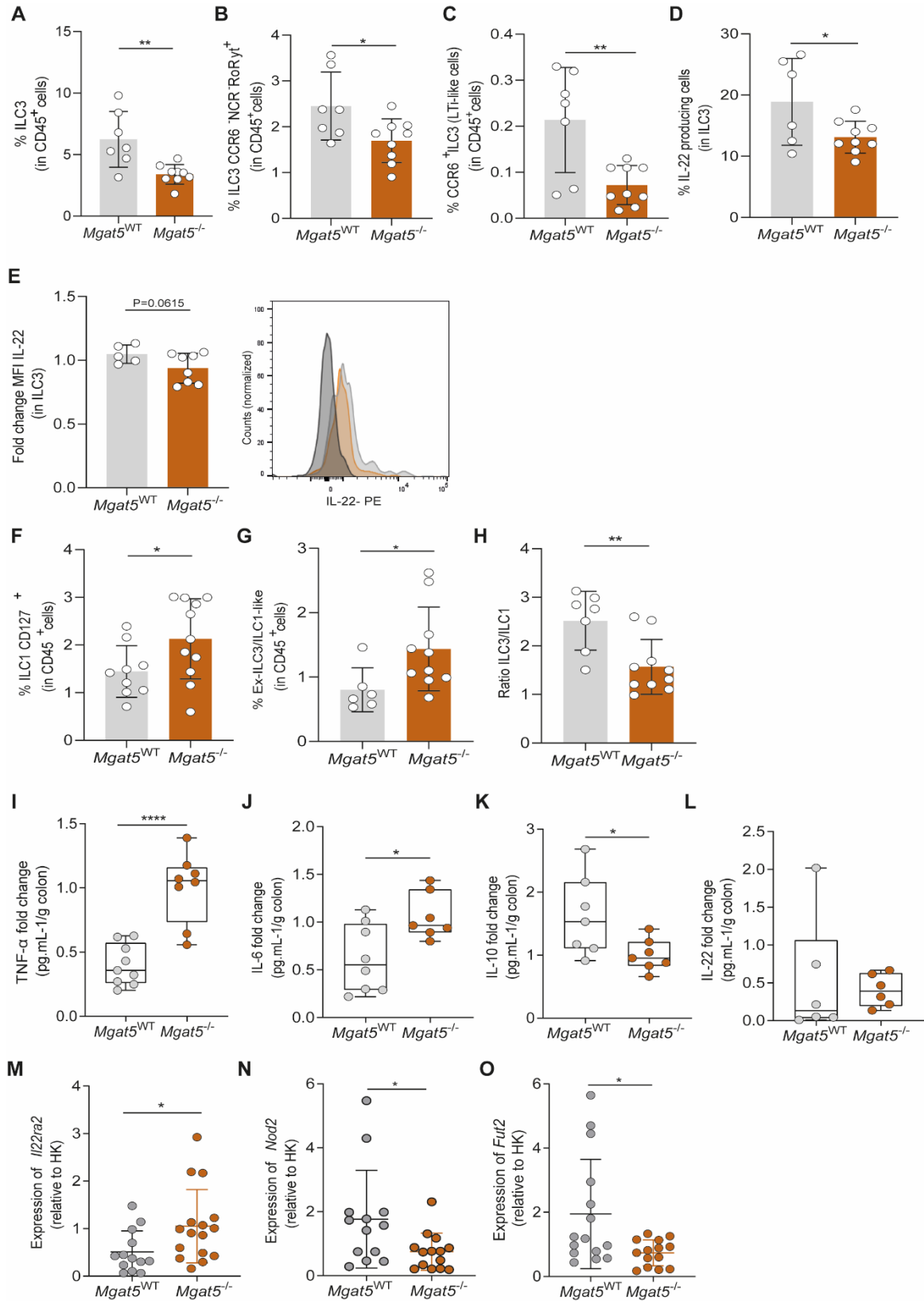


Figure 3. Deficiency in mucosa β 1,6-GlcNAc complex branched *N*-glycosylation leads to an impaired ILC3-IL-22-mediated immune response. Frequency of (A) ILC3, (B) CCR6-NCR-RoRyt⁺ ILC3, and (C) CCR6⁺ILC3 (LTi-like) subsets in CD45⁺ cell population in *Mgat5*^{-/-} and *Mgat5*^{WT} mice, at steady state. Frequency of (D) IL-22-producing ILC3 and (E) Mean fluorescence intensity (MFI) of intracellular IL-22 in ILC3 of *Mgat5*^{-/-} and *Mgat5*^{WT} mice at steady state. Frequency of (F)

CD127⁺ILC1, and (G) ex-ILC3/ILC1-like cell subsets in CD45⁺ cell population in *Mgat5*^{-/-} and *Mgat5*^{WT} mice, at steady state. (H) Ratio between the frequencies of ILC3 and CD127⁺ILC1 in *Mgat5*^{-/-} mice and *Mgat5*^{WT}, at steady state. (I-L) Cytokine concentrations in supernatants of colonic explants cultured for 24 hours. The concentrations are normalized to tissue weight. (M-O) The mRNA expression levels at steady state of genes encoding for (M) Il22bp, (N) Nod2 and (P) Fut2 in the colonic tissue measured by RT-qPCR. Expression of target gene mRNAs was calculated based on housekeeping gene (*Gapdh*). mRNA expression levels were normalized for the average of mRNA levels of *Mgat5*^{-/-} mice. (A) n = 7–9 per group. (B, C, F, G and H) n = 6–11 per group. (D and E) n = 6–9 per group. (I-L) n = 6–8 per group. (M-O) n = 13–16 per group. Each datapoint represents an individual animal. Data is represented as mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 using an unpaired two-tailed Student's *t*-test or Mann-Whitney test.

Prophylactic remodelling of mucosa glycosylation through metabolic supplementation with glycans controls immune response by repairing ILC3-IL22 homeostatic immune pathway.

To gain mechanistic insights into the microbial-derived pathways underlying the protective colitis phenotype that was observed in CH-*Mgat5*^{-/-} mice, an unstratified pathway output of Picrust2 analysis based on 16S rRNA data of SH-*Mgat5*^{-/-} and CH-*Mgat5*^{-/-} mice was performed (Figure 4A). The results demonstrated that CH-*Mgat5*^{-/-} mice exhibited the clear upregulation of the general microbial-associated traits involved in glycosylation biosynthetic pathways, particularly the UDP-*N*-acetylglucosamine biosynthesis pathway. In fact, UDP-GlcNAc is the preferential substrate for the activity of GnT-V that results in the synthesis of β1,6-GlcNAc complex branched *N*-glycans (Figure 4A). The upregulation of this selected microbial-derived trait involved in UDP-*N*-acetylglucosamine biosynthesis pathway upon mouse cohousing, associated with prevention of inflammation and control of dysbiosis (Figure 2), highlights the potential regulatory/protective involvement of complex branched *N*-glycans in host-microbial relationship associated with gut homeostasis.

To further test this hypothesis, we metabolically supplemented colitis-susceptible *Mgat5*^{-/-} mice with GlcNAc before disease induction (Figure 4B). This was specifically aimed at testing the prophylactic effect of the glycosylation remodeling in preventing inflammation. Notably, we observed that the supplementation of *Mgat5*^{-/-} mice with GlcNAc for 8 weeks was able to re-establish the levels of ILC3 (Figure 4C) and, importantly, of the homeostatic cytokine IL-22 produced by ILC3 (Figure 4D and E) at steady state. The prophylactic supplementation with GlcNAc also led to the upregulation of CCR6⁺ILC3 (LTi-like cells) and NCR-RORγt⁺ILC3 sub-populations (Figure 4F and Supplementary Figure 4A). Notably, GlcNAc supplementation was also able to significantly reduce the frequency of ILC1 (Figure

4G) and ex-ILC3/ILC1 like (Figure 4H), as well as decrease IFN γ produced by these cells in the colon (Figure 4I-L). Indeed, GlcNAc supplementation significantly increased the ILC3/ILC1 ratio in *Mgat5*^{-/-} mice (Figure 4M). No differences were found in ILC3 NCR⁺RoRyt⁺ and Treg cells (Supplementary Figure 4B and 4C). Nevertheless, we observed a decrease in the frequency of Th17 cells after glycan supplementation, with no impact on its IL-22 production (Supplementary Figures 4D-F). Accordingly, the effect of GlcNAc supplementation was further demonstrated by the reduced levels of proinflammatory cytokines IL-6 and TNF α in colonic supernatants, comparing treated *versus* non-treated mice, at baseline (Figure 4N and O). No alterations were found for IL-10 and IL-22 production (Figure 4P and Q). Remarkably, we also showed that GlcNAc treatment was able to reduce the expression of *IL22ra2* (Figure 4R). Importantly, the prophylactic effect of glycan supplementation was also observed in the rescue of the expression levels of *Nod2* and *Fut2* (Figure 4S and T). GlcNAc supplementation was also tested in *Mgat5*^{WT} mice, and no major alterations were found between *Mgat5*^{WT} treated and non-treated with GlcNAc in terms of ILC3 abundance and ILC3-derived IL22 production (Supplementary Figure 4G-I); however, GlcNAc supplementation significantly decreased IL-22 production in Th17 cells (Supplementary Figure 4J-L). Additionally, *Mgat5*^{WT} treated mice also display a trend decrease in IL-10 levels in colonic explants (Supplementary Figure 4M) with no major significant differences observed for the production of IL-6, TNF α and IL-22 (Supplementary Figure 4N-P). No changes were observed in the expression of *IL22ra*, *Nod2* and *Fut2* in *Mgat5*^{WT} of GlcNAc treated mice (Supplementary Figure 4R-T).

Overall, these data demonstrate the biological effect of prophylactically remodeling mucosa glycosylation as a promising strategy to control a key homeostatic intestinal module mediated by ILC3 and IL-22 dynamics.

Chapter 3 | Alterations in mucosa branched *N*-glycans lead to dysbiosis and downregulation of ILC3: a key driver of intestinal inflammation

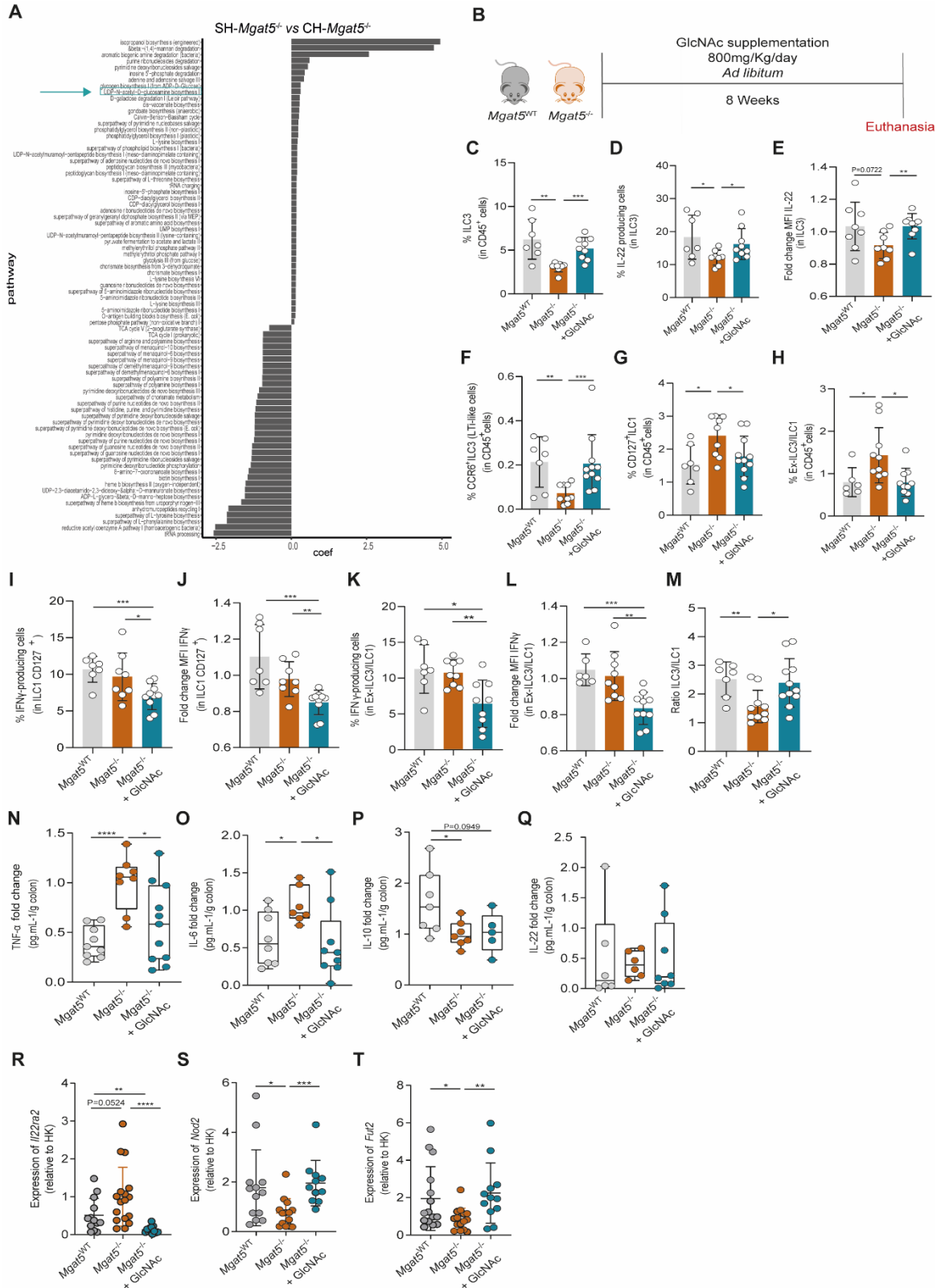


Figure 4. Prophylactic GlcNAc supplementation modulates immune response of *Mgat5*^{-/-} mice rescuing ILC3-IL22- homeostatic axis. (A) Unstratified pathway output of Picrust2 based on 16S rRNA sequencing of single- and co-housed (SH and CH) *Mgat5*^{-/-} mice. (B) Schematic representation of 800 mg/Kg/day *N*-acetylglucosamine (GlcNAc) supplementation, *ad libitum*, of *Mgat5*^{-/-} mice for 8 weeks. Frequency of (C) ILC3 in CD45⁺ population in *Mgat5*^{WT}, *Mgat5*^{-/-}, and GlcNAc-supplemented

Mgat5^{-/-} mice. (D) Frequency of IL22-producing ILC3 and (E) Mean fluorescence intensity (MFI) of intracellular IL-22 in ILC3, in *Mgat5*^{WT}, *Mgat5*^{-/-}, and GlcNAc-supplemented *Mgat5*^{-/-} mice. Frequency of (F) CCR6⁺ILC3 (LTi-like), (G) CD127⁺ILC1 (G), and (H) ex-ILC3/ILC1-like cells in CD45⁺ cell population in *Mgat5*^{WT}, *Mgat5*^{-/-}, and GlcNAc-supplemented *Mgat5*^{-/-} mice. Frequency of (I) IFN γ -producing CD127⁺ILC1 and (J) Mean fluorescence intensity (MFI) of intracellular IFN γ in CD127⁺ILC1, in *Mgat5*^{WT}, *Mgat5*^{-/-}, and GlcNAc-supplemented *Mgat5*^{-/-} mice. MFI were normalized for the average of *Mgat5*^{-/-} mice MFI. Frequency of (K) IFN γ -producing ex-ILC3/ILC1-like cells and (L) Mean fluorescence intensity (MFI) of intracellular IFN γ in ex-ILC3/ILC1-like cells, in *Mgat5*^{WT}, *Mgat5*^{-/-}, and GlcNAc-supplemented *Mgat5*^{-/-} mice. MFI were normalized for the average of *Mgat5*^{-/-} mice MFI. (M) Ratio between the frequencies of ILC3 and CD127⁺ ILC1 in *Mgat5*^{WT}, *Mgat5*^{-/-}, and GlcNAc-supplemented *Mgat5*^{-/-} mice. (N-Q) Cytokine concentrations in culture supernatants of colonic explants. The concentrations are normalized to tissue weight. (R-T) The mRNA expression levels at steady state of genes encoding for (R) Il22bp, (S) Nod2 and (T) Fut2 in the colonic tissue measured by qPCR. Expression of target gene mRNAs was calculated based on housekeeping gene (*Gapdh*). mRNA expression levels were normalized for the average of mRNA levels of *Mgat5*^{-/-} mice. (C-E) n = 8–10 per group. (F-H) n = 9–11 per group. (I-M) n = 8–11 per group (N, Q) n= 6–11 per group. (R-T) n=11–17 per group. Each datapoint represents an individual animal. Data is represented as mean $\pm$ SD. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 using an unpaired two-tailed Student's *t*-test or Mann-Whitney test.

GlcNAc prophylactic supplementation is able to prevent severe colitis

Finally, we tested whether the prophylactic effect of GlcNAc supplementation is effective in preventing disease development. Thus, after 8 weeks of glycan supplementation, colitis was induced by DSS (Figure 5A). Remarkably, prophylactic glycan supplementation significantly reduced disease susceptibility and severity in comparison with non-treated *Mgat5*^{-/-} mice (Figure 5B). Histopathological analysis of colon section, after DSS-colitis induction, revealed that treated animals showed a reduced inflammatory infiltrate when compared with non-supplemented mice (Figure 5C and D). To understand if the decreased susceptibility of supplemented mice was also due to a remodelling of the intestinal epithelial barrier, expression of claudin-encoding genes and intestinal permeability was assessed prior colitis induction. No significant alterations were found in intestinal permeability or in claudin-2 expression between non-supplemented and glycan-supplemented *Mgat5*^{-/-} mice (Supplementary Figures 5A-B), however a tendency for an upregulation of claudin-4 was observed (Figure 5E). Consistent with a protective phenotype imposed by supplementation with glycans, SCFAs levels were markedly increased upon glycan-supplemented *Mgat5*^{-/-} mice (Figure 5F), which is in accordance with increased levels of *Smct1* transporter (Figure

5G). No differences were observed for the *Mct1* transporter and receptors (Supplementary Figure 5C-E). We wanted to assess whether the immunomodulatory effect of GlcNAc in ILC3 dynamics was maintained upon intestinal inflammation. We found that, upon DSS-induced colitis, *Mgat5*^{-/-} supplemented mice exhibited enhanced ILC3 levels concomitantly with an increase in IL-22 production (Figure 5H-J).

Overall, these results pinpoint the immunomodulatory properties of GlcNAc supplementation in a colitis-susceptible mouse model. This is supported by the ability of glycans in shaping a tolerogenic gut immune profile by activating the protective ILC3-IL-22 module and thus preventing health-to-intestinal disease transition.

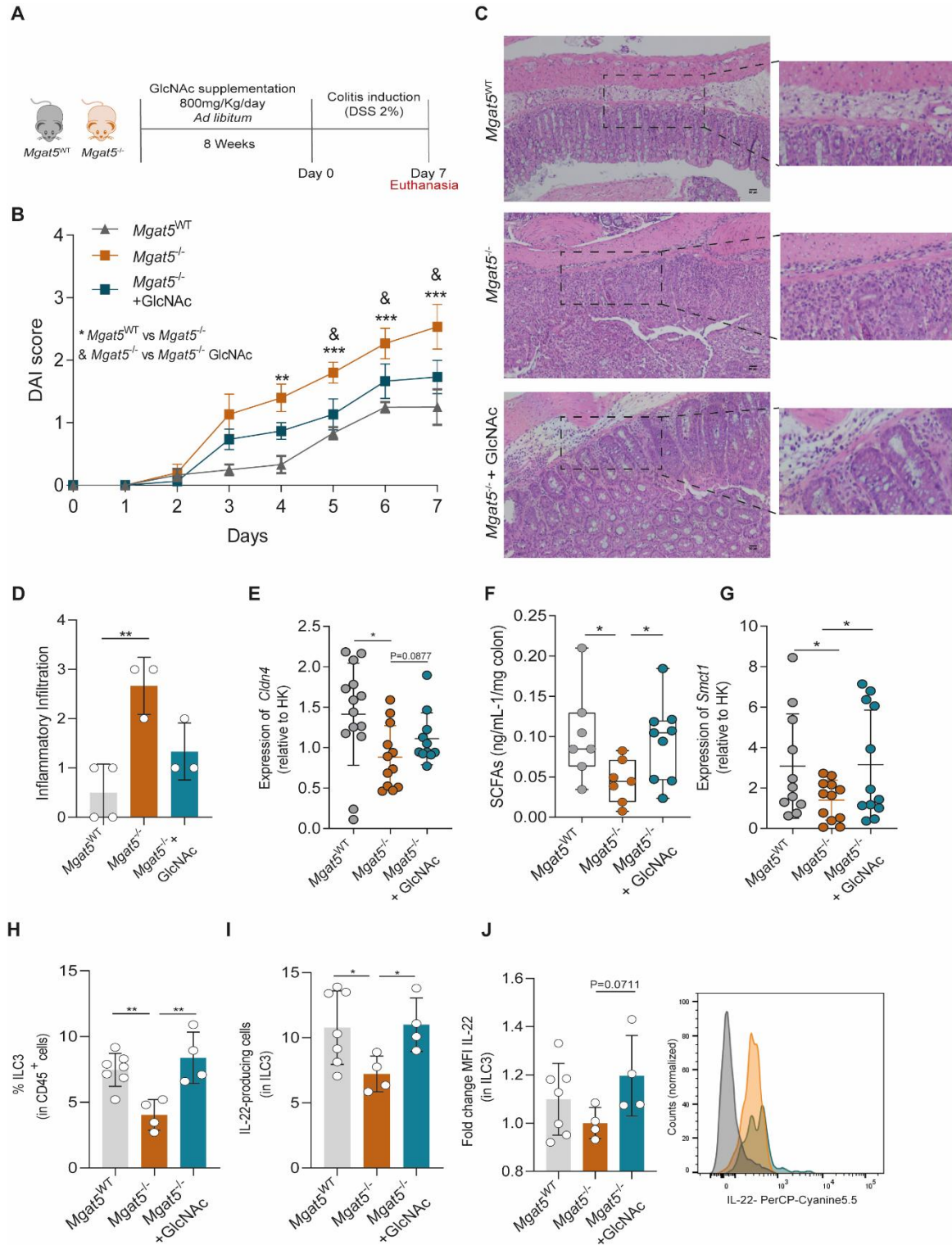


Figure 5. Glycan supplementation has a protective effect against colitis development and restores the levels of SCFAs. (A) Schematic representation of 800 mg/Kg/day *N*-acetylglucosamine (GlcNAc) supplementation of *Mgat5*^{-/-} mice for 8 weeks followed by 2% DSS treatment for 7 days. (B) Disease activity score (DAI) of *Mgat5*^{WT}, *Mgat5*^{-/-}, and GlcNAc-supplemented *Mgat5*^{-/-} mice upon DSS-induced colitis. (C and D) Histological analysis of haematoxylin & eosin staining and qualitative scores of intestinal immune infiltration of colon sections from *Mgat5*^{WT}, *Mgat5*^{-/-}, and GlcNAc-supplemented *Mgat5*^{-/-} mice upon DSS-induced colitis. There is

no statistical significance between *Mgat5*^{-/-} and GlcNAc-supplemented *Mgat5*^{-/-} mice. Zoomed images highlight immune infiltrate. Scale bar = 50 μ m. (E) The mRNA expression levels at steady-state of *Cldn4* in *Mgat5*^{WT}, *Mgat5*^{-/-}, and GlcNAc-supplemented *Mgat5*^{-/-} mice. Expression of target gene mRNAs was calculated based on housekeeping gene (*Gapdh*). mRNA expression levels were normalized for the average of mRNA levels of *Mgat5*^{-/-} mice. (F) Quantification of SCFAs measured by ELISA in the colon of *Mgat5*^{WT}, *Mgat5*^{-/-}, and GlcNAc-supplemented *Mgat5*^{-/-} mice at steady-state. (G) The mRNA expression levels at steady-state of SCFAs transporter *Smct1* in *Mgat5*^{WT}, *Mgat5*^{-/-}, and GlcNAc-supplemented *Mgat5*^{-/-} mice. Expression of target gene mRNAs was calculated based on housekeeping gene (*Gapdh*). mRNA expression levels were normalized for the average of mRNA levels of *Mgat5*^{-/-} mice. (H) Frequency of ILC3 in CD45⁺ population in *Mgat5*^{WT}, *Mgat5*^{-/-}, and GlcNAc-supplemented *Mgat5*^{-/-} mice upon DSS-induced colitis. Frequency of (I) IL22-producing ILC3 and (J) Mean fluorescence intensity (MFI) of intracellular IL-22 in ILC3, in *Mgat5*^{WT}, *Mgat5*^{-/-}, and GlcNAc-supplemented *Mgat5*^{-/-} mice upon DSS-induced colitis. (B) n = 5 per group. (D) n = 3-4 per group. (E) n=11-14 per group; (F) n=7-9 per group. (G) n= 11-12 per group. (H-J) n=4-7 per group. Each datapoint represents an individual animal. Data is represented as mean $\pm$ SD. *p < 0.05; **p < 0.01; using an unpaired two-tailed Student's *t*-test or Mann-Whitney test.

Impaired mucosa *N*-glycosylation occur concomitantly with decreased frequency of intestinal ILC3 in human IBD patients.

To analyze further the specific impact of an altered intestinal glycome in the regulation of ILC-mediated gut immunity in IBD immunopathogenesis, we investigated whether changes in gut mucosa glycosylation may be linked to alterations in ILC3 module in human IBD. To do so two publicly available databases (GSE134809; Martin et al. (2019)) and Häsler et al. (2017) were analysed. Based on the study of Häsler et al., we found that the expression of *MGAT5* glycoprotein, involved in the production of complex branched *N*-glycans, appears to be impaired in inflamed colonic tissue of CD patients when compared to controls (Figure 6A). Interestingly, we also demonstrated that the expression of the transcription factor *RORC* (associated with ILC3) was significantly downregulated in inflamed CD biopsies (Figure 6B; study Häsler et al), and the levels of ILC3 also seem to be decreased in inflamed CD biopsies (Supplementary Figure 6; study Martin et al.). This evidence supporting the link between *MGAT5* glycoprotein and ILC3 modulation was further validated in clinical samples obtained from mucosal biopsies of CD and UC patients, as well as healthy individuals (controls) (S. Table 1). Accordingly, the results showed that inflamed biopsies from IBD patients displayed a significant decreased frequency of ILC3 compared to healthy controls (Figure 6C). Concomitantly, the expression of less complex, mannose-enriched *N*-glycans (GNA binding) at the epithelial cells was inversely correlated with frequency of ILC3,

suggesting that a deficiency in complex branched *N*-glycosylation and decreased frequency of gut ILC3 in IBD are intricately related (Figure 6D). Altogether, these findings support the association between loss of complex *N*-glycans (through downregulation of *MGAT5*) with impaired levels of ILC3 at intestinal mucosa, associated with IBD.

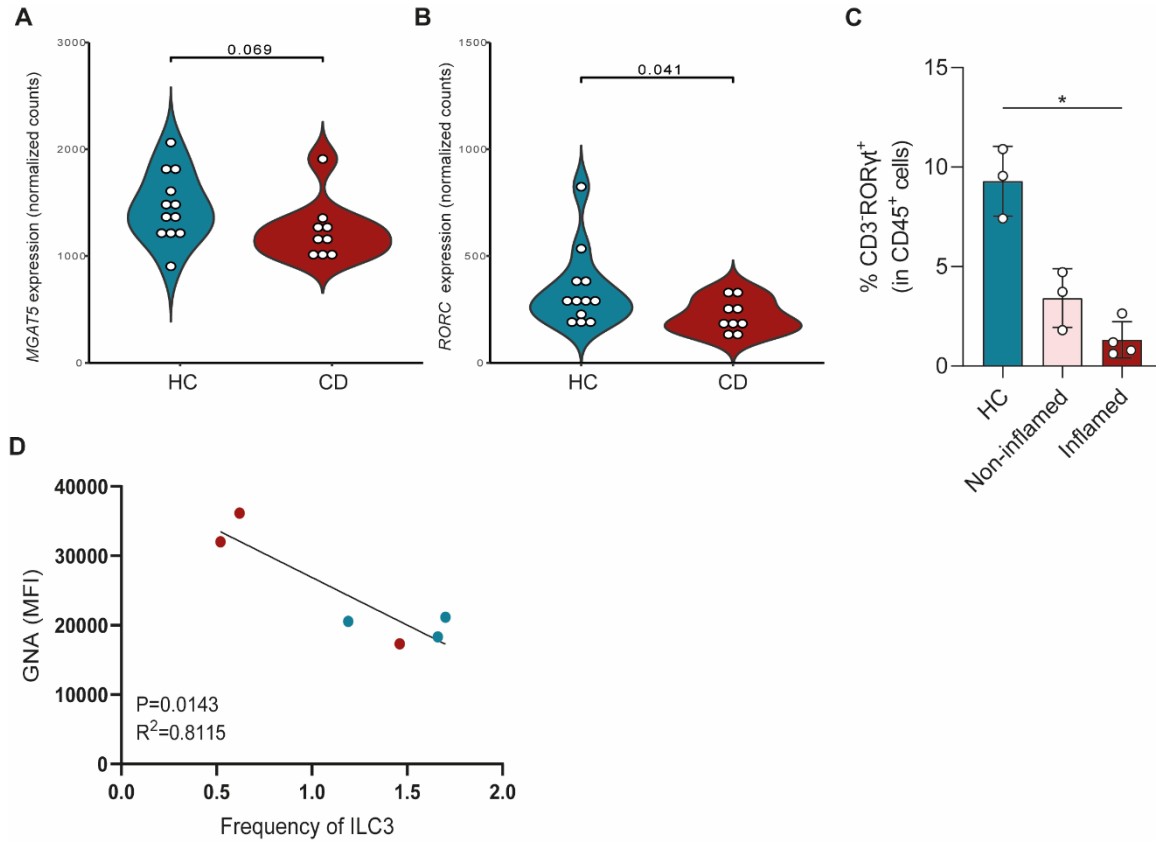


Figure 6. Impaired *N*-glycan-related glycoprotein expression in IBD patients associates with reduced frequency of intestinal mucosal ILC3. The gene expression of (A) *MGAT5* glycoprotein and (B) *RORC* in the inflamed colonic tissue from CD patients and healthy controls. (C) Frequency of ILC3 (CD3⁺RORγt⁺) in CD45⁺ population in inflamed and non-inflamed mucosal tissue from IBD patients and healthy controls. (D) The coefficient correlation between frequency of ILC3 and GNA expression in inflamed mucosal tissue from IBD patients (red dots) and healthy controls (blue dots). (A and B) n=9-12 per group. Data were derived from dataset published by Häsler et al. (2017). (C) n=3-4 per group. Data are represented as mean ± SD *p < 0.05; using a One-way ANOVA test. (D) n=3 per group; Pearson correlation test.

Discussion

The loss of equilibrium between microbes and the intestinal immune system is one of the main drivers of the onset of IBD [4, 53, 54]. In fact, recent evidence points towards altered host-microbiome interactions as one of the earliest events in the pathogenesis of intestinal inflammation [28].

Protein glycosylation has emerged as a possible link between host microbiome and immune response. In fact, we and others have previously demonstrated the implications of glycans in gut immunity and in the immunopathogenesis of IBD [28], even years before IBD diagnosis [23]. However, whether and how an altered host glycocalyx contribute to the loss of immune tolerance, by imposing alterations in microbiota composition (dysbiosis) and the activation of immune response, is a fundamental question that remains unanswered.

In this study, we demonstrated that alterations in host mucosa *N*-glycans are at the basis of the crosstalk between the gut microbiome and the host immune response associated with health to intestinal inflammation transition. We revealed that a deficiency in the expression of complex branched *N*-glycans leads to a clear dysbiosis, together with the perturbation of the gut permeability, that was associated with colitis severity. This dysbiotic phenotype was characterized by a significant reduction in protective bacteria from Firmicutes phylum, namely *Blautia*, *Ruminococcaceae* *NK4A214* group and *Lachnospirillum* genera, concomitantly with the overgrowth of selective pathobionts such as families from the Actinobacteriota, Proteobacteria and Bacteroidota phyla, considered general glycan degraders [55, 56]. *Blautia*, *Ruminococcaceae* *NK4A214* group and *Lachnospirillum* genera are known for producing SCFAs, mainly propionate and butyrate, thus playing a significant role in strengthening the intestinal barrier, maintaining intestinal homeostasis, and preventing inflammation [57]. Several species included in the Firmicutes phylum, such as *Blautia*, are considered as specialists due to their limited capacity for glycan degradation. Therefore, we hypothesize that the altered glycocalyx in *Mgat5*^{-/-} might have a detrimental impact on the abundance and function of these elements, leading to the reduced production of SCFAs, which are by-products of glycan fermentation. Importantly, in line with our observations, a significant decreased abundance of these species has been reported in IBD patients [53, 58]. The mucosa glycan switch, imposed by a deficiency in complex branched *N*-glycans and the abnormal exposure of less complex/mannose-enriched glycans alongside with dysbiotic microbiota, was also found to compromise the intestinal barrier, as observed by changes in the expression of claudins and tight junctions. This impact of changes in mucosa glycome in gut permeability is in fact in line with previous evidence revealing that increased intestinal permeability may predict later development of CD [59].

We further demonstrated that changes in mucosa glycosylation not only led to dysbiosis, but directly perturb a key homeostatic module in the intestine mediated by ILC3 immune response. In fact, ILC3 are known to be a central player in orchestrating tissue and gut immune homeostasis at steady state [60]. ILC3 control the immune response against pathogens and opportunistic commensals [52, 61-63], representing the main source of protective IL-22 in the intestine [64]. Diet and commensal microbiota-derived signals are described to regulate ILC3 recruitment and function in the intestine [64-66]. Here we showed, for the first time that a deficiency in complex branched *N*-glycans at gut mucosa results in a significant decrease of ILC3 and ILC3-producing IL-22 revealing mucosa glycans as a new source of ILCs regulators. This downregulation of ILC3, imposed by the deficiency in complex branched *N*-glycans, further promoted its plasticity towards an ILC1 phenotype. Specifically, we showed that the deficiency in mucosa complex branched *N*-glycans in *Mgat5*^{-/-} mice induced the downregulation of CCR6⁺ILC3 (LTi-like ILC3) and NCR⁻CCR6⁻RoRγt⁺ cell subsets. LTi-like ILC3 were described to contribute to gut homeostasis, tight junction expression and IL-22 production [67-69], being the prevailing ILC3 subset in colonic lamina propria of healthy mice [70, 71]. CCR6⁻NCR⁻ILC3 can transform into CCR6⁻NCR⁺ILC3 through the upregulation of T-bet and Notch [72]. These results may suggest that the development of these ILC3 precursors may be impaired through changes in mucosa glycosylation associated with altered microbiota and respective signals. Furthermore, this effect of the mucosa glycan switch in the perturbation of ILC3 module is also in line with the impact observed in *Nod2* expression and in *Fut2*-mediated glycosylation. *Nod2* is an intracellular pattern recognition receptor present in most intestinal immune and epithelial cells and known to recognize glycans, such as bacterial peptidoglycans, being thus involved in IBD immunopathogenesis [51]. Accordingly, we also observed that the downregulation of IL-22-producing ILC3 imposed by glycan alterations was associated with decreased expression of *Fut2*-mediated fucosylation associated with susceptibility to inflammation, despite we found only a slight decrease in UEA-I binding. This is in accordance with previous evidence suggesting that IL-22 produced by ILC3 can act as a modulator of intestinal epithelial fucosylation by *Fut2* expression associated with homeostasis [52]. Importantly from the clinical point of view, we explored a possible association between an altered mucosa branching glycosylation and ILC3 module in human IBD clinical samples. Our findings suggest that impaired complex branched *N*-glycosylation may be associated with deficient intestinal ILC3, which could reinforce the clinical impact of host mucosa glycome and ILC3 dynamics associated with IBD. Nevertheless, further studies are required to validate this association. To further explore the intestinal environment in context of deficiency in complex branched *N*-glycans, we assessed some inflammatory markers in the colonic samples at the basal level. Notably, the colonic proinflammatory cytokines found in

IBD development, namely, TNF α [73-74] and IL-6 [75-78], were substantially increased in *Mgat5*^{-/-} mice, whereas IL-10 was significantly reduced. The overproduction of pro-inflammatory mediators, such as TNF α and IL-6, by immune and epithelial cells promotes the exacerbation of the inflammatory process and contributes to the epithelial barrier damage. This may lead to the upregulation of claudin-2 [79, 80], which is in line with our observations. Furthermore, pro-inflammatory cytokines, including TNF α , are recognised to either increase the permeability of the intestinal epithelial cell barrier or impair the expression of SCFA transporters and receptors. This results in the disruption of essential host–commensal mutualistic pathways [81-83]. Notably, the relevance of TNF α in IBD pathogenesis is clearly demonstrated by the efficacy of anti- TNF α biologics in reducing disease severity in some patients, being one of the most common biologics administered in the clinical management of IBD [84]. In contrast, IL-10 is a critical mediator for the maintenance of intestinal immune homeostasis. Previous studies have found that mice deficient in IL-10 are highly susceptible to intestinal inflammation triggered by the presence of commensal bacteria [85-88]. Thus, the reduced levels detected in *Mgat5*^{-/-} at steady-state could potentially result from the disruption of immune tolerance and from the dysregulation of regulatory immune circuits towards a proinflammatory milieu.

Taken together, we have here disclosed, at a mechanistic level, the biological effects of changing the intestinal *N*-glycome composition in gut permeability and triggering gut dysbiosis and the downregulation of protective ILC3/IL22 axis, associated with *Nod2* downregulation and the activation of immune pathways that culminate in intestinal inflammation.

Evidence have pinpointed the critical role of intestinal glycocalyx to the maintenance of composition and function of gut microbiota [89]. Intestinal commensal-derived signals are of pivotal importance in maintaining a healthy intestinal barrier and in regulating the differentiation and proliferation of both intestinal epithelial and immune cells [90]. Among the ILC population, ILC3s have been found to be most strongly regulated by commensal microbial signals, while ILC1s are potentially promoted under proinflammatory cues [91]. Specifically, mounting body of evidence indicates that ILC3 can be directly regulated by SCFAs, promoting ILC3 proliferation and IL-22 production [65]. Indeed, ILC3 was found to express the SCFAs receptors such as *Gpr109a* and *Gpr43* [92]. An elegant study by Sepahi and colleagues demonstrated that mice fed with diets containing dietary fibre exhibited significantly elevated ILC3 numbers via the production of varying levels of SCFAs in the colon [65]. Therefore, we hypothesize that the reduction in complex branched *N*-glycans might alter the dynamics of the gut microbiota, leading to a reduction in SCFAs production and a significant impairment of gut environment homeostasis. This might occur via an imbalance of the intestinal epithelial barrier and intestinal immune response, through a shift

in ILC3 function towards a ILC1 proinflammatory profile. The identification of a specific immunological axis controlled by mucosa *N*-glycans pave the way for exploiting whether the prophylactic supplementation with glycans may enhance the biosynthesis of “tolerogenic” complex *N*-glycans and, in this way, prevent the health to intestinal inflammation transition. Remarkably, the prophylactic supplementation of glycoengineered colitis-susceptible mice with GlcNAc suppressed disease severity, by significantly ameliorating clinical symptoms. This clinical effect of GlcNAc supplementation was due to the restoration of the expression of the homeostatic ILC3-IL-22 module, with increased ILC3 frequency and blockade of ILC3-to-ILC1 transition. Moreover, the protective effect of GlcNAc supplementation was also highlighted by rescuing protective levels of SCFAs and increased *Nod2* expression.

Altogether, our findings bring to light the potential beneficial effects of targeting mucosa glycocalyx, as a promising strategy to preserve a healthy crosstalk between microbiome and gut immunity and to prevent IBD development (Figure 7). Accordingly, the evidence suggesting the clinical and biological impact of glycome alterations in the preclinical phase of CD [23, 93] brings to light the prominent impact of glycans and mucosa glycosylation as a putative trigger of health to intestinal inflammation transition with promising clinical applications for IBD prediction and prevention.

Overall, this study unlocks a new mechanism in gut immunity tightly regulated by host mucosa *N*-glycans as master shapers of microbiome composition and ILC3/ILC1 immune regulation. The remodelling of mucosa glycome through glycan supplementation showed clear prophylactic effects in preserving gut homeostasis by promoting the activation of ILC3-IL-22 protective module. These results emphasize the impact of mucosa glycans in the crosstalk between gut microbiota and immune regulation, revealing the potential use of glycans on precision nutrition approaches and therapeutic interventions for preventing health to intestinal inflammation transition and for IBD prevention.

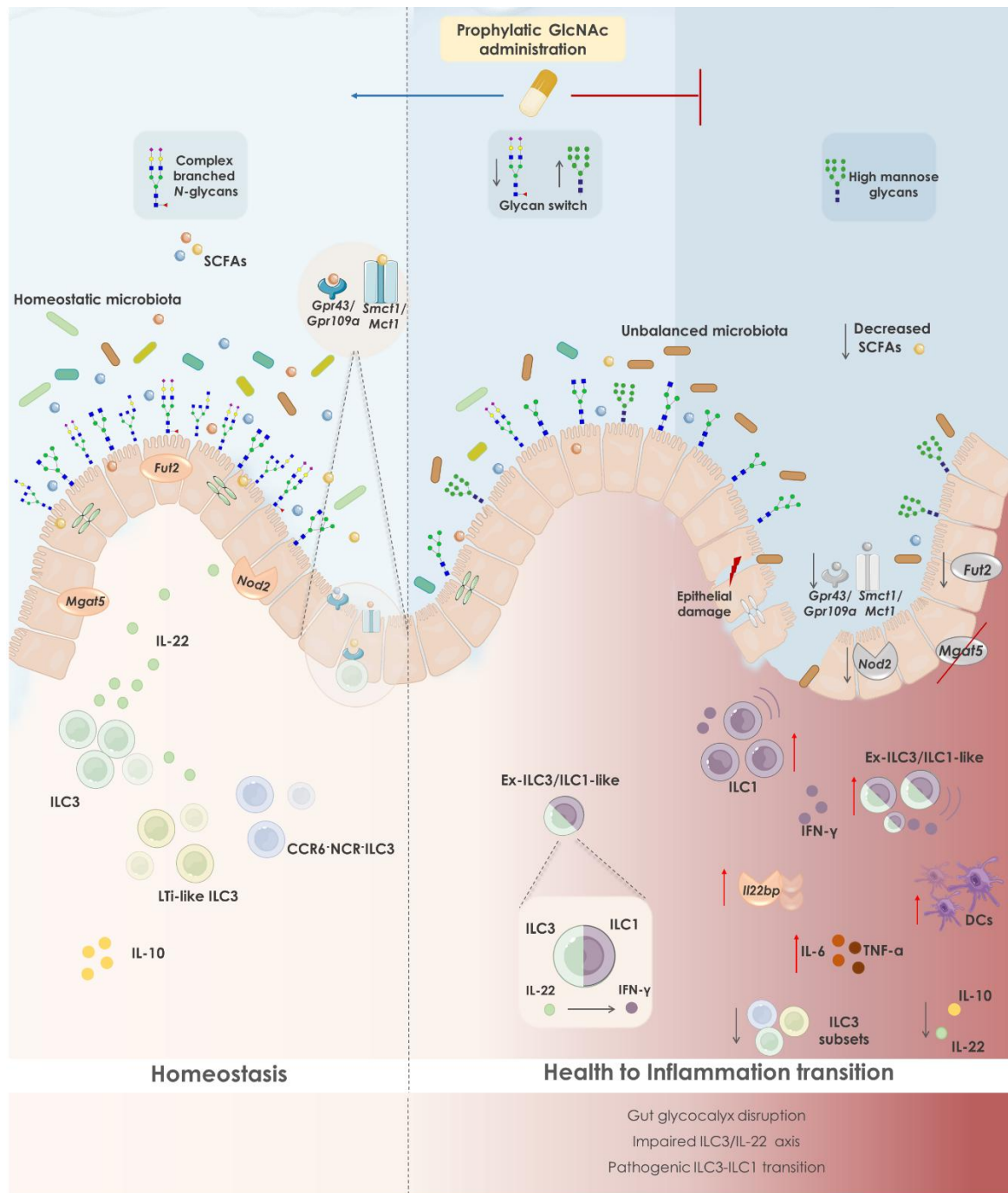


Figure 7. Changes in mucosa branched *N*-glycosylation impacts gut microbial balance, leading to an intestinal inflammatory response. In a healthy gut glycocalyx, epithelial branched *N*-glycans sustain commensal gut microbiota composition, contributing to the integrity of the epithelial barrier. ILC3 subsets, including LTi-like ILC3 and CCR6⁺NCR-ILC3, promote gut homeostasis through IL-22 release. Short-chain fatty acids (SCFAs) produced by commensal microbiota regulate mucosal homeostasis through activation of *Gpr43*/*Gpr109a* signaling pathways or entering cells through *Mct1* and *Smct1* transport. Intestinal epithelial fucosylation (via *Fut2* expression) and microbiota recognition (through *Nod2* expression) also support the symbiotic relationship between microbes and the intestinal immune system in homeostasis. During health to intestinal inflammation

transition, there is an altered host glycome profile, characterized by a deficiency in the mucosa expression of complex branched *N*-glycans and exposure of mannose-enriched glycans, that perturb mucosal integrity. This imbalanced glycoenvironment leads to dysbiotic gut microbiota and deficient SCFAs release. Signalling and transport of SCFAs is also impaired. Concomitantly, this mucosa glycan switch hampers a protective immune response, through decreased frequency of ILC3 subsets, reduction in homeostatic cytokines (IL-22 and IL-10) and downregulation of *Nod2* and *Fut2* expression in the intestinal mucosa. An ILC3-ILC1 plasticity occurs imposed by the mucosa glycosylation alteration, leading to a pathogenic ILC1 proinflammatory phenotype, with increased TNF α and IL-6 production and expression of inhibitory IL-22 binding protein, which has the ability to block IL-22. This culminates in a pro-inflammatory gut environment. The frequency of dendritic cells (DCs) is also increased upon deficiency in *Mgat5*-mediated branched *N*-glycans. Prophylactic supplementation with GlcNAc, a key metabolite in the hexosamine biosynthetic pathway, restores the expression of the protective ILC3-IL-22 module, suppressing health to intestinal inflammation transition. This ultimately highlights the beneficial impact of mucosa glycosylation remodelling through nutritional intervention in order to promote intestinal homeostasis.

Acknowledgments

Co-funded by the European Union (GlycanTrigger, Grant Agreement N°: 101093997). Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or the European Research Council Executive Agency. Neither the European Union nor the granting authority can be held responsible for them. Salomé S. Pinho and Harry Sokol acknowledge funding from European Crohn's and Colitis Organisation (ECCO) Pioneer Award 2021; Salomé S. Pinho also acknowledges the International Organization for the study of Inflammatory Bowel Disease (IOIBD).

Cláudia Rodrigues thanks the Portuguese Foundation for Science and Technology (FCT) for funding (2020.08422.BD). Joana Gaifem acknowledges funding from European Society of Clinical Microbiology and Infectious Diseases (ESCMID Research Grant, 2022), European Crohn's and Colitis Organisation (ECCO Grant, 2023) and FCT (DOI 10.54499/2020.00088.CEECIND/CP1608/CT0001). Márcia S. Pereira, Mariana Silva, Bruno Cavadas and Inês Alves acknowledge funding from FCT (SFRH/BD/110148/2015, SFRH/BD/136388/2018, CEECINST/00123/2021/CP1772/CT0001 and 2022.00337.CEECIND, respectively).

Author Contributions

C.S.R., J.G., M.S.P. and S.S.P. designed and conceived the study. C.S.R., J.G., M.S.P., M.F.A., M.S., N.P. and I.A. performed all experiments. C.S.R., J.G. and M.S.P. analyzed the experimental data. A.L. and H.S. performed the 16S rRNA sequencing bioinformatic analysis. B.C. analyzed the RNA sequencing data. C.M.B., R.M.P. and J.T. collected human biological samples and clinical data from inaugural CD cases and healthy controls. J.F.C. contributed to critical revisions of the paper. C.S.R., J.G. and S.S.P wrote the manuscript. All authors contributed to the manuscript revision. J.G., H.S. and S.S.P were responsible for funding acquisition.

Conflict of Interest Statement

The authors disclose no conflicts.

Data availability

Data, reagents, materials and protocols are available from the corresponding author upon request.

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Supplementary Material for

Alterations in mucosa branched *N*-glycans lead to dysbiosis and downregulation of ILC3: a key driver of intestinal inflammation.

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Supplementary Table 1. Demographic and clinical characteristics of the samples from inaugural IBD and healthy controls.

	Patients with IBD (UC; CD)		Healthy control individuals
Number of individuals	5 (2; 3)		3
Number of samples	8		3
Age^a years; mean $\pm$ SD	43.0 $\pm$ 16.2		79.0 $\pm$ 8.0
Sex	Female	5	2
n	Male	0	1

^aAge at sample collection

Supplementary Table 2. Disease activity index used to evaluate mice disease severity.

DAI	Weight loss	Stool consistency	Bleeding
0	No loss	WF	No blood in the swab
1	1-5%	WF/P	Traces of blood in the swab
2	6-10%	P	Clear presence of blood in the swab
3	11-18%	P/L	Red-stained, clear presence of blood in the swab
4	>18%	L	Blood in anus at naked eye

WF – well-formed; P – pasty; L – liquid.

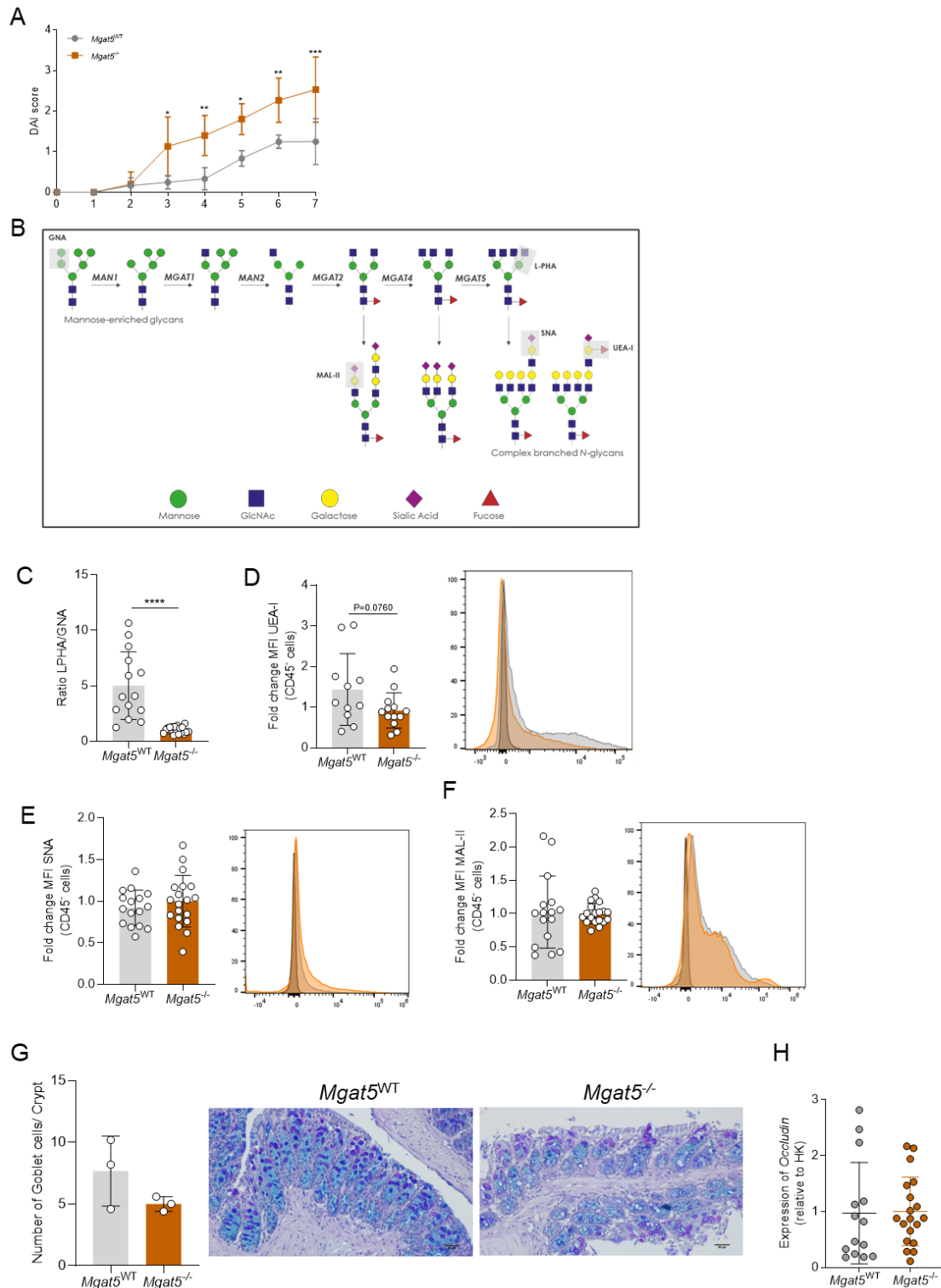
Supplementary Table 3. Antibodies and lectins used to flow cytometry.

Antibodies	Source	Identifier
APC/Fire™ 810 anti-mouse CD45 (clone 30-F11)	BioLegend	Cat#: 103173
Brilliant Violet 605 anti-mouse CD3 (clone 17A2)	BioLegend	Cat#: 100237
eFluor 506 Anti Hu/Mo CD45R (B220) (clone RA3-6B2)	eBioscience	Cat#: 69-0452-82
eFluor 506 Anti-Mo CD11c (clone N418)	eBioscience	Cat#: 69-0114-80
eFluor 506 Anti-Mo Ly-6G/Ly-6C (clone RB6-8C5)	eBioscience	Cat#: 69-5931-80
eFluor 506 Anti-Mo TER-119 (TER-119)	eBioscience	Cat#: 69-5921-80
eFluor 506 Anti-Mo CD11b (clone M1/70)	eBioscience	Cat#: 69-0112-80
PE/Cyanine5 anti-mouse CD335 (NKp46) (clone 29A1.4)	BioLegend	Cat#: 137647
BV786 Rat anti-mouse CD90.2 (clone 53-2.1)	BD	Cat#: 564365
Brilliant Violet™ 711 Anti-Mo CD127 (clone A7R34)	eBioscience	Cat#: 407-1271-82
eFluor™ 660 Anti-Mo CD196 (CCR6) (clone sirx6)	eBioscience	Cat#: 50-7196-82
APC Anti-Hu/Mo T-bet (clone 4B10)	eBioscience	Cat#: 17-5825-80
PE-Cyanine7 Anti-Mo ROR gamma (t) (clone B2D)	eBioscience	Cat#: 25-6981-82
PE-CF594 Mouse anti-EOMES (clone X4-83)	BD	Cat#: 567167
FITC anti-mouse IL-17 (clone TC11-8H4)	Life Technologies	Cat#: A15377
PE anti-mouse IL-22 (clone Poly5164)	BioLegend	Cat#: 516404
Brilliant Violet™ 421 Anti-Mo GM-CSF (clone: MP1-22E9)	BD	Cat#: 564747
PerCP-Cyanine5.5 Anti-Mo IFN gamma (clone: XMG1.2)	eBioscience	Cat#: 45-7311-80
FITC Anti-Mouse CD45 (clone: 30-F11)	eBioscience	Cat#: 53-1071-82
PE-Cyanine5 Anti-Mi MHC class II (I-A/I-E) (clone: M5/114.15.2)	eBioscience	Cat#: 15-5321-81
PE Anti-Mo CD24 (clone: M1/69)	eBioscience	Cat#: 12-0242-81
APC Anti-Mo CD64 (clone: X54-5/7.1)	eBioscience	Cat#: 17-0641-80
eFluor 450 Anti-Mo CD11c (clone: N418)	eBioscience	Cat#: 48-0114-82
PE-Cyanine7 Anti-Mo CD45 (clone: 30-F11)	eBioscience	Cat#: 25-0451-82
APC/Fire 810 Anti-Human CD45 (clone: HI30)	BioLegend	Cat#: 304076
Brilliant Violet 605 Anti-Human CD3 (clone: OKT3)	BioLegend	Cat#: 317322
Alexa Fluor 647 Anti-Human Rorγt (clone: Q21-559)	BD	Cat#: 563620
FITC Anti-Human CD19 (clone: HIB19)	BioLegend	Cat#: 302206
PE Streptavidin	BioLegend	Cat#: 405203
PE Spark Blue 550 Streptavidin	BioLegend	Cat#: 405363
LPHA (495/515)	Vector Laboratories	FL-1111
SNA (Cy5)	Vector Laboratories	CL-1305
GNA (495/515)	Vector Laboratories	FL-1241
MAL-II (biotinylated)	Vector Laboratories	B-1265
UEA-I (biotinylated)	Vector Laboratories	B-1065

Supplementary Table 4. Mouse oligonucleotides used in quantitative real-time PCR.

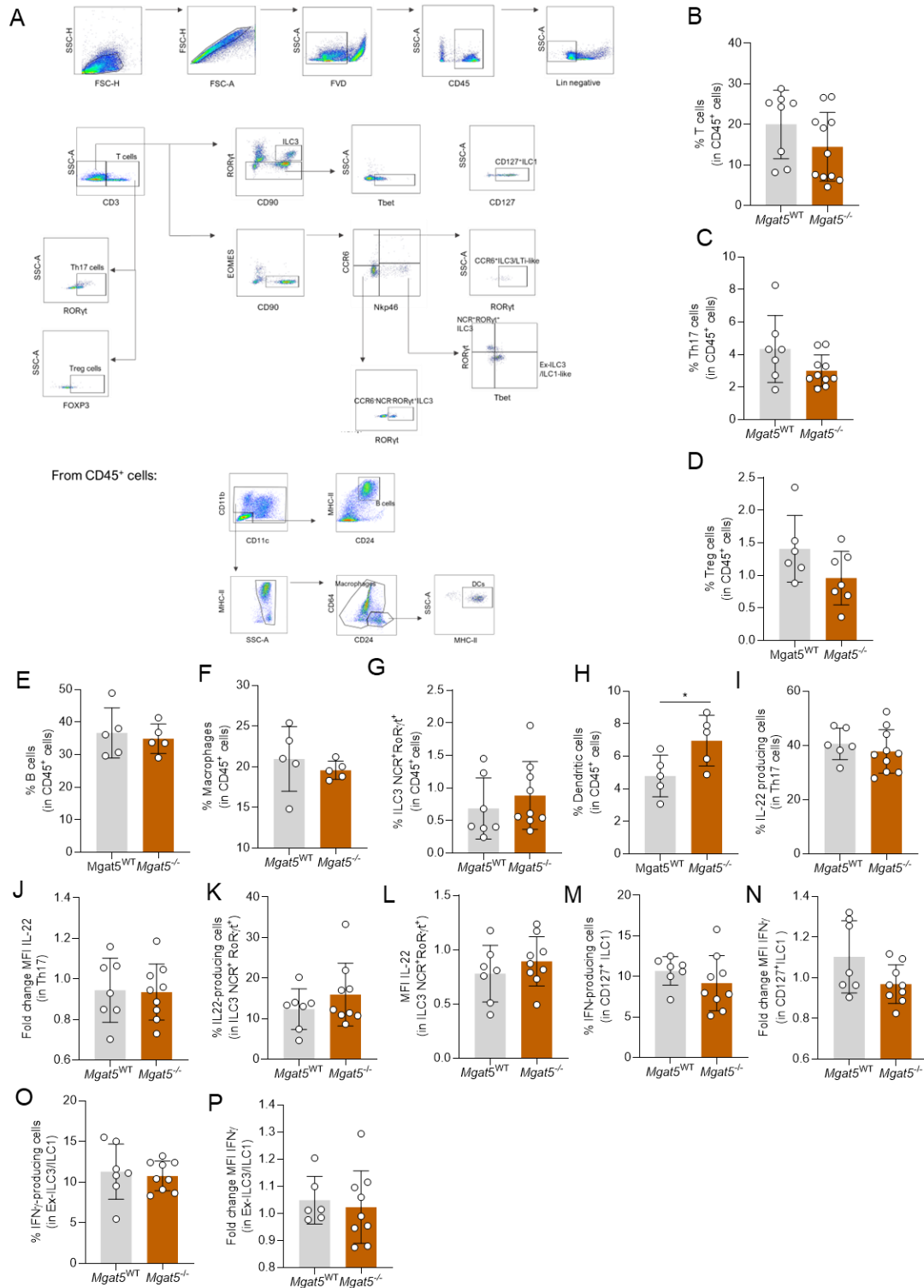
Primers	Forward Sequence	Reverse Sequence
<i>Fut2</i>	AGGCGGTTCAAATGTCCTCA	CGTTGTGCATGGATTCAGGG
<i>Il22ra2</i>	TCAGCAGCAAAGACAGAAGAAAC	GTGTCTCCAGCCCAACTCTCA
<i>Nod2</i>	CTGTCCAACAATGGCATC	GTTCCCTCGAAGCCAAACCT
<i>Gpr43</i>	AATTCCTGGTGTGCTTTGG	ACCAGACCAACTTCTGGGTG
<i>Gpr109a</i>	ATGGCGAGGCATATCTGTGTAGCA	TCCTGCCTGAGCAGAACAAGATGA
<i>Smct1</i>	TTATGGGCGGTGCGAGTA	CAGAGGCCCAACAAGGTTGACAT
<i>Mct1</i>	TGTTGTTGCAAATGGAGTGT	AAGTCGATAATTGATGCCCATGCCAA
<i>Ocludin</i>	GCTGTGATGTGTGTTGAGCT	GACGGTCTACCTGGAGGAAC
<i>Cldn1</i>	GGCTTCTCTGGGATGGATCG	CCCCAGCAGGATGCCAATTA
<i>Cldn2</i>	TTTTGGGGCTGTTAGGCACA	AGAATCCTGGCAGAACACGG
<i>Cldn3</i>	GTACAAGACGAGACGGCCAA	CGTAGTCCTTGCGGTCTGTAG
<i>Cldn4</i>	ACGTCATCCGCGACTTCTAC	TTGTCGTTGCTACGAGGTGG
<i>Gapdh</i>	GAAGGTCGGTGTGAACGGAT	CTCGCTCCTGGAAGATGGTG

Supplementary Figures



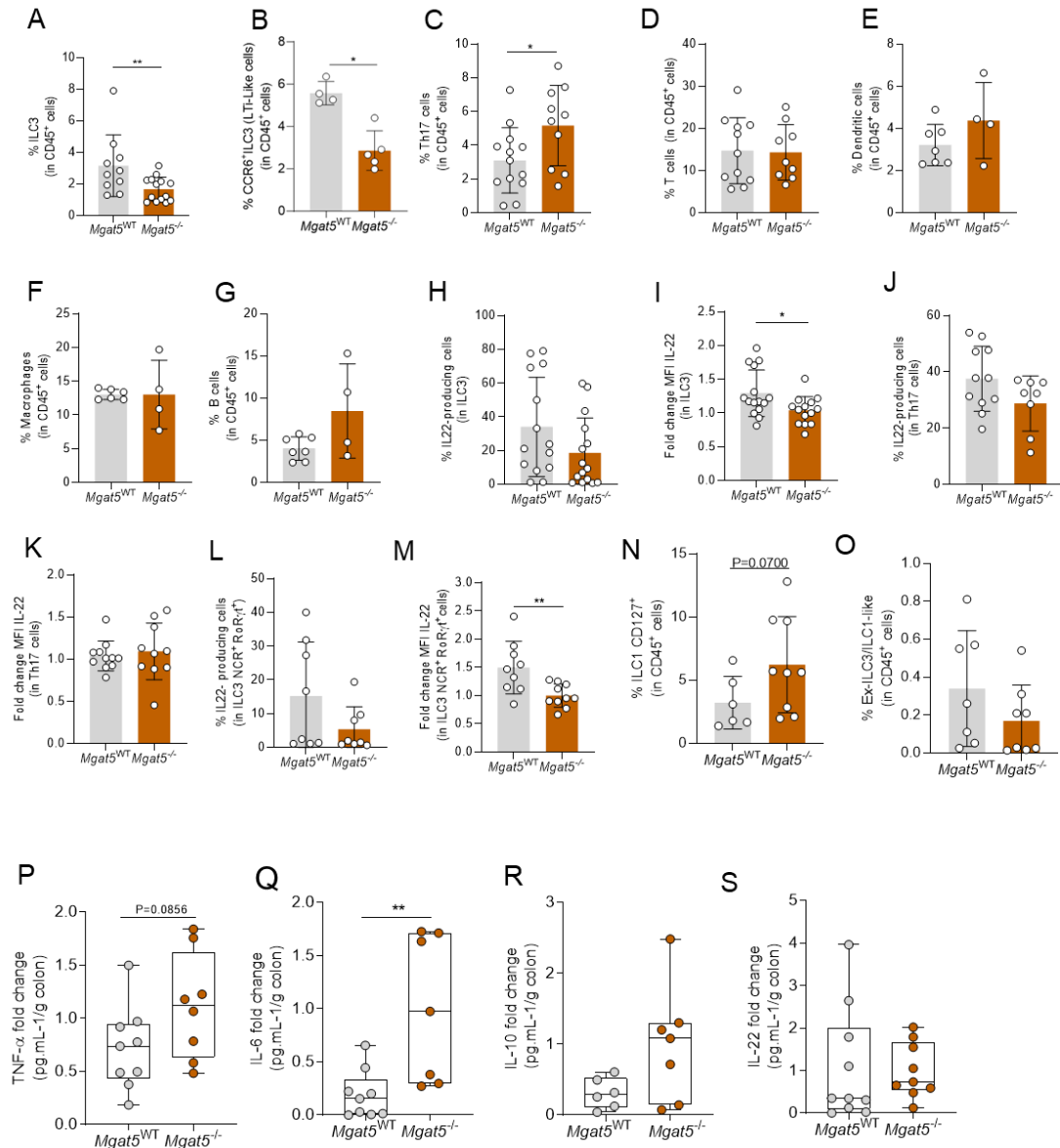
Supplementary Fig. 1. Reduction of branched *N*-glycans in mice promotes gut dysbiosis and intestinal permeability. (A) Disease activity score (DAI) of $Mgat5^{WT}$ and $Mgat5^{-/-}$ upon DSS-induced colitis. Data represents 4 independent experiments. (B) Schematic representation of the *N*-

glycosylation pathway from high-mannose *N*-glycans to complex *N*-glycans. Respective monomers recognized by the different lectins (GNA, LPHA, MAL-II, SNA and UEA-I). (C) Ratio between the MFI of LPHA and GNA in *Mgat5*^{-/-} mice and *Mgat5*^{WT} at steady state. (D-F) Levels of (D) α 1,2-fucose residues (UEA-I), (E) terminally α 2,6-sialylated glycan (SNA) and (F) α 2,3-sialylated glycans (MAIL-II) at steady state in epithelial cells (CD45⁺ cells) from *Mgat5*^{-/-} mice and *Mgat5*^{WT} controls. Median fluorescence intensity (MFI) was determined by flow cytometry. MFI were normalized for the average of *Mgat5*^{-/-} mice MFI. In the representative histograms, dark grey, light grey and orange depicts unstained control, *Mgat5*^{WT} and *Mgat5*^{-/-}, respectively. (G) Alcian Blue/periodic acid Schiff staining of the colonic tissue for goblet cells and mucus analysis. (H) The mRNA expression levels at steady state of gene encoding for occludin in *Mgat5*^{-/-} and *Mgat5*^{WT} mice. Expression of target gene mRNAs was calculated based on housekeeping gene (*Gapdh*). mRNA expression levels were normalized for the average of mRNA levels of *Mgat5*^{-/-} mice. (C) n = 14-19 per group. (D-F) n = 16–19 per group. (G) n= 3 per group (H) n= 14-19 per group. Each datapoint represents an individual animal. Data are represented as mean $\pm$ SD. ****p < 0.0001, using an unpaired two-tailed Student's *t*-test.



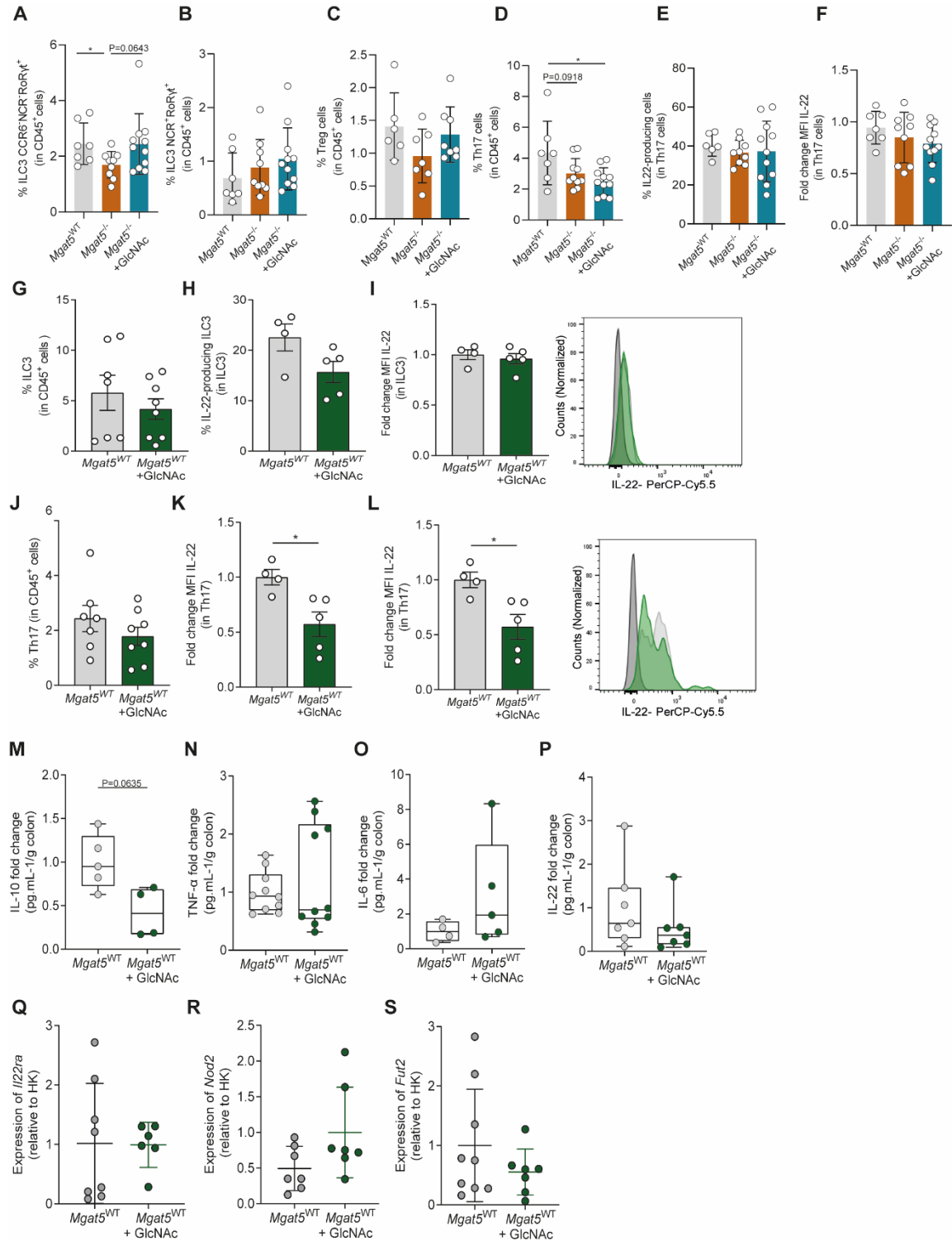
Supplementary Fig. 2. Deficiency in mucosa branched *N*-glycans leads to an impaired ILC3-IL-22 axis and gut inflammation. (A) Gating strategy for different immune populations of colon lamina propria cells. Living cells were gated based on FVD staining and doublets were excluded using an FSC-H/FSC-A gate. ILC3s: CD45⁺, lineage negative, CD3⁻, CD90⁺; RORγt⁺; ILC1: CD45⁺, lineage negative, CD3⁻, CD90⁺, RORγt⁻, Tbet⁺, CD127⁺; CCR6⁺ILC3 (LTi-like): CD45⁺, lineage negative, CD3⁻, CD90⁺, EOMES⁻, CCR6⁺, Nkp46⁻, RORγt⁺; CCR6⁻NCR-RORγt⁺ILC3: CD45⁺, lineage negative, CD3⁻, CD90⁺, EOMES⁻, CCR6⁻, Nkp46⁻, RORγt⁺; Ex-ILC3/ILC1-like: CD45⁺, lineage

negative, CD3⁺, CD90⁺, EOMES⁺, CCR6⁺, Nkp46⁺, RORγt⁺, Tbet⁺; NCR⁺RORγt⁺ILC3: CD45⁺, lineage negative, CD3⁺, CD90⁺, EOMES⁺, CCR6⁺, Nkp46⁺, RORγt⁺); T cells: CD45⁺, lineage negative, CD3⁺; Th17 cells: CD45⁺, lineage negative, CD3⁺, RORγt⁺; Treg: CD45⁺, lineage negative, CD3⁺, FOXP3⁺. For lineage negative, the following markers were used: B220, CD11c, Gr1, Ter119 and CD11b. Dendritic cells (DCs): CD45⁺, CD3⁺, CD11c⁺, CD11b⁺, MHC-II⁺, CD24⁺; Macrophages: CD45⁺, CD3⁺, CD11c⁺, CD11b⁺, MHC-II⁺, CD24⁺, CD64⁺; B cells: CD45⁺, CD3⁺, CD11c⁺, CD11b⁺, MHC-II⁺, CD24⁺; (B-H) Frequency of (B) T cells (CD3⁺) cells, (C) Th17 cells, (D) regulatory T cells, (E) B cells, (F) macrophages, (G) NCR⁺RORγt⁺ ILC3, and (H) dendritic cells in CD45⁺ cell population in *Mgat5*^{-/-} and *Mgat5*^{WT} mice at steady state. (I-J) Frequency of IL-22- producing cells in (I) Th17 cells and (J) Mean fluorescence intensity (MFI) of intracellular IL22 in Th17 cells in *Mgat5*^{-/-} and *Mgat5*^{WT} mice at steady state. MFI were normalized for the average of *Mgat5*^{-/-} mice MFI. (K-L) Frequency of IL22-producing cells in NCR⁺RoRγt⁺ ILC3 and (L) Mean fluorescence intensity (MFI) of intracellular IL22 in NCR⁺RoRγt⁺ ILC3 in *Mgat5*^{-/-} and *Mgat5*^{WT} mice at steady state. MFI were normalized for the average of *Mgat5*^{-/-} mice MFI. (M-N) Frequency of IFNγ-producing cells in CD127⁺ILC1 cells and (N) Mean fluorescence intensity (MFI) of intracellular IFNγ in CD127⁺ILC1 cells in *Mgat5*^{-/-} and *Mgat5*^{WT} mice at steady state. MFI were normalized for the average of *Mgat5*^{-/-} mice MFI. (O-P) Frequency of IFNγ-producing cells in Ex-ILC3/ILC1-like cells in *Mgat5*^{-/-} and (P) Mean fluorescence intensity (MFI) of intracellular IFNγ in Ex-ILC3/ILC1-like cells *Mgat5*^{WT} mice at steady state. MFI were normalized for the average of *Mgat5*^{-/-} mice MFI. The frequencies of different immune populations were determined by flow cytometry. (B) n = 8-11 per group. (C) n = 7–10 per group. (D) n = 6–7 per group. (E, F and H) n = 5 per group. (I, J) n=6-10 per group. (G, K, L, M, N, O and P) n=6-9 per group. Each datapoint represents an individual animal. Data is represented as mean ± SD. *p < 0.05 using an unpaired two-tailed Student's *t*-test.

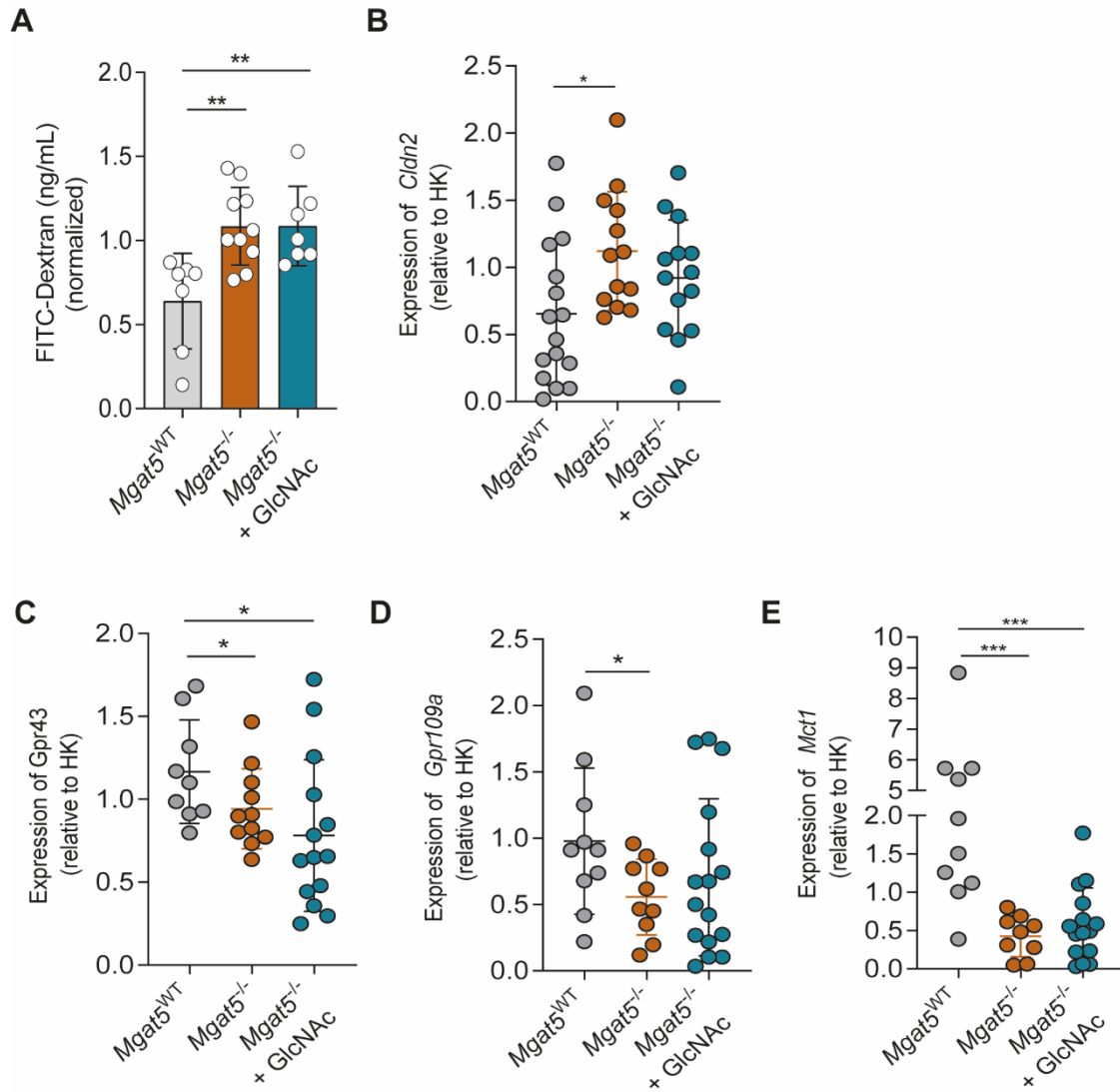


Supplementary Fig. 3. Deficiency in mucosa branched *N*-glycans leads to an impaired ILC3-IL22 axis after disease induction. (A-G) Frequency of (A) ILC3, (B) CCR6+ILC3 (LTi-like cells) (C) Th17 cells, (D) CD3⁺ cells, (E) dendritic cells, (F) macrophages, and (G) B cells in CD45⁺ population in *Mgat5*^{-/-} and *Mgat5*^{WT} mice upon DSS-induced colitis. (H-I) Frequency of IL-22-producing cells in (H) ILC3 and (I) Mean fluorescence intensity (MFI) of intracellular IL-22 in ILC3 in *Mgat5*^{-/-} and *Mgat5*^{WT} mice upon DSS-induced colitis. MFI were normalized for the average of *Mgat5*^{-/-} mice MFI. (J-K) Frequency of IL-22-producing cells in (J) Th17 cells and (K) Mean fluorescence intensity (MFI) of intracellular IL22 in Th17 cells in *Mgat5*^{-/-} and *Mgat5*^{WT} mice upon DSS-induced colitis. MFI were normalized for the average of *Mgat5*^{-/-} mice MFI. (L) Frequency of IL-22-producing cells in NCR⁺RoRγt⁺ ILC3 and (M) Mean fluorescence intensity (MFI) of intracellular IL-22 in NCR⁺RoRγt⁺ ILC3 in *Mgat5*^{-/-} and *Mgat5*^{WT} mice upon DSS-induced colitis. MFI were normalized for the average of *Mgat5*^{-/-} mice MFI. (N-O) Frequency of (N) CD127⁺ILC1 and (O) Ex-ILC3/ILC1-like cells in CD45⁺ population in *Mgat5*^{-/-} mice and *Mgat5*^{WT} upon DSS-induced colitis. (P-S) Cytokine levels upon DSS-induced colitis in culture supernatants of colonic explants. Concentrations are normalized to tissue

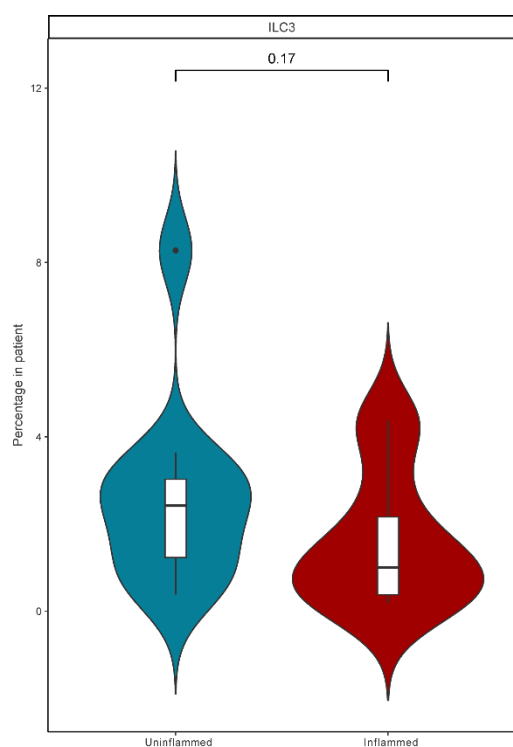
weight. The frequencies and MFIs of different immune populations were determined by flow cytometry. (A) $n = 10-14$ per group. (B) $n = 4-5$ per group (C-D) $n = 9-13$ per group. (E-G) $n = 4-7$ per group. (H-I) $n = 14-15$ per group. (J, K) $n = 11-10$ per group. (L, M) $n = 8-10$ per group. (N, O) $n = 6-9$ per group. (P, S) $n = 6-9$ per group. Each datapoint represents an individual animal. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; using an unpaired two-tailed Student's *t* test or Mann-Whitney test.



Supplementary Fig. 4. GlcNAc supplementation modulates immune response of *Mgat5*^{-/-} mice rescuing *ILC3-IL22* axis. (A) Frequency of NCR⁺RoRγt⁺ ILC3, (B) NCR⁺RoRγt⁺ ILC3, and (C) Treg cells in CD45⁺ population in *Mgat5*^{WT}, non-supplemented *Mgat5*^{-/-} mice and GlcNAc-supplemented. (D-F) Frequency of Th17 cells (D), frequency of IL-22- producing cells in (E) Th17 cells and (F) Mean fluorescence intensity (MFI) of intracellular IL22 in Th17 cells in *Mgat5*^{WT}, non-supplemented *Mgat5*^{-/-} mice and GlcNAc-supplemented. (G) Frequency of ILC3 in CD45⁺ population in GlcNAc-supplemented and non-supplemented *Mgat5*^{WT} mice. (H-I) Frequency of IL-22-producing cells in (I) ILC3 and (J) Mean fluorescence intensity (MFI) of intracellular IL22 in ILC3 in supplemented and non-supplemented *Mgat5*^{WT} mice with GlcNAc. (J-L) Frequency of Th17 (J), (K) Frequency of IL-22-producing cells in Th17 cells and (L) Mean fluorescence intensity (MFI) of intracellular IL22 in Th17 cells in supplemented and non-supplemented *Mgat5*^{WT} mice with GlcNAc. (M-P) Cytokine levels at steady-state in culture supernatants of colonic explants from supplemented and non-supplemented *Mgat5*^{WT} mice with GlcNAc. Concentrations are normalized to tissue weight. (Q-S) The mRNA expression levels at steady state of genes encoding for (R) *Il22bp*, (S) *Nod2* and (T) *Fut2* in the colonic tissue measured by qPCR. Expression of target gene mRNAs was calculated based on housekeeping gene (*Gapdh*). mRNA expression levels were normalized for the average of mRNA levels of *Mgat5*^{WT} mice. The frequencies and MFIs of different immune populations were determined by flow cytometry. (A) n = 9-11 per group. (B and C) n = 9-11 per group. (D-E) n = 9-11 per group. (F, G and J) n = 7-8 per group. (H and I) n = 4 per group. (K-L) n = 4-5 per group. (M-P) n=4-10 per group. (Q-S) n= 7-7 per group. Each datapoint represents an individual animal. *p < 0.05; using an unpaired two-tailed Student's t test or Mann-Whitney test.



Supplementary Fig. 5. Glycan supplementation display a protective effect against colitis development and restores the levels of SFCAs. (A) Intestinal permeability measured by FITC-labelled dextran in *Mgat5*^{WT}, non-supplemented *Mgat5*^{-/-} mice and GlcNAc-supplemented. (B-E) The mRNA expression levels of genes encoding for (B) of claudin-2, (C) Gpr43, (D) Gpr109a and (E) Mct1 in the colonic tissue from in *Mgat5*^{WT}, non-supplemented *Mgat5*^{-/-} mice and GlcNAc-supplemented., measured by RT-qPCR. Expression of target gene mRNAs was calculated based on housekeeping gene (*Gapdh*). mRNA expression levels were normalized for the average of mRNA levels of *Mgat5*^{-/-} mice. (A) n = 7 per group. (B-E) n = 9–16 per group. Each datapoint represents an individual animal. Data is represented as mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001; using an unpaired two-tailed Student's t test or Mann-Whitney test.



Supplementary Fig. 6. Reduced frequency of intestinal mucosal ILC3 in CD patients. Frequency of ILC3 in the inflamed and non-inflamed colonic tissue from CD patients; n=10-11 per group. Data were derived from Gene Expression Omnibus datasets (GSE134809). Wilcoxon test.

Chapter 4

A unique serum IgG glycosylation signature predicts development of Crohn's disease and is associated with pathogenic antibodies to mannose glycan

A unique serum IgG glycosylation signature predicts development of Crohn's disease and is associated with pathogenic antibodies to mannose glycan

Received: 28 June 2023

Accepted: 3 July 2024

Published online: 30 July 2024

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Inflammatory bowel disease (IBD) is characterized by chronic inflammation in the gut. There is growing evidence in Crohn's disease (CD) of the existence of a preclinical period characterized by immunological changes preceding symptom onset that starts years before diagnosis. Gaining insight into this preclinical phase will allow disease prediction and prevention. Analysis of preclinical serum samples, up to 6 years before IBD diagnosis (from the PREDICTS cohort), revealed the identification of a unique glycosylation signature on circulating antibodies (IgGs) characterized by lower galactosylation levels of the IgG fragment crystallizable (Fc) domain that remained stable until disease diagnosis. This specific IgG2 Fc glycan trait correlated with increased levels of antimicrobial antibodies, specifically anti-*Saccharomyces cerevisiae* (ASCA), pinpointing a glycome–ASCA hub detected in serum that predates by years the development of CD. Mechanistically, we demonstrated that this agalactosylated glycoform of ASCA IgG, detected in the preclinical phase, elicits a proinflammatory immune pathway through the activation and reprogramming of innate immune cells, such as dendritic cells and natural killer cells, via an FcγR-dependent mechanism, triggering NF-κB and CARD9 signaling and leading to inflammasome activation. This proinflammatory role of ASCA was demonstrated to be dependent on mannose glycan recognition and galactosylation levels in the IgG Fc domain. The pathogenic properties of (anti-mannose) ASCA IgG were validated in vivo. Adoptive transfer of antibodies to mannan (ASCA) to recipient wild-type mice resulted in increased susceptibility to intestinal inflammation that was recovered in recipient FcγR-deficient mice. Here we identify a glycosylation signature in circulating IgGs that precedes CD onset and pinpoint a specific glycome–ASCA pathway as a central player in the initiation of inflammation many years before CD diagnosis. This pathogenic glyco-hub may constitute a promising new serum biomarker for CD prediction and a potential target for disease prevention.

Inflammatory bowel disease (IBD) is a complex chronic gut inflammatory disorder, comprising Crohn's disease (CD) and ulcerative colitis (UC), which predominantly affect young people in their most productive and active period of life. Despite several advances in IBD research, the etiology of IBD remains unclear, and there is still no cure^{1,2}. Individuals undergoing the most advanced medical therapies frequently fail to achieve remission, losing response over time or displaying intolerance to treatments³. Therefore, efforts need to be directed toward understanding the earlier events that occur before disease onset so that targeted interventions can be developed.

In IBD, and predominantly in CD, like in other immune-mediated diseases, there is mounting evidence of a preclinical phase that starts years before diagnosis and is characterized by immunological changes that precede symptoms and perhaps even mucosal injury⁴. Having a better knowledge of this preclinical period may increase the potential for improved understanding of disease pathogenesis and improved therapies as well as disease prediction and prevention.

Antibodies to *Saccharomyces cerevisiae* (ASCAs) have been widely associated with IBD and are the most accurate biomarker of CD⁵. ASCAs target mannose glycans, which are a common polysaccharide component of the fungal cell wall, such as in *Candida albicans*, but they are also found on other microorganisms, such as bacteria, viruses and host glycoconjugates⁶. In fact, the structural similarity between glycans on microorganisms and those of host surface glycoconjugates may create a glycan mimicry between microorganisms and host cells. This may lead to the generation of glycan-specific antibodies that cross-react with human glycoantigens, thereby contributing to the development of immune-mediated diseases⁶. Initial results from the Proteomic Evaluation and Discovery in an IBD Cohort of Tri-service Subjects (PREDICTS) cohort revealed that pathways involving protein glycosylation, the innate immune response and the complement cascade were the most predictive markers and were detected in the sera of individuals with CD before clinical onset⁷. This evidence set the groundwork for investigating whether and how changes in glycosylation may constitute a primary event that triggers chronic inflammation associated with IBD development.

Glycosylation is a major post-translational modification that consists of the addition of carbohydrate structures (glycans) to essentially all cells⁸. Numerous studies demonstrate that glycosylation is substantially altered in major diseases, such as cancer, and in immune-mediated diseases, including IBD and lupus^{8–14}. We have previously demonstrated that alterations in cellular glycosylation can trigger proinflammatory responses associated with increased susceptibility to severe forms of intestinal inflammation and early-onset colitis^{8,10}. Together, these results highlight the key role of glycans in gut immunity and specifically in IBD immunopathogenesis.

A growing body of evidence has highlighted the prominent role of glycans in the regulation of humoral immune responses. IgG antibodies are the predominant antibody class in the circulation and are key effectors of the humoral immune system as they trigger leukocyte

activation and inflammation^{15,16}. Human IgG isolated from serum is composed of multiple glycoforms owing to the addition of a diverse repertoire of glycan structures on the IgG crystallizable fragment (Fc) region, a conserved domain responsible for modulating IgG effector functions through interactions with Fcγ receptors (FcγRs) on immune cells¹⁷. The presence of post-translational modifications in IgGs has been suggested to shape their activity and function¹⁸. Over 30 different glycan variations have been detected on circulating IgGs in healthy individuals¹⁹, which reflects a tremendous heterogeneity in the IgG Fc glycome. Individuals diagnosed with IBD were shown to display altered glycosylation of IgG Fc antibodies that were associated with disease severity compared to healthy individuals^{20,21}. The role of this IgG glycome heterogeneity or switching among the population in defining risk for the transition from healthy tissue to intestinal inflammation and IBD development years before diagnosis remains completely unknown.

Taking advantage of the unique preclinical PREDICTS cohort²², with multiple longitudinal samples many years preceding IBD diagnosis, we identified a serum glycome signature on circulating antibodies (IgGs) detectable up to 6 years before CD diagnosis. This IgG glycome signature was specific for CD and was not observed in preclinical samples of UC. We further demonstrated that the altered IgG Fc glycome signature (specifically the agalactosylated IgG2 H3N4F1 glycoform), detected in a preclinical phase, was positively correlated with the presence of ASCA, unlocking a unique glycome–ASCA hub that predicts CD development years before diagnosis. In addition, we demonstrated that the preclinical ASCA IgG glycoform was able to activate innate immune cells toward a proinflammatory phenotype via an FcγR-dependent mechanism, promoting NF-κB signaling as well as *CARD9* expression and inflammatory activation in dendritic cells (DCs). These pathogenic properties of ASCA were confirmed by adoptive IgG transfer to wild-type (WT) mice that subsequently developed an increased susceptibility to intestinal inflammation, which was hampered in recipient FcγR-deficient mice.

Results

Changes in IgG Fc glycosylation predate CD diagnosis

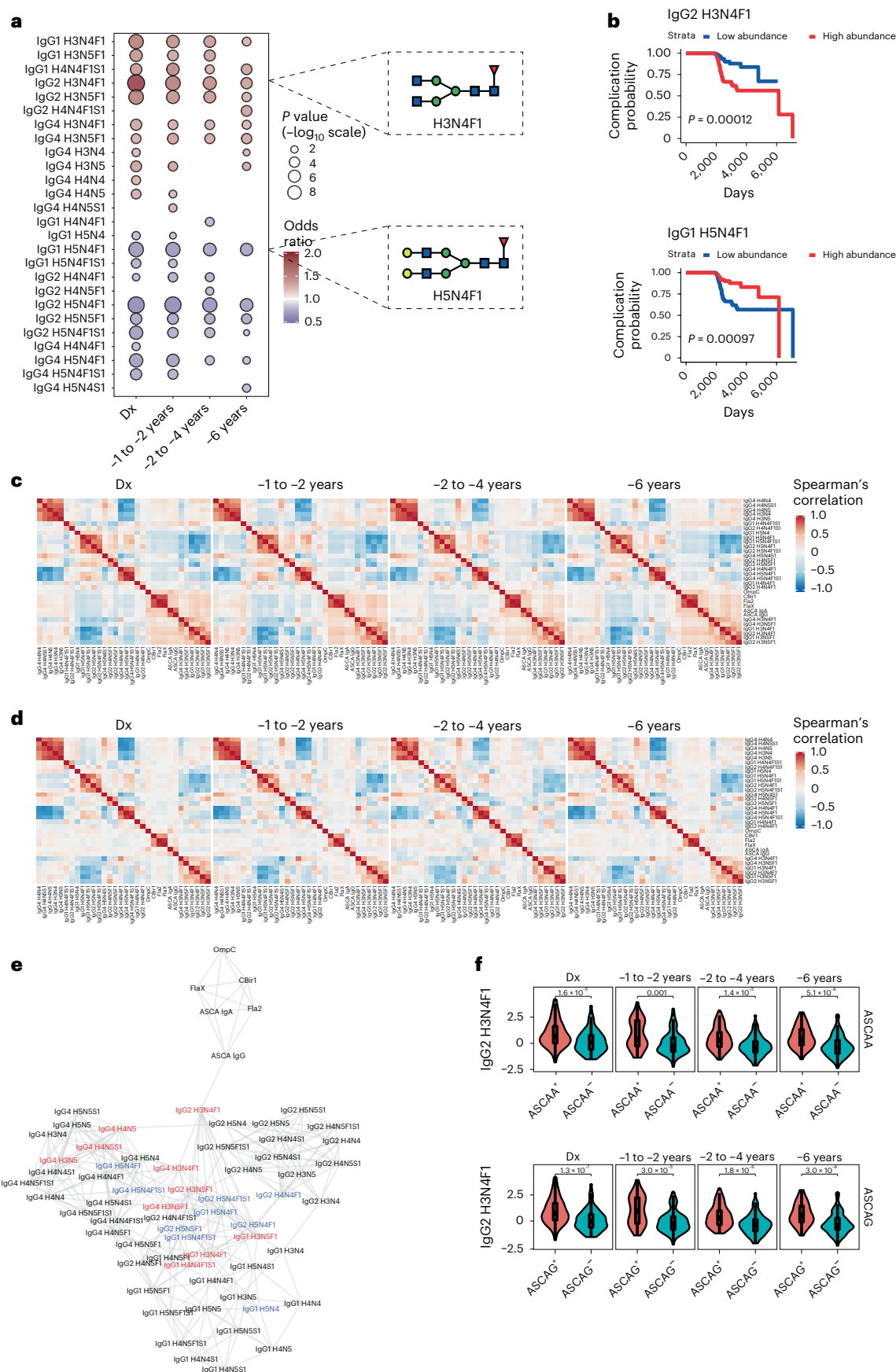
Serum samples from individuals with CD, individuals with UC and healthy control (HC) individuals from four different time points (approximately 6, 4 and 2 years before diagnosis and at the time of diagnosis; Supplementary Table 1) were retrieved from the US Department of Defense serum repository. The glycosylation profile of the Fc region of IgG was evaluated by nano-liquid chromatography–tandem mass spectrometry (nano-LC–ESI–MS; Supplementary Fig. 1a). Glycan structures were grouped according to their glycan traits (Supplementary Table 2). Specific glycoforms of IgG Fc, containing predominantly less complex *N*-glycan structures, were consistently detected in the circulation in all preclinical CD samples up to 6 years before diagnosis (Fig. 1a). Individuals that later developed CD displayed a distinct and unique IgG Fc glycoprofile, characterized by an overall Fc agalactosylation of serum IgG1, IgG2 and IgG4 (predominantly the H3N4F1

Fig. 1 | Alterations in IgG Fc glycosylation precede CD development and are associated with complicated disease. **a**, Total IgGs from serum samples from $n = 251$ individuals with CD and $n = 250$ HC individuals at different time points (preclinical phase: 1–2, 4–6 and 6 years before CD diagnosis and at CD diagnosis matched for HCs) were isolated and analyzed by mass spectrometry. The association between glycan traits measured at different time points and CD onset is shown. The size of the bubble corresponds to the P value from two-sided t -tests from a logistic regression model ($-\log_{10}$ scale) after adjusting for sex, race and age, whereas bubble color corresponds to odds ratio. Only associations significant at a 10% false discovery rate are reported. **b**, Association between different glycan traits and the development of CD complications (depicted as days to CD complication development) assessed by Kaplan–Meier analysis. P values from two-sided log-rank tests are reported. **c**, Spearman's correlations between serologic markers and glycan traits associated with CD onset. Correlations are reported considering only individuals with CD for different time

points before diagnosis. **d**, Spearman's correlations between serologic markers and glycan traits associated with CD onset. Correlations are reported considering only HC individuals for different time points before diagnosis. **e**, Estimated coexpression networks based on individuals with CD capturing the association between glycan traits and serologic markers. **f**, Box plot of IgG2 H3N4F1 stratified by ASCA IgA (ASCAA) and ASCA IgG (ASCAG) positivity for individuals with CD for different time points before diagnosis. The number of individuals with CD in **f** for each time point is 200 at diagnosis (Dx), 116 at –1 to –2 years, 165 at –2 to –4 years and 201 at –6 years before diagnosis. In the box plot, the bottom and top hinges correspond to the first and third quartiles (the 25th and 75th percentiles), with median levels represented by a horizontal line. The top (bottom) whisker extends from the hinge to the largest (smallest) value no farther than 1.5× interquartile range from the hinge. Data beyond the end of the whiskers are defined as outliers and are plotted individually. P values from two-sided t -tests are reported.

glycoform; GOF), compared to HC individuals, which exhibited an overall increase in digalactosylation of serum IgG Fc (H5N4F1; G2F; Fig. 1a). These distinct glycoprofiles of IgG Fc were detected at the farthest time

point to the diagnosis (Fig. 1a). We also demonstrated that a specific agalactosylated IgG2 Fc glycoform (namely H3N4F1), present in the sera of individuals with CD up to 6 years before diagnosis, significantly



correlated with a complicated CD phenotype at diagnosis, in contrast to a digalactosylated IgG Fc glycoform (Fig. 1b and Supplementary Fig. 1b). This agalactosylated profile seems to be specific for serum IgG because no major alterations were found for agalactosylated forms of serum IgA at the preclinical stage (Supplementary Fig. 1c). By contrast, the IgG Fc glycoprofile in individuals with UC only changed at the time of diagnosis (Supplementary Fig. 2).

We then correlated the levels and profiles of IgG Fc glycans with serum antimicrobial antibodies that have previously been described in the preclinical phase of CD, namely ASCA, anti-flagellin (Fla2 and FlaX) and anti-*Escherichia coli* outer membrane porin (OmpC)⁷. We observed that the IgG Fc glycosignature that precedes CD development clustered with antimicrobial antibodies in a stable and consistent way and up to 6 years before diagnosis (Fig. 1c). This correlation between the altered serum IgG Fc glycome and antimicrobial antibodies was only observed in individuals with CD and not in HC individuals (Fig. 1d). In addition, using a network analysis of the association between IgG glycoprofiles and serum antimicrobial antibodies in individuals with CD at the farthest time from diagnosis (6 years before), we demonstrated a unique connection between the IgG2 H3N4F1 glycoform and ASCA IgGs, pinpointing a serum glycome–ASCA hub that predates clinical CD development (Fig. 1e). Indeed, IgG2 H3N4F1 levels were significantly higher among individuals positive for ASCA IgA and ASCA IgG for all time points before diagnosis (Fig. 1f), supporting its potential as a marker of subsequent CD development.

Preclinical ASCA elicits proinflammatory immune responses

ASCA levels from a subset of samples from the PREDICTS cohort were compared to those from individuals with full-blown, established CD (Supplementary Table 3). ASCA levels gradually increased throughout the preclinical phase of disease until reaching established CD (Supplementary Fig. 1d). The same profile is found for ASCA titers, specifically analyzed for a subset of samples (Supplementary Fig. 1e).

To explore whether ASCA IgG Fc glycosylation may contribute to a proinflammatory response years before diagnosis, we tested the ability of ASCA IgG to activate immune cells such as DCs (Supplementary Fig. 3a) because DCs express FcγRIIa, which is known to interact with Fc glycans on IgG²³. The ASCA IgG glycoform from individuals with CD before diagnosis induced a clear DC activation, as demonstrated by the upregulation of CD86 and *FCGR2A* expression (the latter of which encodes FcγRIIa). This innate immune cell activation induced by the ASCA IgG glycoform was found to occur up to 6 years before CD diagnosis (Fig. 2a,b). To further validate that the interaction between ASCA IgG Fc and DCs was mediated by FcγR, incubation with a FcγRIIa-blocking

antibody (anti-CD32a) was performed (Supplementary Fig. 3b), and a decrease in DC activation was observed (Fig. 2c). To exclude any potential effect of IgG aggregation in FcγR binding and activity, we measured IgG aggregation after isolation in the different (pre)clinical conditions. No major differences were observed for all the groups (Supplementary Fig. 3c,d).

This effect observed in innate immune cell activation was accompanied by a significant increase in the production of proinflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, IL-8 and tumor necrosis factor (TNF), by DCs compared to IgGs from HC individuals (Fig. 2d–h). No differences were found in the production of the anti-inflammatory cytokine IL-10 (Fig. 2i). Moreover, FcγRIIa blockade resulted in a general decrease in cytokine production, particularly IL-6 and TNF, in preclinical individuals with CD (Supplementary Fig. 3e–i). Together, these results reveal the proinflammatory properties of ASCA IgG glycoforms in shaping innate immune cell activation through Fc–FcγRIIa interactions during the preclinical phase of CD. The proinflammatory properties of ASCAs were also observed in natural killer (NK) cells, with increased granzyme B secretion and CD107a surface expression (Supplementary Fig. 4a–c). A slight nonsignificant increase in interferon-γ (IFNγ) production was also observed (Supplementary Fig. 4d).

To gain further insights into the immunological remodeling of DCs, NK cells and B cell-derived plasma cells that is involved in antibody production that occurs in the preclinical phase, we analyzed the cell-type signatures from the report by Newman and colleagues²⁴ using somalogics data generated within our consortium⁷. This analysis showed that markers associated with activated DCs and NK cells were found to be increased at the farthest time points from diagnosis in individuals with CD compared to other immune populations (Supplementary Fig. 5a,b). We also found that the expression levels of markers for plasma cells were significantly increased in the 2- to 4-year timeframe before diagnosis, supporting the existence of a potential remodeling of B cell-derived plasma cells, within the context of a sub-clinical inflammation, which may contribute to autoantibody production associated with intestinal inflammation (Supplementary Fig. 5c). This was complemented by a significant increase in the frequency of B cells and plasmablasts observed in first-degree relatives of individuals with CD compared to individuals diagnosed with CD, concomitant with an increasing trend in the frequency of plasma cells (Supplementary Fig. 5d–f and Supplementary Table 4). No major alterations were found in IgG-producing plasma cells in these individuals (Supplementary Fig. 5g). Together, these results support the remodeling of immunological pathways related to immune cell activation occurring during the transition from a healthy intestine to intestinal inflammation.

Fig. 2 | ASCAs generated years before CD diagnosis can trigger proinflammatory immune responses.

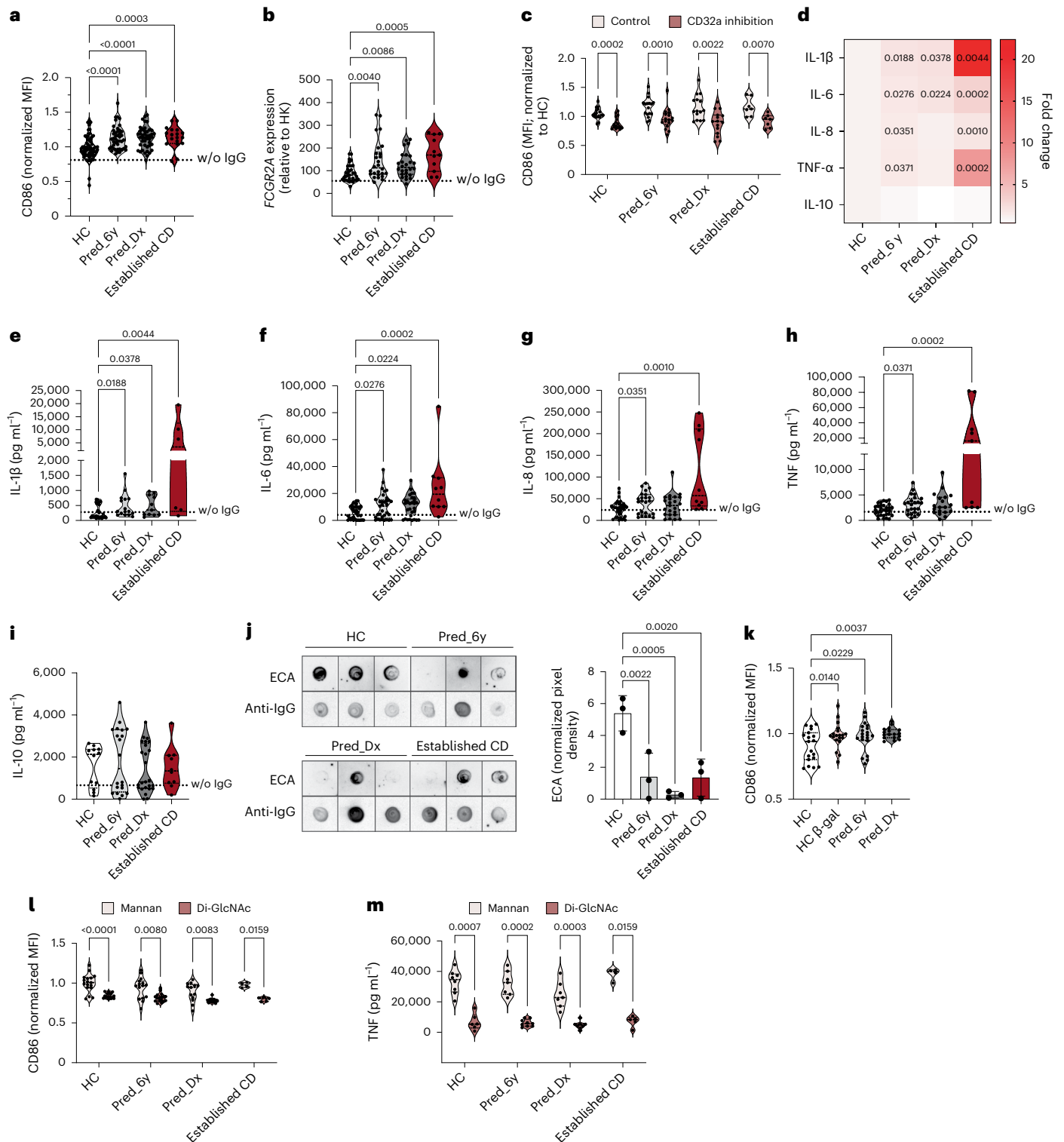
a, DCs incubated with ASCAs from preclinical individuals (Pred_6y, *n* = 44) and individuals diagnosed with CD (Pred_Dx, *n* = 44; established CD (full-blown), *n* = 18) displayed increased CD86 expression (data are normalized to HC individuals; HC, *n* = 62); MFI, mean fluorescence intensity; w/o, without. **b**, *FCGR2A* expression is also increased in DCs incubated with ASCAs from preclinical individuals (Pred_6y, *n* = 26) and individuals diagnosed with CD (Pred_Dx, *n* = 26; established CD, *n* = 11) compared to HC individuals (*n* = 34). *FCGR2A* expression is shown normalized to the expression of the housekeeping (HK) gene 18S. **c**, CD86 expression on DCs was decreased after blockade of FcγRIIa (data are normalized to that of HC individuals; control: HC *n* = 17, Pred_6y *n* = 17, Pred_Dx *n* = 15, established CD *n* = 7; with CD32a inhibition: HC *n* = 16, Pred_6y *n* = 17, Pred_Dx *n* = 15, established CD *n* = 7). **d–i**, ASCAs from individuals before and after diagnosis of CD promoted a distinct proinflammatory profile on DCs compared to ASCAs from HC individuals (*n* = 6–36 per group). Data in **d** corresponds to the fold change compared to HC. Data in **e** show HC (*n* = 23), Pred_6y (*n* = 16), Pred_Dx (*n* = 11) and established CD (*n* = 6). Data in **f** show HC (*n* = 35), Pred_6y (*n* = 33), Pred_Dx (*n* = 29) and established CD (*n* = 10). Data in **g** show HC (*n* = 36), Pred_6y (*n* = 23), Pred_Dx (*n* = 23) and established CD (*n* = 10). Data in **h** show HC (*n* = 31), Pred_6y (*n* = 23), Pred_Dx (*n* = 19) and established CD (*n* = 9). Data in **i** show HC (*n* = 12),

Pred_6y (*n* = 20), Pred_Dx (*n* = 25) and established CD (*n* = 10). **j**, ASCAs from individuals before and after diagnosis with CD show decreased galactosylation (by ECA reactivity) compared to ASCAs from HC individuals (*n* = 3 per group; independent replicates). **k**, Ablation of galactose residues on IgGs from HC individuals (HC β-gal; *n* = 19) leads to increased CD86 expression similar to that imposed by ASCAs from individuals with CD (normalized to HC; HC, *n* = 19; Pred_6y, *n* = 20; Pred_Dx, *n* = 17). **l**, CD86 expression on DCs is lower when in contact with di-GlcNAc-specific IgGs than ASCAs (normalized to HC mannan; mannan: HC *n* = 17, Pred_6y *n* = 15, Pred_Dx *n* = 13, established CD *n* = 4; di-GlcNAc: HC *n* = 17, Pred_6y *n* = 17, Pred_Dx *n* = 12, established CD *n* = 5). **m**, DCs cocultured with di-GlcNAc-specific IgGs also display a decrease in TNF production (mannan: HC *n* = 8, Pred_6y *n* = 7, Pred_Dx *n* = 7, established CD *n* = 4; di-GlcNAc: HC *n* = 6, Pred_6y *n* = 9, Pred_Dx *n* = 8, established CD *n* = 5). Data in **c**, **l** and **m** were analyzed comparing treatments within each group by Mann–Whitney *t*-test. Data in **j** and **k** were analyzed comparing each condition with the control (HC) by one-way analysis of variance with an uncorrected Fisher's least significant difference test. Data on the remaining graphs were analyzed comparing each condition with the control (HC) by Kruskal–Wallis test with an uncorrected Dunn's test. *P* values are shown in the graphs. Each data point represents the data from a single individual (biological replicates).

Next, our observation that serum IgG2 Fc agalactosylation (H3N4F1) was the only IgG glycosignature that specifically correlated with ASCAs predicting CD development (Fig. 1e) raised the question about the particular impact of the agalactosylated form of ASCAs in inducing early immune activation. To address this, we modulated the galactose levels on IgGs from HC individuals and evaluated the specific impact on DC activation. Levels of ASCA IgG galactosylation were confirmed by *Erythrina cristagalli* lectin (ECA) reactivity, which specifically recognizes galactose glycan residues (Fig. 2j). Total IgGs (including ASCAs) isolated from HC individuals ('healthy' galactosylated Fc profile)

were digested with β -galactosidase (β -gal) to mimic an agalactosylated ('pathogenic') IgG Fc glycosignature (Supplementary Fig. 4e). Degalactosylated ASCA IgG Fc from HC individuals led to a significant increase in DC activation, with CD86 expression reaching similar levels as those observed using ASCA IgG from individuals with CD (Fig. 2k). A similar activation profile was also observed for NK cells, in which removal of galactose from ASCA IgGs from HC individuals resulted in higher granzyme B secretion and LAMP-1 expression (Supplementary Fig. 4b,c).

Together, these findings suggest that reducing levels of galactosylation on ASCA IgG Fc switches its function toward a proinflammatory



phenotype by triggering the activation of the innate immune response mediated by DCs and NK cells. This highlights a key role of ASCA IgG glycosylation as an important inducer of proinflammatory responses years before CD diagnosis.

We then explored whether the proinflammatory response was dependent not only on ASCA Fc galactosylation but also its specificity for mannose glycan–antigen recognition and not for other glycan epitopes. Mannan-unbound IgGs from preclinical and diagnosed CD had no impact on DC activation compared to those from HC individuals, demonstrating the specific impact of mannan-specific ASCAs in activating DCs (Supplementary Fig. 6a). Furthermore, we observed that DC activation was significantly decreased in cocultures with anti-di-GlcNAc (a nonmannose glycan) compared to ASCA IgGs (Fig. 2l and Supplementary Fig. 6b,c), as was the secretion of TNF and IL-8 (Fig. 2m and Supplementary Fig. 6d). Incubation with other microbial patterns, in particular the yeast/bacteria-derived β -glucan, demonstrated that antibodies to β -glucan caused no differential activation of DCs (Supplementary Fig. 6e). The selective activation imposed by ASCA IgGs was also observed for NK cells (Supplementary Fig. 4f,g).

Overall, these results support the proinflammatory function of the ASCA–glycome hub detected years before disease diagnosis by demonstrating that mannan recognition through the IgG Fc agalactosylated profile triggers innate immune cell activation, initiating a proinflammatory process years before CD diagnosis.

Preclinical ASCAs reprogram glycan-binding proteins in DCs

To assess, using an unbiased approach, the impact of ASCAs on the reprogramming of immunological pathways during preclinical CD, RNA-sequencing analysis of DCs cocultured with preclinical ASCA IgG glycoforms from different settings (HC, 6 years before diagnosis and at diagnosis) showed clear differential gene expression profiles compared to DCs incubated with ASCA IgGs from HC individuals and individuals with CD at the time of diagnosis (Fig. 3a). We found a significant increase in the expression of genes involved in proinflammatory signaling pathways, such as TNF, NF- κ B and C-type lectin receptor pathways (Fig. 3b), up to 6 years before diagnosis compared to DCs incubated with ASCAs from HC individuals. Indeed, *TNF* was found to be one of the top genes significantly upregulated by ASCA IgGs in the preclinical phase (Fig. 3c), supporting the premise that ASCA IgGs act as an early trigger for the transition between a healthy intestine and intestinal inflammation. Additionally, one of the pathways shown to be upregulated by preclinical ASCA IgGs is the complement cascade

(Fig. 3b), in particular *CIQA* and *CIQB* (Fig. 3c), which matches our results demonstrating that ASCA IgGs from individuals with CD exhibit an increase in C1q binding (Supplementary Fig. 3j) and agrees with the proinflammatory environment imposed by ASCA IgGs in pre-CD. This complement activation is apparently independent of galactose levels on ASCA IgGs (Supplementary Fig. 3k).

Taking into consideration the data provided by the RNA sequencing of DCs, we analyzed the impact of ASCA glycoforms on the modulation of DC activation programs. DCs are equipped with glycan-binding proteins (C-type lectins), such as DC-SIGN and dectin-2, which enable them to recognize glycan structures such as mannose glycans, thus shaping their immune response^{25–27}.

Agalactosylated ASCA IgGs from individuals before CD diagnosis imposed a significant overexpression of DC-SIGN on DCs at the cell surface (Fig. 3d). Dectin-2 also showed a significant overexpression on DCs at the time of diagnosis (Fig. 3e). The increased expression of these glycan-binding proteins on DCs after interaction with ASCA IgG glycoforms appears to occur very early in disease development and is maintained until CD onset, likely shaping innate immune function.

The NF- κ B signaling pathway (a major downstream player of DC-SIGN and dectin-2 and Fc γ R activation on DCs) was also identified as significantly upregulated in the signaling pathway analysis (Fig. 3b). We also showed increased phospho-NF- κ B activation in DCs cocultured with ASCA IgGs, predominantly up to 6 years before diagnosis (Fig. 3f), suggesting that the NF- κ B signaling pathway is modulated by ASCA IgG glycoproteins in the preclinical phase of CD. This is in accordance with the increased production of several cytokines known to be produced after NF- κ B activation (Fig. 2d–h). For individuals with CD with established disease, no major alterations were found (Fig. 3f), which can be associated with the fact that these individuals are not naive to therapy, and thus the immunomodulatory treatment may be influencing their immune behavior.

Importantly, Fc γ RIIa on DCs can activate the NF- κ B pathway and its intermediate partners, such as the NLRP3 inflammasome²⁶. Indeed, *NLRP3* expression in DCs was significantly increased after interaction with ASCA IgGs from individuals with CD up to 6 years before diagnosis and was maintained until the establishment of CD (Fig. 3g). Accordingly, the increased production of IL-1 β by DCs (Fig. 2e) also corroborates the activation of the *NLRP3* inflammasome. Fc γ RIIa and several C-type lectins expressed on DCs can engage CARD9 for NF- κ B activation and proinflammatory gene transcription^{26,28–30}. In fact, genetic mutations in *CARD9* are described in CD, in which predisposing variants are associated with increased expression of *CARD9* mRNA and

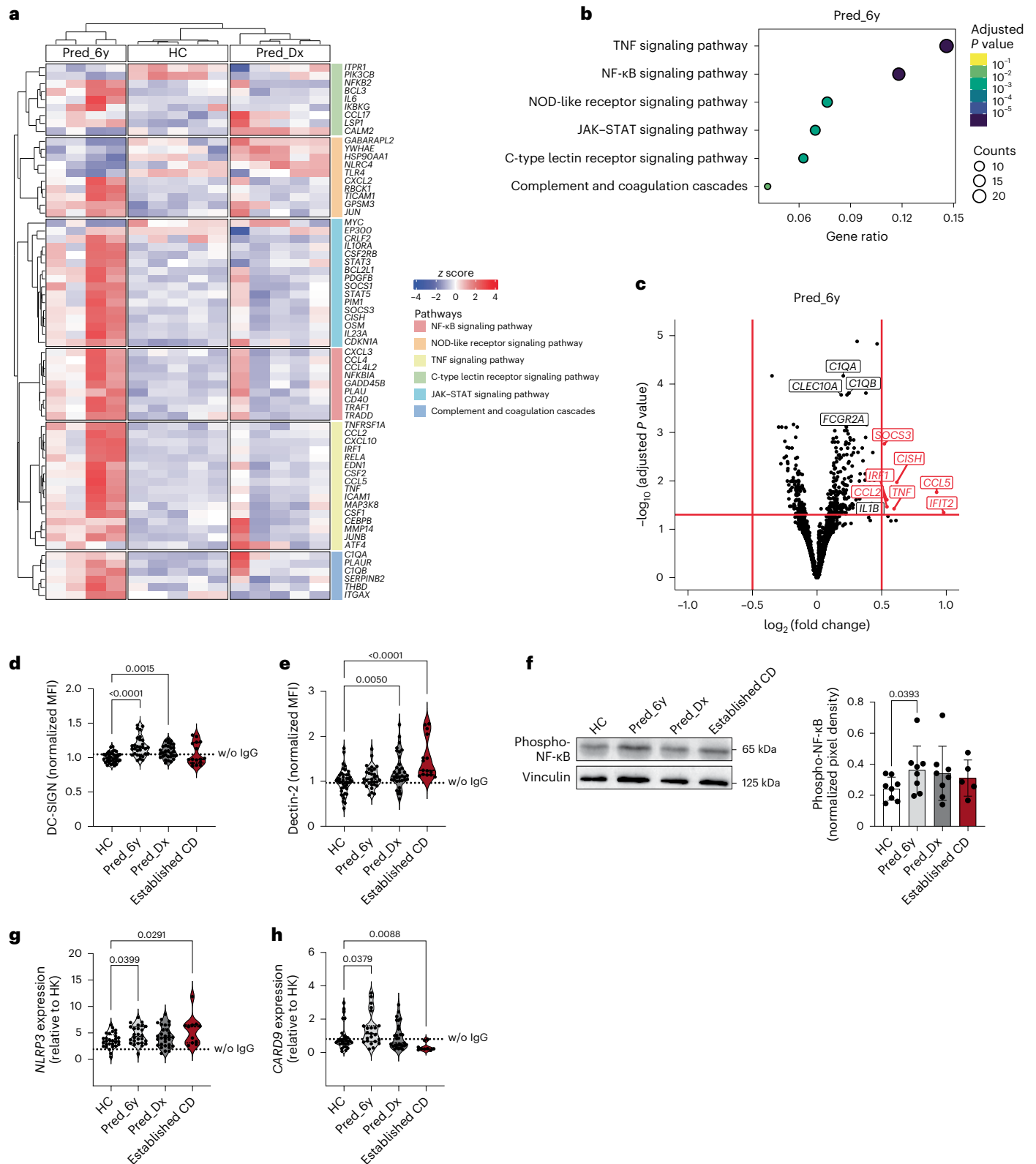
Fig. 3 | ASCA Fc glycoforms from individuals before CD diagnosis shape the expression of proinflammatory signaling pathways and glycan-binding proteins in DCs, leading to *NLRP3* and *CARD9* expression. a, Heat map of the relative expression values (z score of each gene across samples) for the top six upregulated KEGG pathways found in DCs cocultured with ASCA IgGs from individuals at the preclinical phase (Pred_6y, $n = 4$) versus ASCA IgGs from HC individuals ($n = 5$). In addition to Pred_6y and HC individuals, the heat map also shows the expression of these genes in DCs cultured with ASCA IgGs from individuals at diagnosis (Pred_Dx, $n = 5$). Only differentially expressed genes are shown, excluding genes belonging to more than one pathway. **b**, Dot plot of the overrepresentation analysis (KEGG ontology) for Pred_6y versus HC. The plot represents the top six enriched pathways (false discovery rate < 0.05), excluding human disease pathways. The diameter of the dot indicates the number of upregulated genes (false discovery rate < 0.05) belonging to the pathway. The color code indicates the adjusted *P* value for the pathway. Overrepresentation uses a hypergeometric test corrected for multiple testing using the Benjamini–Hochberg method to determine the statistical significance of the upregulated differentially expressed genes in each Gene Ontology term. **c**, Volcano plot depicting differentially expressed genes in DCs cocultured with ASCAs from Pred_6y ($n = 4$) versus HC ($n = 5$) individuals. Red dots represent genes expressed at higher levels in Pred_6y individuals. Black dots represent genes below the cutoff of significance ($|\log_2(\text{fold change})| \geq 0.5$ and adjusted

P values of ≤ 0.05). DESeq2 uses negative binomial generalized linear models for the differential analysis of count data and the Wald test with multiple correction (Benjamini–Hochberg method) for significance testing. The $\log_2(\text{fold change})$ values were shrunk with the aplegm method to increase the signal-over-noise ratio of the effect size. **d**, ASCAs from preclinical (Pred_6y, $n = 30$) and at-diagnosis CD samples (Pred_Dx, $n = 30$; established CD, $n = 16$) can induce the surface expression of DC-SIGN on DCs (HC, $n = 36$). **e**, DCs display increased expression of dectin-2 after incubation with ASCAs from individuals with CD (Pred_Dx, $n = 35$; established CD, $n = 15$; HC, $n = 43$); no major alterations were found for Pred_6y ($n = 31$). **f**, DCs cultured with ASCAs from preclinical samples show increased expression of phospho-NF- κ B (HC, $n = 8$; Pred_6y, $n = 8$; Pred_Dx, $n = 8$; established CD, $n = 5$; independent replicates). **g**, ASCAs from preclinical individuals and individuals with established CD promoted the upregulation of *NLRP3* expression in DCs (HC, $n = 29$; Pred_6y, $n = 26$; Pred_Dx, $n = 30$; established CD, $n = 11$). **h**, Similar profiles were found for *CARD9* expression (HC, $n = 29$; Pred_6y, $n = 23$; Pred_Dx, $n = 29$; established CD, $n = 9$). Data are presented as mean $\pm$ s.d. and were analyzed comparing each condition to the control (HC). Data presented in **f** were analyzed by paired one-way ANOVA with uncorrected Fisher's least significant difference test compared to HC and are shown as mean $\pm$ s.d.; data on the remaining graphs were analyzed by a Kruskal–Wallis test with an uncorrected Dunn's test. *P* values are shown in the graphs. Each data point represents the data from a single individual (biological replicates).

activation of the immune response^{31,32}. We showed that *CARD9* expression was significantly increased in DCs cocultured with ASCA IgGs (Fig. 3h). This increase in expression was more robust after stimulation with preclinical ASCAs (Fig. 3h), suggesting the relevance of ASCA IgG glycans in early DC activation and in triggering of inflammatory processes, whereas other pathways may have greater contributions in fully established CD.

Anti-mannan IgGs increase susceptibility to mouse colitis

To further validate the pathogenic properties of ASCA IgGs in inflammation initiation and disease development, we performed a proof-of-concept experiment to evaluate the ability of anti-mannan to trigger IBD in vivo. WT mice were immunized with mannan to stimulate the production of anti-mannan (ASCA-like) IgGs (Fig. 4a). Mannan immunization resulted in significantly increased levels of serum ASCA



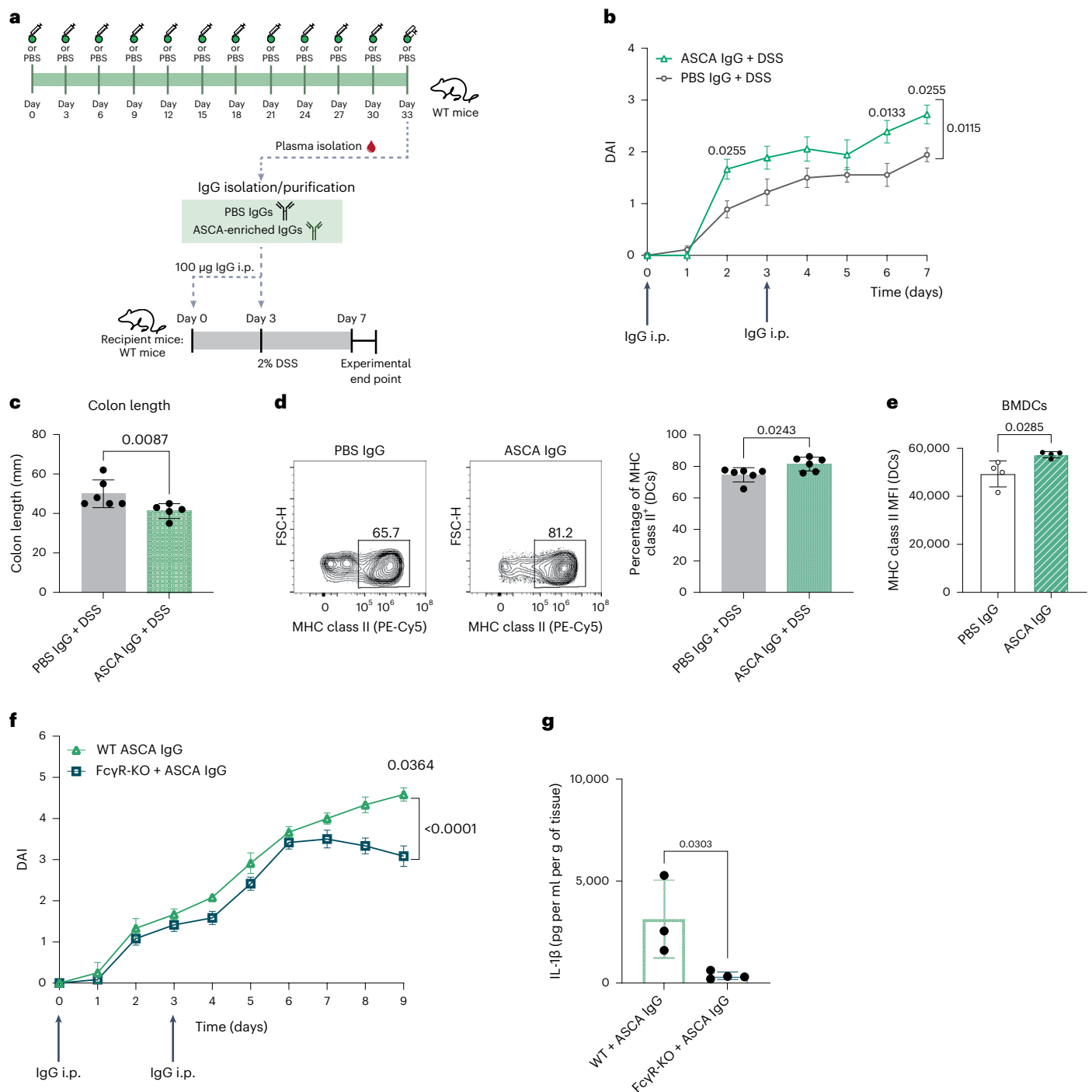


Fig. 4 | Antibodies to mannan impose an increased susceptibility to colitis in mice. a, WT mice were inoculated subcutaneously with mannan to promote the generation of antibodies to mannan (ASCA-like). Control mice were injected with PBS. Total IgGs were collected from both groups, and recipient mice were inoculated twice with 100 µg of total IgGs (ASCA enriched or from PBS-injected mice (control)), while colitis was chemically induced by administration of 2% DSS in drinking water; i.p., intraperitoneal. **b**, Mice inoculated with ASCA IgGs ($n = 6$) display increased susceptibility to colitis by showing increased DAI compared to PBS IgG-inoculated mice ($n = 6$). Data are presented as mean $\pm$ s.e.m. **c**, ASCA IgG-inoculated mice ($n = 6$) also display shortening of the colon compared to control animals ($n = 5$). **d**, ASCA-inoculated mice ($n = 6$) display increased frequencies of MHC class II⁺ cells in the colonic tissue after the induction of colitis compared to control animals ($n = 6$). **e**, BMDCs from WT mice were cocultured with

mannan-specific IgGs (mouse ASCA IgGs; $n = 4$) or nonspecific IgGs (mouse PBS IgGs; $n = 4$), and MHC class II expression (activation) was assessed by fluorescence-activated cell sorting (FACS). **f**, FcγR-KO mice inoculated with ASCA IgGs ($n = 4$) showed decreased susceptibility to colitis compared to WT mice ($n = 4$). Data are presented as mean $\pm$ s.e.m. **g**, FcγR-KO mice ($n = 4$) displayed decreased levels of IL-1β in the supernatants of colonic explants compared to WT mice ($n = 3$). Scatter dot plots are presented as mean $\pm$ s.d. Data presented in **b** and **f** were analyzed by two-way ANOVA with a Šidák's post-test, data presented in **c** were analyzed by two-tailed Mann-Whitney test, and data in the remaining graphs were analyzed by two-tailed unpaired *t*-test. *P* values are shown in the graphs. In **e**, each data point represents a technical replicate. For the remaining figures, each data point represents the data from a single individual (biological replicates).

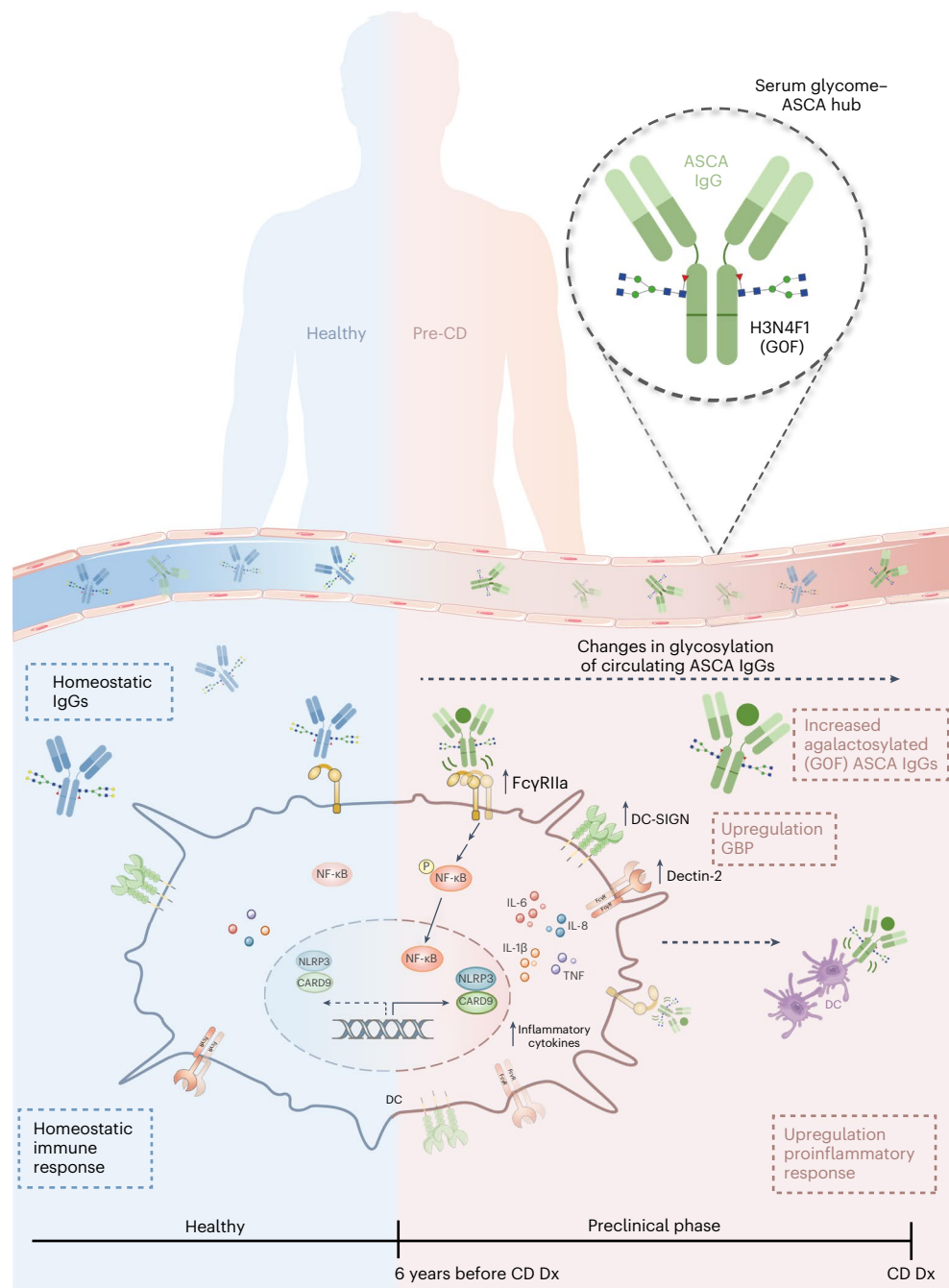


Fig. 5 | ASCA agalactosylation precedes CD development, contributing to the initial steps of intestinal inflammation. Alterations in IgG Fc glycosylation were found up to 6 years before CD diagnosis. This altered glycosignature (IgG2 H3N4F1; GOF) was identified as significantly associated with antimicrobial antibodies, in particular ASCAs. These antibodies to mannan, even those from years before diagnosis, also contribute to intestinal inflammation by shaping the profile of immune cells toward proinflammation. ASCAs were also demonstrated

to modulate the expression of glycan-binding proteins (GBP) on the surface of DCs. Moreover, these antimicrobial antibodies found 6 years before diagnosis induced phosphorylation of NF-κB, as well as the expression of *NLRP3* and *CARD9*. This work identified a new biomarker for CD prediction, as this altered glycoform of IgG Fc is detected in the circulation many years before disease diagnosis. Additionally, we detailed an axis mediated by ASCA IgG glycoforms that mechanistically is able to trigger an inflammatory event.

IgGs (Supplementary Fig. 7a). No significant alterations in intestinal permeability after mannan immunization were observed (Supplementary Fig. 7b). Serum was collected from immunized mice, and ASCA-enriched IgGs were isolated (along with PBS-immunized IgGs from the control group). Analysis of the serum IgG glycome showed that no major differences were found in terms of IgG glycan composition between immunized and nonimmunized mice (Supplementary Fig. 7c). Moreover, no differences were observed in circulating ASCA IgGs after dextran sulfate sodium (DSS) treatment for colitis induction

(Supplementary Fig. 7d). This was expected because WT mice, with no basal genetic alterations in terms of glycosylation machinery on B cells, were used for mannan immunization and ASCA production. ASCA-enriched IgGs were then transferred into recipient C57BL/6 WT mice, followed by DSS-induced colitis (Fig. 4a).

Mice inoculated with ASCA IgGs displayed higher susceptibility to and severity of colitis than mice in the control group (Fig. 4b). Indeed, ASCA-treated mice developed an early disease onset and exhibited a more severe phenotype with a higher disease activity index (DAI;

Fig. 4b and Supplementary Table 5) and a more marked shortening of the colon (Fig. 4c). Analysis of lamina propria leukocytes demonstrated an increase in the expression of major histocompatibility complex class II⁺ (MHC class II⁺) DCs (Fig. 4d), suggesting the impact of ASCA IgGs on the activation profile of DCs in vivo. Additionally, coculture of ex vivo ASCA IgGs from mannan-immunized mice with bone marrow-derived DCs (BMDCs) from WT mice showed a similar increase in the expression of MHC class II (Fig. 4e). A similar trend in activation imposed by ASCA IgGs was found in NK cells, with increased expression of granzyme B (Supplementary Fig. 7e). The frequency of IFN γ -producing NK cells was also increased; however, this finding did not reach statistical significance (Supplementary Fig. 7f).

To explore the biological effect of ASCA IgGs on imposing an inflammatory environment in an Fc–Fc γ R-dependent manner, we used a mouse model deficient for Fc γ R1 (*Fcgr1/Fcgr2/Fcgr3/Fcgr4*-KO; hereafter referred to as Fc γ R-KO). WT and Fc γ R-KO mice were inoculated with ASCA IgGs isolated from mannan-immunized mice, and colitis was induced. Fc γ R-KO mice inoculated with ASCA displayed a significantly reduced susceptibility to colitis, with decreased DAI values compared to WT mice (Fig. 4f and Supplementary Fig. 7g) and lower levels of IL-1 β in the supernatants of colonic explants (Fig. 4g). TNF levels were also decreased, despite not reaching statistical significance (Supplementary Fig. 7h). Overall, we demonstrated the relevance of ASCA IgG Fc–Fc γ R interactions in imposing a proinflammatory environment associated with intestinal inflammation, supporting the pathogenic effects of ASCA IgGs in vivo.

We further assessed whether oral supplementation with mannan would promote similar susceptibility to colitis. Oral immunization with mannan was not effective in inducing increased serum ASCA IgG levels compared to nontreated mice (Supplementary Fig. 8a,b), and, expectedly, no major alterations were found in colitis susceptibility (Supplementary Fig. 8c) nor colon length (Supplementary Fig. 8d), demonstrating that increased levels of ASCA IgGs are intricately related to increased susceptibility to colitis. Overall, our data suggest that ASCA IgGs may not only act as a classical biomarker for IBD but also play a key role in the inceptive stages of initiation of a proinflammatory response in the gut, potentially triggering a more aggressive inflammatory response.

Discussion

Recent evidence obtained from the PREDICTS cohort revealed that changes in glycosylation pathways can be detected in serum years before CD diagnosis, with protein glycosylation being among the top three markers/pathways that appeared to be activated in the preclinical phase⁷. Moreover, we previously demonstrated that changes in the expression of complex branched *N*-glycans at the surface of the gut mucosa regulate the immune response associated with IBD immunopathogenesis^{8,10,12,33}. We also recently described that neutralizing antibodies to granulocyte–macrophage colony-stimulating factor (GM-CSF) were found to be specific for a glycosylated form of GM-CSF, which is detectable years before CD diagnosis³⁴.

A seminal observation in the 1990s described that glycan structures from *S. cerevisiae*, specifically mannosylated glycans, elicit an antibody response associated with CD development³⁵. Elevated levels of antibodies to yeast glycans (that is, ASCAs) are still the most sensitive and specific serologic marker for CD and are detectable years before diagnosis³⁶. Accordingly, findings from the Crohn's and Colitis Canada Genetics Environment Microbial Project cohort (a study on first-degree relatives of individuals with CD) showed that ASCAs are highly predictive of CD development and can be detected years before diagnosis³⁷. In addition, data from the PREDICTS cohort also showed that ASCA levels predict complicated disease at diagnosis³⁸.

Here, we disclose the proinflammatory properties and disease-initiating capacity of an altered serum IgG glycome years before diagnosis. We identified a specific glycome–ASCA hub that appears to

act as an early event that triggers innate immune response activation, reprogramming immunological pathways toward inflammation many years before clinical CD diagnosis (Fig. 5). Importantly, this glycome–ASCA pathway was validated in vivo, in which the adoptive transfer of ASCA IgGs led to early-onset disease and a remarkable increased susceptibility to colitis.

We also pinpointed a precise agalactosylated IgG signature in CD (IgG2 H3N4F1) as one of the top IgG Fc glycan traits that uniquely connects with increased ASCA levels up to 6 years before diagnosis. Although our understanding of the regulation of protein glycosylation is limited, recent studies demonstrated that gene networks that regulate glycosylation of different proteins can be very different³⁹. A large study on over 12,000 individuals identified 27 genetic loci that associate with IgG glycosylation, including some that are known risk factors for IBD⁴⁰. Therefore, specific glycovariants on IgG that associate with IBD development are revealed in this study.

We demonstrated that ASCA IgG is more than a bystander biomarker of IBD and plays an effective immunomodulatory function in the initial steps of inflammation years before CD diagnosis. We showed that ASCA IgG glycoforms interact with DCs and NK cells through Fc–Fc γ R interactions, instructing a proinflammatory response. Mechanistically, this interaction was demonstrated to reprogram DCs toward a proinflammatory phenotype, promoting the expression of genes associated with several proinflammatory signaling pathways such as TNF, NF- κ B and C-type lectin receptor signaling pathways. Concomitantly, it also led to increased expression of glycan-binding proteins such as DC-SIGN, together with increased NF- κ B signaling and *NLRP3* and *CARD9* expression and increased production of IL-1 β and IL-8. Remarkably, this proinflammatory response imposed by ASCA IgG glycoforms is mannose glycan specific, dependent on galactosylation levels of ASCA IgG Fc and, even more importantly, occurs in a preclinical phase (Fig. 5). The in vivo pathogenic effect of ASCA-like IgGs was also demonstrated. This susceptibility is considerably ameliorated in Fc γ R-KO mice, supporting the biological relevance of ASCA Fc–Fc γ R interactions in triggering the inflammatory process. Overall, our results highlight the pathogenic properties of ASCAs in preclinical CD.

Agalactosylated IgGs have been associated with many immune-mediated and infectious diseases, such as coronavirus disease 2019 (refs. 15,16,41–43), which is consistent with our results. In addition, the effect of ASCA Fc–Fc γ R1a interactions associated with DC activation and modulation of glycan-binding proteins may represent a synergistic mechanism imposed by ASCA IgG Fc glycoforms toward equipping DCs with proinflammatory functions, an issue that should be further explored.

Together, here, we identified an immunological program underlined by an ASCA–glycome hub that occurs years before CD diagnosis and can trigger an inflammatory response associated with the transition from healthy tissue to intestinal inflammation. This study not only highlights a new preclinical biomarker of CD development associated with CD pathogenesis but also helps define a new target for future preventive strategies for IBD and other immune-mediated diseases.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41590-024-01916-8>.

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Methods

Cohort description and selection criteria

This study was approved as nonhuman subjects research (PJT 19-08) by the Naval Medical Research Command. Serum samples from 251 CD, 249 UC and 250 HC cases were retrieved from the US Department of Defense Serum Repository. From almost all individuals (741 of 750), four serum samples were retrieved comprising four specific time points, one corresponding to the nearest sample available closest to diagnosis (approximately 1 year; identified here as Dx) and three more samples collected before the diagnosis of IBD and separated by approximately 2-year intervals. Complicated disease was categorized as previously described³⁸. Samples from HC individuals with no medical record of IBD, rheumatoid arthritis, celiac disease or colon cancer were selected to match the individuals with IBD and were collected as controls (Supplementary Table 1)²². As a positive control for established disease, 33 serum samples from individuals with established CD were obtained at Centro Hospitalar Universitário Santo António, Porto (Supplementary Table 3). For the analysis of plasma cells, peripheral blood mononuclear cells (PBMCs) from individuals with CD (collected at the time of diagnosis (inaugural disease)), first-degree relatives of individuals with CD and HC individuals were obtained both at Centro Hospitalar Universitário Santo António, Porto, Hospital Beatriz Ângelo, Loures, and Hospital da Luz, Lisbon (Supplementary Table 4). Ethical approval was obtained from the Ethical Committees of all hospitals.

Isolation of IgG from human and mouse sera and ASCA quantification in human samples

Human and mouse serum samples were thawed and centrifuged at 1,620g for 10 min. Samples were then diluted in PBS and filtered through 0.45- μ m Supor AcroPrep 96-well filter plates. Serum samples were applied to the protein G plate and washed to remove unbound proteins. IgG was eluted from protein G using 0.1 M formic acid (pH 2.5) into a 96-deep-well plate and immediately neutralized with 1 M ammonium bicarbonate.

ASCA levels and titers (serial dilutions) were measured in human serum samples using a QUANTA Lite ASCA IgG enzyme-linked immunosorbent assay (ELISA; QUANTA), according to manufacturer's instructions.

Glycopeptide preparation and nano-LC-ESI-MS

Human and mouse IgGs were treated with TPCK-treated trypsin (Promega) and purified using solid-phase extraction on C18 beads (Chromabond, Macherey-Nagel). IgG glycopeptides were eluted into a 96-well PCR plate with 200 μ l of 20% acetonitrile by centrifugation at 105g for 5 min. Eluates were dried by vacuum centrifugation and stored at -20 °C until analysis by MS. For human IgA, plasma samples were transferred into corresponding wells of Orochem filter plates containing 40 μ l of IgA beads slurry (Thermo Scientific, IgA Affinity Matrix). Following 1 h of incubation and several steps of washing with 1 $\times$ PBS and MilliQ water, captured IgA was eluted with 100 mM formic acid. Samples were then dried in a speedvac at 60 °C for 2.5 h and resuspended in 25 mM ammonium bicarbonate for the subsequent reduction and alkylation. For the nano-LC-ESI-MS of human IgG, human IgA and mouse IgG N-glycopeptides, purified tryptic human IgG, human IgA and mouse IgG glycopeptides were analyzed on a Waters Acquity M Class UPLC system. The detected glycopeptides are presented in Supplementary Table 2 and in accordance with previous reports^{44–46}. Detailed methodology can be found in Supplementary Information.

Degalactosylation of human IgGs and dot blot analysis

IgGs from each individual were incubated with β 1,4-galactosidase S (New England BioLabs), according to manufacturer's instructions. The efficiency of sugar removal was verified by lectin dot blot. Briefly, 100 ng of IgG was placed on a nitrocellulose membrane and incubated for 1 h at room temperature. After, nonspecific sites were blocked with

4% bovine serum albumin (BSA) in PBS and 0.05% Tween 20 for 1 h before incubation with biotinylated ECA (diluted in blocking buffer with 0.1 mM CaCl₂; Vector Laboratories) for 1 h. After lectin incubation, the membrane was incubated with streptavidin conjugated with horseradish peroxidase (HRP) for 30 min. All incubations were performed at room temperature. Dots were then detected using ECL reagent (GE Healthcare, Life Sciences).

IgG aggregation assay

IgG aggregation was assessed using a protein aggregation assay kit (Abcam), according to manufacturer's instructions. Detailed methodology can be found in Supplementary Information.

Isolation of PBMCs

PBMCs were obtained from buffy coats from healthy donors as well as peripheral blood from inaugural individuals with CD (collected at the time of diagnosis) and first-degree relatives of individuals with CD. The separation of PBMCs was achieved by density centrifugation using Lymphoprep (density of 1.077 g ml⁻¹; Stem Cell Technologies). Anticoagulated blood was diluted in PBS (ratio of 1:1), slowly layered onto Lymphoprep (ratio of 1:2) and centrifuged on a gradient at 900g for 30 min at room temperature. After centrifugation, the interface containing PBMCs was transferred to a conical centrifuge tube and washed with PBS. For isolation of natural killer cells, detailed methodology is provided in Supplementary Information.

Isolation of CD14⁺ cells and generation of monocyte-derived DCs

CD14⁺ monocytes were purified from PBMCs from healthy donors using an immunomagnetic positive selection bead kit (Miltenyi Biotec) conjugated with a monoclonal antibody to human CD14 (isotype mouse IgG2a), according to the manufacturer's instructions.

CD14⁺ cells were then cultured in six-well cell culture plates in culture medium comprising RPMI-1640 and HEPES (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 0.1% gentamicin, 50 ng ml⁻¹ human GM-CSF and 50 ng ml⁻¹ human IL-4 (Peprotech) for 7 days. Cells were maintained at 37 °C in a humidified incubator with 5% CO₂, and medium was replenished every 3 days.

IgG-dependent immune cell activation

Ninety-six-well Nunc MaxiSorp high-binding plates were precoated with mannan from *S. cerevisiae* (Sigma-Aldrich) at 10 μ g ml⁻¹ in 50 mM carbonate-bicarbonate coating buffer (pH 9.6) overnight at 4 °C. Afterward, a blocking step with 5% BSA was performed. Total IgGs (6 μ g ml⁻¹) were added to plates previously coated with mannan, β -glucan or di-GlcNAc (10 μ g ml⁻¹) for 90 min at 37 °C in a humidified incubator with 5% CO₂. To assess the effect of galactosylation, 6 μ g ml⁻¹ total IgGs treated with β -gal was also used. Monocyte-derived DCs were suspended in complete RPMI medium and plated in anti-mannan IgG-coated wells at 1.5 $\times$ 10⁵ cells per well for 6 h. Additionally, for functional blockade of Fc γ RIIa, cells were preincubated for 1 h at 37 °C with 5 μ g ml⁻¹ InVivoMab anti-human CD32a (Fc γ RIIa) blocking antibody (Clone IV.3) before incubation in anti-mannan IgG-coated plates. After incubation, the culture supernatants were collected and frozen at -80 °C for further cytokine analysis. Cells were washed with FACS buffer and stained for viability and surface markers (Supplementary Table 6). All incubations were performed for 30 min at 4 °C. Samples were fixed in 2% paraformaldehyde (Sigma-Aldrich), and cells were acquired by flow cytometry on a FACSCanto II system using FACSDiva software. Data were analyzed using FlowJo version 10.5.3 (Tree Star) using the gating strategy shown in Supplementary Fig. 9a. For IgG-dependent NK cell degranulation, detailed methodology is provided in Supplementary Information, and flow cytometry data analysis was performed using the gating strategy shown in Supplementary Fig. 9b.

Plasma cell characterization in human PBMcs

PBMcs from inaugural individuals with CD (collected at the time of diagnosis), first-degree relatives of individuals with CD and HC individuals were collected, and plasma cell characterization was performed by flow cytometry using surface and intracellular markers (Supplementary Table 6), as described above. Cells were acquired by flow cytometry on a Cytex Aurora system with SpectraFlow v3.2.1, and data were analyzed in FlowJo version 10.5.3 (Tree Star) using the gating strategy shown in Supplementary Fig. 9c.

Cytokine quantification

NK cells were cultured in a 96-well Nunc MaxiSorp high-binding plate precoated with mannan and total IgGs at 2×10^5 cells per well for 48 h at 37 °C in a humidified incubator with 5% CO₂. IFN γ levels in the supernatants were quantified by ELISA using a human IFN γ DuoSet ELISA (R&D Systems), according to the manufacturer's instructions.

Supernatants from 6-h DC cultures were analyzed by flow cytometry (BD Accuri C6 Plus) using a BD Cytometric Bead Array (CBA) Human Inflammatory Cytokine kit (BD Biosciences), following the manufacturer's instructions.

For mouse experiments, TNF and IL-1 β levels were measured in supernatants from colonic explants (in culture for 24 h) by ELISA using commercially available kits (Invitrogen) according to the manufacturer's instructions. Levels of cytokines (measured in pg ml⁻¹) were normalized by explant weight (g).

Bulk RNA-sequencing data processing and analysis

Sequenced reads were aligned to the human GRCh38 (Ensembl version 94) reference genome using HISAT2 (v2.0.5) with default settings. Counts per gene were generated from the alignment files by FeatureCounts (v1.5.0-p3). Differential expression analysis between pairwise conditions was conducted in R (v4.1.2) using DESeq2 (v1.34). For each comparison, log₂ (fold change) results were shrunk using the aplem (v1.23.1) package to remove noise (differentially expressed genes with low counts and/or high dispersion values) while preserving significant differences.

Overrepresentation pathway analysis was conducted in clusterProfiler (v4.11.0.001) to the human KEGG ontology database for the set of upregulated and downregulated genes (false discovery rate ≤ 0.05) separately. Pathways with an adjusted *P* value of <0.05 were regarded as significant. Bubble diagrams were constructed for the visualization of the top six enriched pathways, after excluding those belonging to human diseases.

A heat map of the differentially expressed genes between conditions for the top six upregulated KEGG pathways was plotted using complexHeatmap (v2.10.0). Volcano plots representing differentially expressed genes were generated using ggplot2 (v3.4.0). Data were deposited in the European Nucleotide Archive with the dataset identifier [PRJEB76671](https://www.ebi.ac.uk/ena/record/PRJEB76671).

C1q (complement) binding assay

C1q binding was assessed by incubation with complement component C1q to IgGs added to plates previously coated with mannan (10 μ g ml⁻¹) for 90 min at 37 °C in a humidified incubator with 5% CO₂ and detected with HRP sheep anti-human C1q. Detailed methodology can be found in Supplementary Information.

Gene expression analysis using quantitative PCR with reverse transcription

Total RNA from DCs was extracted using TRIzol reagent (Thermo Fisher Scientific). Total RNA was quantified and retrotranscribed to cDNA using Superscript IV Reverse Transcriptase (Invitrogen), according to the instructions of the kit. cDNA was amplified using Taqman Universal PCR Master Mix (Applied Biosystems) and Taqman probes (Supplementary Table 7). Amplification data were acquired with a 7500 Fast

Real-Time PCR System (Applied Biosystems); 18S was used as the housekeeping gene. Relative quantification values for gene expression were calculated based on the change in cycling threshold (ΔC_t) method using the following equation: $2^{-(\text{target gene mRNA expression} - \text{housekeeping gene mRNA expression})}$.

Phospho-NF- κ B immunoblotting

After 3 h of culture with IgGs, DCs were lysed in RIPA buffer (150 mM sodium chloride, 50 mM Tris-HCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and 10% SDS) with 1% sodium orthovanadate (Sigma), 1% phenylmethylsulfonyl fluoride (Thermo Fisher Scientific) and 4% cOmplete protease inhibitor cocktail (Roche). Protein lysates were separated on 10% resolving polyacrylamide gels. Proteins were then transferred to nitrocellulose membranes (GE Healthcare, Amersham) and blocked with 4% BSA in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h. Membranes were incubated with rabbit anti-human phosphorylated NF- κ B (93H1, Cell Signaling Technology) diluted 1:1,000 in 4% nonfat dry milk in TBS-T. For protein loading (control), membranes were blocked with 4% nonfat dry milk for 1 h and incubated with mouse anti-human vinculin (V284, Santa Cruz Biotechnology) diluted 1:500 in 1% nonfat dry milk in TBS-T. Bound anti-phospho-NF- κ B and anti-vinculin were detected using goat anti-rabbit IgG HRP and chicken anti-mouse IgG HRP, respectively. Chemiluminescence was determined using ECL prime (GE Healthcare, Amersham).

Animal model of colitis

All mouse procedures were approved by the Institute for Research and Innovation in Health (i3S) Animal Ethics Committee for animal experimentation under Portuguese regulations (DGAV license number 009268/2022-06-02). Mice were housed at the Association for Assessment and Accreditation of Laboratorial Animal Care-accredited i3S animal facility in a temperature-controlled (20–24 °C) room maintained at a humidity of 45–55% under a 12-h light/12-h dark period.

C57BL/6 WT mice aged between 6 and 10 weeks were inoculated with 12 mg of mannan subcutaneously every 3 days over 5 weeks. The control group received PBS by the same route at similar time points. Levels of ASCA were monitored after 5 weeks by collecting a small amount of blood by the tail vein and analyzing by ELISA (QUANTA Lite ASCA IgG ELISA, QUANTA; goat anti-mouse IgG HRP (Bethyl) was used as the detection antibody). Sera from mannan-treated and control mice were collected upon cardiac puncture at the experimental endpoint, and total IgGs were isolated and quantified (as previously described for human samples).

In vivo intestinal permeability was assessed by administration of fluorescein isothiocyanate-labeled dextran. Food and water were withdrawn for 8 h. Mice were administered 44 mg per 100 g (body weight) fluorescein isothiocyanate-labeled dextran (TdB Consultancy; 4 kDa) by oral gavage. Serum was collected from the tail vein 4 h later, and fluorescence intensity was measured by spectrophotofluorimetry (excitation: 485 nm; emission: 528 nm).

WT and Fc γ R-KO (C57BL/6 background) mice aged 10–16 weeks (recipient) were intraperitoneally injected with 100 μ g of ASCA-enriched IgG pool or, as a control, 100 μ g of PBS-derived IgGs on days 0 and 3. At day 0, colitis was induced chemically by treatment with 2% DSS (36,000–50,000 Da; MP Biomedicals) via the drinking water (provided ad libitum) from day 0 to day 7, which was then switched to tap water. Mice were monitored daily, and the DAI was assessed according to the parameters specified in Supplementary Table 5. Animals were killed according to the humane endpoints.

For oral immunization with mannan, mice were treated with 12 mg of mannan by oral gavage in the first 3 days of each week over 3 weeks (total of nine administrations), followed by DSS-induced colitis.

To isolate lamina propria leukocytes, colonic fragments 0.5–1 cm in size were incubated in DMEM supplemented with 1 mM CaCl₂, 1 mM MgCl₂, 1.5 mg ml⁻¹ collagenase IV (Sigma) and 0.4 mg ml⁻¹ dispase (Gibco) under agitation at 100 rpm and 37 °C for 40 min. Tissues were dissociated and filtered through a 70- μ m cell strainer (BD Biosciences).

Cell suspensions were resuspended in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin and layered on Lymphoprep solution at a ratio of 1:2 (Lymphoprep:cell suspension). After gradient centrifugation at 800g for 20 min at 20 °C (without acceleration or break), immune cells (retained in the interface) were collected for staining using the antibodies listed in Supplementary Table 6. Cells were acquired by flow cytometry on a Cytex Aurora system with SpectraFlow v3.2.1, and data were analyzed in FlowJo version 10.5.3 (Tree Star) using the gating strategy shown in Supplementary Fig. 9d.

BMDCs and coculture with mouse ASCA IgGs

Bone marrow was extracted from the femurs and tibias of C57BL/6 WT mice, and isolated cells were then cultured in RPMI supplemented with 10% FBS, penicillin/streptomycin (100 U ml⁻¹) and mouse recombinant proteins IL-4 and GM-CSF (50 ng ml⁻¹; *Escherichia coli*, PeproTech). On the third day of culture, the medium was replaced, and the cells were cultured for additional 4 days. Differentiated DCs were then incubated with mouse ASCA IgGs and PBS IgGs (control), similar to the protocol described for IgG-dependent human DC activation. Cells were acquired by flow cytometry on a Cytex Aurora system with SpectraFlow v3.2.1, and data were analyzed using FlowJo version 10.5.3 (Tree Star).

Statistical analysis

Data were obtained from at least two independent experiments and processed using GraphPad Prism 10.2.3 (GraphPad Software). Statistical significance was determined by Kruskal–Wallis test, ANOVA, unpaired *t*-test or Mann–Whitney test, according to normality of the samples. For animal experiments, a two-way ANOVA was applied to the analysis of DAI. The robust regression and outlier removal test was performed to identify outliers, and those values were eliminated from the analysis. Flow cytometry data were analyzed using FlowJo v10. The number of samples is indicated in each figure legend.

Batch correction of glycan traits. Glycome profiles for 2,991 samples were obtained using 40 plates. Batch correction across different plates was performed using the Combat function from the ‘sva’ package available in R Cran⁴⁷.

Association between glycan traits and disease onset. The association between each glycan marker and disease onset was assessed via logistic regression after adjusting for age, race and sex. Logistic regression was estimated for each time point before diagnosis separately. *P* values from logistic regressions were adjusted for multiple comparisons via the Benjamini–Hochberg method⁴⁸, and only markers with an adjusted *P* value smaller than 10% were reported as significant.

Predictive performance. The predictive performance of different glycan traits was evaluated based on leave-one-out cross-validation. Specifically, for each sample, we used the remaining *n* – 1 samples to fit a logistic regression where the disease status (that is, HC versus CD and HC versus UC) was modeled as a function of each glycan trait, sex, race and age. The estimated model was then used to predict disease status of the left-out sample. The predictive performance of each glycan was assessed via area under the receiver operating characteristic curve (ROC). Predictive performance was evaluated for each time point before diagnosis separately. The R package pROC was used to compute area under the ROC values and to plot the ROC⁴⁹.

Coexpression network analysis. We performed coexpression network analysis to identify the associations across glycan traits and antimicrobial antibodies for individuals with CD and HC individuals jointly via joint random forest⁵⁰. Coexpression networks were estimated at the furthest time point before diagnosis. Permutation-based techniques were used to find associations significant at a false discovery rate of 5% (ref. 50).

Deriving cell-type score from proteomic data. Based on prior knowledge of cell-type markers, we leveraged the LM22 signature matrix including the expression of different genes for 22 cell types²⁴. Leveraging cell-type signatures from this study, we computed single-sample gene set enrichment analysis via the R package GSVA⁵¹ (method = ‘zscore’) using proteomic data available for different PRE-DICT samples. We then correlated the estimated cell-type score with CD/HC status via the Wilcoxon rank-sum test.

All analyses were performed using R statistical software (version 3.6.3, R Foundation for Statistical Computing).

Figure preparation

Some schematic figures were drawn using pictures from Servier Medical Art, licensed under a Creative Commons Attribution 4.0 Unported License (<https://creativecommons.org/licenses/by/4.0/>).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The RNA-sequencing data have been deposited in the EMBL Nucleotide Sequence Database, European Nucleotide Archive, with the dataset identifier [PRJEB76671](https://www.ebi.ac.uk/ena/record/PRJEB76671). Participant-related data may be subject to donor confidentiality. The data displayed in Fig. 1 and Supplementary Figs. 1a–c, 2 and 5a–c are available from the corresponding author upon request. Reagents, materials and protocols are available from the corresponding author upon request. Source data are provided with this paper.

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Acknowledgements

We wish to acknowledge the Gastroenterology Department of Centro Hospitalar Universitário de Santo António, in particular P. Lago, for providing samples from individuals with established CD. We kindly thank J. Rojo from the Instituto de Investigaciones Químicas (Universidad de Sevilla) for providing us with the di-GlcNAc glycodendrimer. We would also like to acknowledge J. V. Ravetch (Rockefeller University) and M. S. Cragg (University of Southampton) for kindly providing us with the FcγR-deficient mice used for the in vivo studies. S.S.P. acknowledges funding from the US Department of Defense, US Army Medical Research Acquisition Activity and FY18

Peer-Reviewed Medical Research Program Investigator-Initiated Research Award (award number W81XWH1920053). S.S.P. also acknowledges funding from the European Crohn's and Colitis Organisation (ECCO) Pioneer Award 2021, the International Organization for the study of Inflammatory Bowel Disease (IOIBD) and the Portuguese Foundation for Science and Technology (FCT; EXPL/MED-ONC/0496/2021). J.G. acknowledges funding from European Society of Clinical Microbiology and Infectious Diseases (ESCMID Research Grant 2022), European Crohn's and Colitis Organisation (ECCO Grant 2023) and FCT (2020.00088.CEECIND). C.S.R. thanks FCT for funding (2020.08422.BD). I.A. acknowledges funding from FCT (2022.00337.CEECIND) and the BIAL Foundation and Portuguese Medical Association (Maria de Sousa Award 2023). E.L.-G. thanks FCT for funding (UI/BD/152866/2022). F.P. and Z.H.G. were partially supported by the Kenneth-Rainin Foundation (20210021). B.C. acknowledges funding from FCT (CEECINST/00123/2021/CP1772/CT0001). J.T. acknowledges funding from the Portuguese Society of Gastroenterology and from Luz Saúde (Grupo de Investigações em Patologia Digestiva LUz Saúde LH.INV.F2019015). This study was also cofunded by the European Union (GlycanTrigger, grant agreement number 101093997). The views and opinions expressed are, however, those of the author(s) only and do not necessarily reflect those of the European Union or the European Research Council Executive Agency. Neither the European Union nor the granting authority can be held responsible for them. This study was conducted under support of Peer-Reviewed Medical Research Program (PR180831P1). The views expressed in this article reflect the results of the research conducted by the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, the Henry M. Jackson Foundation for the Advancement of Military Medicine or the US Government. There are no restrictions on its use. This article was prepared while R.M.L. was employed at Henry M. Jackson Foundation for the Advancement of Military Medicine. The opinions expressed in this article are those of the authors and do not reflect the view of the National Institutes of Health, the Department of Health and Human Services or the US Government. C.K.P. is an employee of the US Government. This work was prepared as part of official duties. Title 17 U.S.C. §105 provides that 'Copyright protection under this title is not available for any work of the United States Government'. Title 17 U.S.C. §101 defines a US Government work as a

work prepared by a military service member or employee of the US Government as part of that person's official duties.

Author contributions

J.G. and C.S.R. designed the study, performed the experiments and data analysis and wrote the paper. F.P., B.T. and Z.H.G. performed the bioinformatic analyses on IgG glycome data. I.A. and E.L.-G. designed, performed and analyzed the animal experiments. B.C. analyzed the RNA-sequencing data. A.M.D. contributed to in vitro experiments. C.M.-B. and J.R. collected human biological samples and clinical data from first-degree relatives and inaugural CD cases. R.M.L. prepared biological samples and demographic data from the PREDICTS cohort. M.N., J.Š. and S.H. performed the IgG/IgA glycome characterizations. R.U. contributed in scientific discussions. J.T., G.L., J.-F.C. and C.K.P. participated in the discussion of the study design and contributed to critical revisions of the paper. S.S.P. designed the study, acquired funding and wrote the paper.

Competing interests

G.L. is the founder and owner of Genos, Ltd., a private research organization that specializes in high-throughput glycomic analysis, and has several patents in this field. M.N. and J.Š. are employees of Genos, Ltd. The other authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41590-024-01916-8>.

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Peer review information *Nature Immunology* thanks Falk Nimmerjahn and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available. Primary Handling Editor: S. Houston, in collaboration with the *Nature Immunology* team.

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SUPPLEMENTARY METHODS

Glycopeptide preparation

To isolated human and murine IgGs, 0.2 µg of TPCK-treated trypsin (Promega, Madison, WI) was added, followed by an overnight incubation at 37°C. Tryptic digests were purified using a solid phase extraction (SPE) on C18 beads (Chromabond, Macherey-Nagel, Duren, Germany). 10 µg of C18 beads was applied to each well of an OF1100 96-well polypropylene filter plate (Orochem, Naperville, IL). The RP stationary phase was activated 3 times with 200 µl of 80 % ACN and conditioned with 3 times with 200 µl 0.1 % TFA (Sigma-Aldrich/Merck, Darmstadt, Germany). The IgG digests were diluted with 0.1 % TFA (Sigma-Aldrich/Merck) loaded onto the C18 beads and washed 3 times 200 µl 0.1 % TFA. The entire procedure was performed on a vacuum manifold under pressure reduction of 2 inches of mercury. IgG glycopeptides were eluted into a PCR 96 well plate with 200 µl of 20 % ACN by centrifugation at 105 g for 5 min. Eluates were dried by vacuum centrifugation and stored at - 20 °C until analysis by MS.

Human IgA enrichment and digestion

20 µL of plasma samples were transferred to wells of PCR plate and diluted with 110 µL of 1xPBS, pH 7.4. Diluted plasma samples were transferred into corresponding wells of Orochem filter plate containing 40 µL of IgA beads slurry (Thermo Scientific, IgA Affinity Matrix). Following 1 hour of incubation and several steps of washing with 1x PBS and MQ water, captured IgA was eluted with 100 mM formic acid. The collected eluate was dried in a speedvac at 60°C for 2.5 hours and resuspended in 25 mM ammonium bicarbonate for the subsequent reduction and alkylation with 200 mM DTT (Sigma-Aldrich) and 400 mM IAA (Sigma-Aldrich). Finally, to the reduced and alkylated sample, 1 µL of 0.2 µg/ µL TPCK-treated trypsin stock (Promega, Madison, WI) was added, followed by an overnight incubation at 37°C. Samples were stored at -20°C until the analysis by LC-MS.

nanoLC-ESI-MS of human IgG, human IgA and murine IgG N-glycopeptides

Purified tryptic human IgG, human IgA and murine IgG glycopeptides were analyzed on Waters ACQUITY M Class UPLC system (Waters, Milford, MA), consisting of binary pump, auxiliary pump, trap valve manager and autosampler maintained at 10 °C with column compartment set to 30°C. 10 µL of sample was applied to a PepMap 100 C18 (5 mm × 300 µm i.d., 5µm, Thermo) SPE trap column conditioned with 0.1 % TFA (mobile phase A) for 1 min at 40 µl/min. For human IgGs: after sample loading, the trap column was switched in-line with the gradient and C18 nano-481 LC column (150 mm x 100 µm i.d., 2.7 µm HALO fused core particles; Advanced Materials Technology, Wilmington, DE) for 9.5 minutes. Trap column was cleaned with two full loop injections containing 20 µl of 95 % ACN and 50% ACN in IPA respectively. C18 nano-LC column was equilibrated with 100 % mobile phase A (0.1% TFA), for 2 minutes. IgG glycopeptides were reconstituted in 100 µl of ultrapure water before injection. Separation was achieved at 1 ml/min using the following gradient of mobile phase A and mobile phase B (80 % ACN and 20 % 0.1 % TFA respectively): 0.5 min 12 % B, 0.5 – 4 min 12 % B - 17 % B, 4 - 5 min 17 % B. The ACQUITY M-Class system was coupled with Compact mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with Captive Spray ion source and nano Booster for introduction of acetonitrile vapor into the source. Nitrogen was used as a drying gas (4 l/min) and nebulizer (0.4 bars). Quadrupole and collision energies were set to 4 eV. Spectra were recorded from m/z 800 to 2000 with 2 averaged scans at a frequency of 0.5 Hz. Acquity M-class UPLC system was operated under MassLynx software 4.1 while Bruker Compact Q-TOF-MS was operated under HyStar software version 4.1. For human IgA, and murine IgGs: after sample loading, the trap column was switched in-line with the gradient and C18 nano LC column (150 mm x 100 µm i.d., 2.6 µm SunShell core shell particles; Chromanik Technologies Inc.) for 9 minutes. Trap column was cleaned with two full loop injections containing 20 µl of 95 % ACN and 50% ACN in IPA respectively. C18 nano-LC column was equilibrated with 100 % mobile phase A (0.1% TFA), for 2 minutes. IgG glycopeptides were reconstituted in 30 µl of ultrapure water before injection. Separation was achieved at 1 ml/min using the

following gradient of mobile phase A and mobile phase B (80 % ACN and 20 % 0.1 % TFA respectively): 1 – 1.5 min 0 % B – 18.5 % B, 1.5 - 5 min 18.5 % B – 26 % B, 5 - 9 min 26 % B. The ACQUITY M-Class system was coupled with Compact mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with Captive Spray ion source and nano Booster for introduction of acetonitrile vapor into the source. Nitrogen was used as a drying gas (4 l/min) and nebulizer (0.2 bars). Quadrupole and collision energies were set to 4 eV. Spectra were recorded from m/z 600 to 2500 with 2 averaged scans at a frequency of 0.5 Hz. Acquity M-class UPLC system was operated under MassLynx software 4.1 while Bruker Compact Q-TOF-MS was operated under HyStar software version 4.1. The detected glycopeptides are presented in **Supplementary Table 2**, and in accordance with previous reports^{1, 2, 3}.

IgG aggregation assay

To assess the degree of IgG aggregation, a subset of IgG samples from healthy controls and CD patients (preclinical phase, at diagnosis, or full-blown disease) were analyzed using a protein aggregation assay kit (Abcam), according to manufacturer's instructions. Besides the standards from the kit, a commercial IgG antibody was incubated at 65°C for 45 minutes to aggregate (positive control). Fluorescence was measured at Ex/Em 440/500 nm and compared with the standard curve of the kit.

Isolation of Natural Killer (NK) cells

NK cells were purified by negative selection from freshly PBMCs using EasySep™ Human NK Cell Isolation Kit, according to the manufacturer's instructions (Stem Cell Technologies). The purity (>94%) of NK cells was determined by flow cytometry analysis on FACSCanto™ II system (BD Biosciences, San Jose, CA). Monoclonal antibodies used for staining of NK cells are shown in **Supplementary Table 6**. Cells were cryopreserved in freeze medium (90% of fetal bovine serum (FBS) and 10% of dimethyl sulfoxide (DMSO)) at -80°C. After defrosting, cryo-preserved PBMCs and cryo-preserved NK cells were maintained in complete culture medium, RPMI 1640 Medium, GlutaMAX™ Supplement, HEPES (Thermo

Fisher Scientific), supplemented with 10% of FBS, 1% penicillin/streptomycin and 0,1% gentamicin overnight at 37°C in a humidified incubator with 5% of CO₂.

IgG-dependent NK cell degranulation

Purified NK cells were suspended in complete RPMI medium supplemented with IL-2 (100 U/mL, Pepotrech) and plated in wells previously coated with mannan, β -glucan or di-GlcNAc (as described above for DCs), at 1×10^5 cells/well in the presence of anti-human CD107a (LAMP-1; **Supplementary Table 6**). Following 1 hour, 10 μ g/ml of Brefeldin A (Sigma–Aldrich) and GolgiStop™ Protein Transport Inhibitor were added and incubated 5 hours in similar conditions. After incubation, cells were stained for viability, as well as for surface markers (**Supplementary Table 6**). Cells were fixed and permeabilized with Transcription Factor Staining Buffer Set (eBioscience), followed by intracellular staining (**Supplementary Table 6**). Samples were acquired by flow cytometry on a FACSCanto™ II system (BD Biosciences) using the FACSDiva™ software (BD), and data were analyzed in FlowJo version 10.5.3 (Tree Star, Inc., Ashland, OR), using gating strategy shown in **Supplementary Fig. 9b**.

C1q (complement) binding assay

96-well Nunc® MaxiSorp™ high-binding plates were pre-coated with mannan as previously described. A blocking step with 5% of BSA was performed. 6 μ g/mL of total IgGs were added to plates previously coated with mannan (10 μ g/mL), for 90 minutes at 37°C in a humidified incubator with 5% of CO₂. After three washing steps with PBS 3% BSA 0.05% Tween20, complement component C1q was added (10 μ g/mL) for 1 hour at room temperature. After washing (3x), HRP sheep anti-human C1q was added for incubation for 1 hour at room temperature. Plate was washed four times (as previously), and 3,3',5,5'-

tetramethylbenzidine (substrate solution) was added for 15 min. Reaction was stopped with 1 N H₂SO₄ and absorbance was measured at 450 nm.

1. Momčilović, A. *et al.* Simultaneous Immunoglobulin A and G Glycopeptide Profiling for High-Throughput Applications. *Anal Chem* **92**, 4518-4526 (2020).
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3. Zaytseva, O.O. *et al.* Fc-Linked IgG N-Glycosylation in FcγR Knock-Out Mice. *Front Cell Dev Biol* **8**, 67 (2020).

SUPPLEMENTARY TABLES

Supplementary Table 1. Demographic and clinical characteristics of the samples from the PREDICTS cohort.

		Patients with Crohn's disease	Patients with ulcerative colitis	Healthy control individuals
Number of individuals		251	249	250
Number of samples		1002	992	997
Age^a years; mean $\pm$ SD		31.70 $\pm$ 6.53	29.77 $\pm$ 5.78	29.67 $\pm$ 5.65
Sex n (%)	Female	184 (18.36)	102 (10.28)	83 (8.32)
	Male	818 (81.64)	890 (89.72)	914 (91.68)

^aAge at sample A (healthy individuals) or diagnosis (inflammatory bowel disease).

Supplementary Table 2. Glycan traits of IgG and IgA characterized in serum samples.

IgG glycan traits			
Glycan Trait	Galactosylation profile	Additional glycan structures	
H3N4	agalactosylated (G0)	-	
H3N4F1		fucosylated	
H3N5F1		fucosylated; bisecting	
H3N5		bisecting	
H4N4	monogalactosylated (G1)	-	
H4N4F1		fucosylated	
H4N4F1S1		fucosylated; sialylated	
H4N5F1		fucosylated; bisecting	
H4N5F1S1		fucosylated; sialylated; bisecting	
H4N5		bisecting	
H5N4	digalactosylated (G2)	-	
H5N4F1		fucosylated	
H5N4F1S1		fucosylated; sialylated	
H5N4S1		sialylated	
H5N5F1		fucosylated; bisecting	
H5N5F1S1		fucosylated; sialylated; bisecting	
IgA glycan traits			
Glycan Trait	Galactosylation profile	Additional glycan structures	Cluster ^a
H3N5	agalactosylated (G0)	bisecting	LSL
H4N4S1	monogalactosylated (G1)	sialylated	LSL
H5N5S2F1	digalactosylated (G2)	fucosylated; sialylated; bisecting	LAGcb
H5N4S1		sialylated	LAGy
H5N4S1F1		fucosylated; sialylated	LAGy
H5N4S2		sialylated	LAGy
H5N5S2F1		fucosylated; sialylated; bisecting	LAGy
H5N2		high-mannose	LSL

^aCluster names for IgA are referring to the first three letters of the peptide sequence.

Supplementary Table 3. Demographic and clinical characteristics of the samples from established CD patients.

Patients with established Crohn's disease		
Number of patients	10	
Number of samples	33	
Age years; mean $\pm$ SD	53.50 $\pm$ 15.98	
Time since diagnosis years; mean $\pm$ SD	19.04 $\pm$ 8.65	
Sex n (%)	Female	3 (30)
	Male	7 (70)
Clinical symptoms <i>samples</i> n (%)	Remission	20 (60.61)
	Activity	13 (39.39)
Therapy <i>samples</i> n (%)	Infliximab	16 (48.48)
	Adalimumab	16 (48.48)
	Vedolizumab	1 (3.03)

Supplementary Table 4. Demographic and clinical characteristics of the samples from inaugural CD patients, first-degree relatives (FDR) of CD patients and healthy controls.

	Patients with Crohn's disease	First-degree relatives	Healthy control individuals
Number of individuals	6	7	6
Number of samples	6	7	6
Age^a years; mean $\pm$ SD	31.83 $\pm$ 12.97	25.71 $\pm$ 3.99	27.17 $\pm$ 1.94
Sex n (%)	Female 1 (16.67) Male 5 (83.33)	5 (71.43) 2 (28.57)	5 (83.33) 1 (16.67)

^aAge at sample collection.

Supplementary Table 5. Disease activity index (DAI). The DAI is obtained by the average score in each category.

DAI	Weight loss (%)	Stool consistency	Blood
0	0	WF	No blood
1	1-5	WF/P	-
2	6-10	P	Vestiges in the swab
3	11-15	P/L	-
4	16-20	L	Blood in the swab
5	21-25	-	-
6	>25	-	-

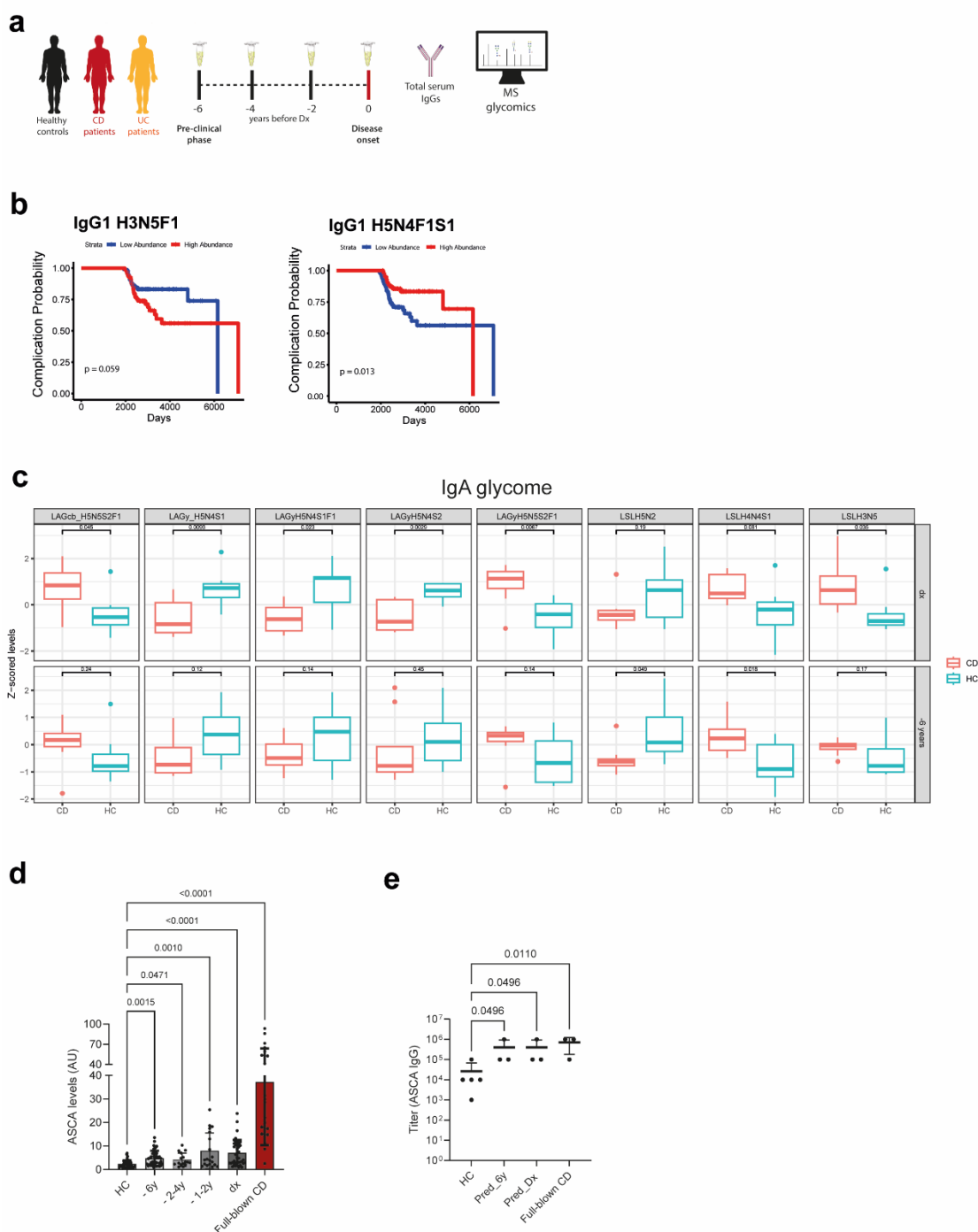
Supplementary Table 6. Antibodies and dyes used in flow cytometry.

Antibody	Catalog number	Company	Company
eBioscience™ Fixable Viability Dye eFluor™ 780	65-0865-18	1:2000	Invitrogen
Brilliant Violet 510 anti-human CD3, clone OKT3	317332	1:100	BioLegend
PE anti-human CD56, clone HCD56	318306	1:100	BioLegend
APC anti-human Granzyme B, clone GB14	MHGB05	1:100	Invitrogen
FITC anti-human CD107a (LAMP-1), clone H4A3	328606	1:150	BioLegend
PE anti-human CD14, clone 61D3	12-0149-42	1:100	Invitrogen
APC anti-human CD11c, clone B015	17-0128-42	1:100	Invitrogen
PE-Cyanine5 anti-human CD86, clone IT2.2	15-0869-42	1:100	Invitrogen
Rabbit anti-human CD209	AHP627	1:100	Bio-Rad
Polyclonal Swine anti rabbit Immunoglobulins/FITC	F0205	1:20	Dako
Anti-hDectin2 Affinity Purified Goat IgG	AF3114	1:100	R&D Systems
Polyclonal Rabbit anti-Goat Immunoglobulins/Biotinylated	E0466	1:100	Dako
Streptavidin PE-Cyanine7	25-4317-82	1:200	Invitrogen
PE-CF594 anti-human CD138, clone MI15	564606	1:100	BD Biosciences
PE-Cyanine5 anti-human CD38, clone HIT2	303507	1:400	BioLegend
BV605 anti-human CD3, clone 17A2	100237	1:300	BioLegend
cFR685 anti-human CD19, clone HIB19	R7-20118	1:300	Cytex
BB700 anti-human IgG, clone G18-145	742235	1:300	BD Biosciences
PE-Cyanine7 anti-human IgM, clone MHM-88	314531	1:200	BioLegend
BV605 anti-mouse CD45, clone 30-F11	103139	1:400	BioLegend
eF450 anti-mouse CD11c, clone N418	48-0114-82	1:100	Invitrogen
PE anti-mouse NKp46, clone 29A1.4	12-3351-80	1:200	Invitrogen
PerCP-Cy5.5 anti-mouse CD3	100328	1:100	BioLegend
PE-Cy5 anti-mouse MHCII, clone M5/114.15.2	15-5321-81	1:800	Invitrogen
PE-Cy7 anti-mouse CD45, clone 30-F11	25-0451-82	1:400	Invitrogen
FITC anti-mouse IFN γ , clone XMG1.2	11-7311-82	1:100	Invitrogen
PE-Cy7 anti-mouse CD16/32, clone 93	101318	1:100	BioLegend
Purified anti-mouse CD16/32, clone 93	101302	1:200	BioLegend

Supplementary Table 7. Probes used for qRT-PCR analysis.

Taqman probe	Reference	Company
<i>18S</i>	Hs99999901_s1	Applied Biosystems
<i>NLRP3</i>	Hs00918082_m1	Thermo Fisher Scientific
<i>CARD9</i>	Hs00364485_m1	Thermo Fisher Scientific
<i>FCGR2A</i>	Hs01013401_g1	Thermo Fisher Scientific

Supplementary Figure 1

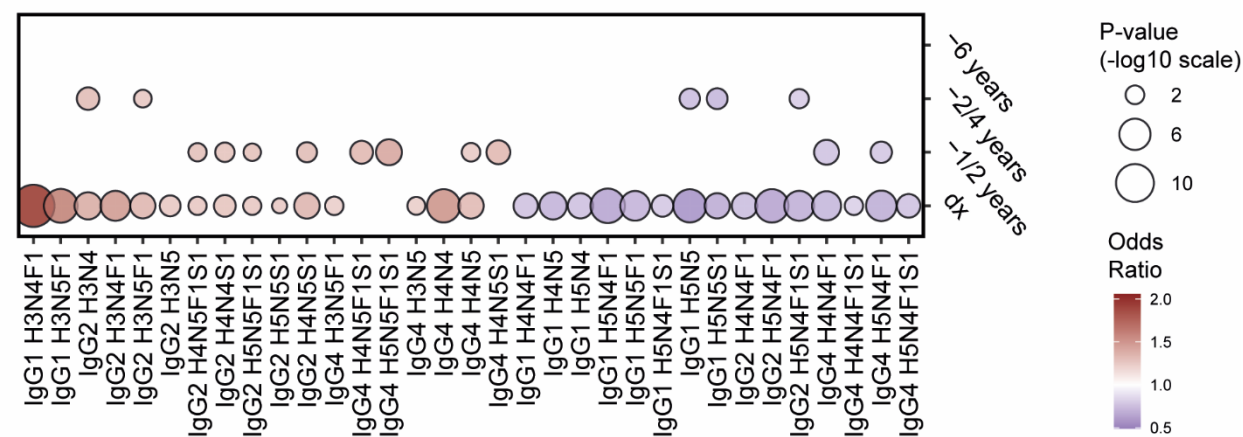


Supplementary Figure 1. Glycosylation signatures of CD patients and ASCA quantification.

(a) Total IgGs from serum samples from $n=251$ CD patients, $n=249$ UC patients, and $n=250$ healthy controls (HC), at different time points (preclinical phase: 1-2, 4-6, and 6 years before diagnosis; and at diagnosis; matched for HC), were isolated and analyzed by mass spectrometry. **(b)** Association between different IgG glycan traits and the development of Crohn's disease complication (days to CD complication development) via Kaplan Meier. **(c)** IgA glycosylation profiles for a subset of CD

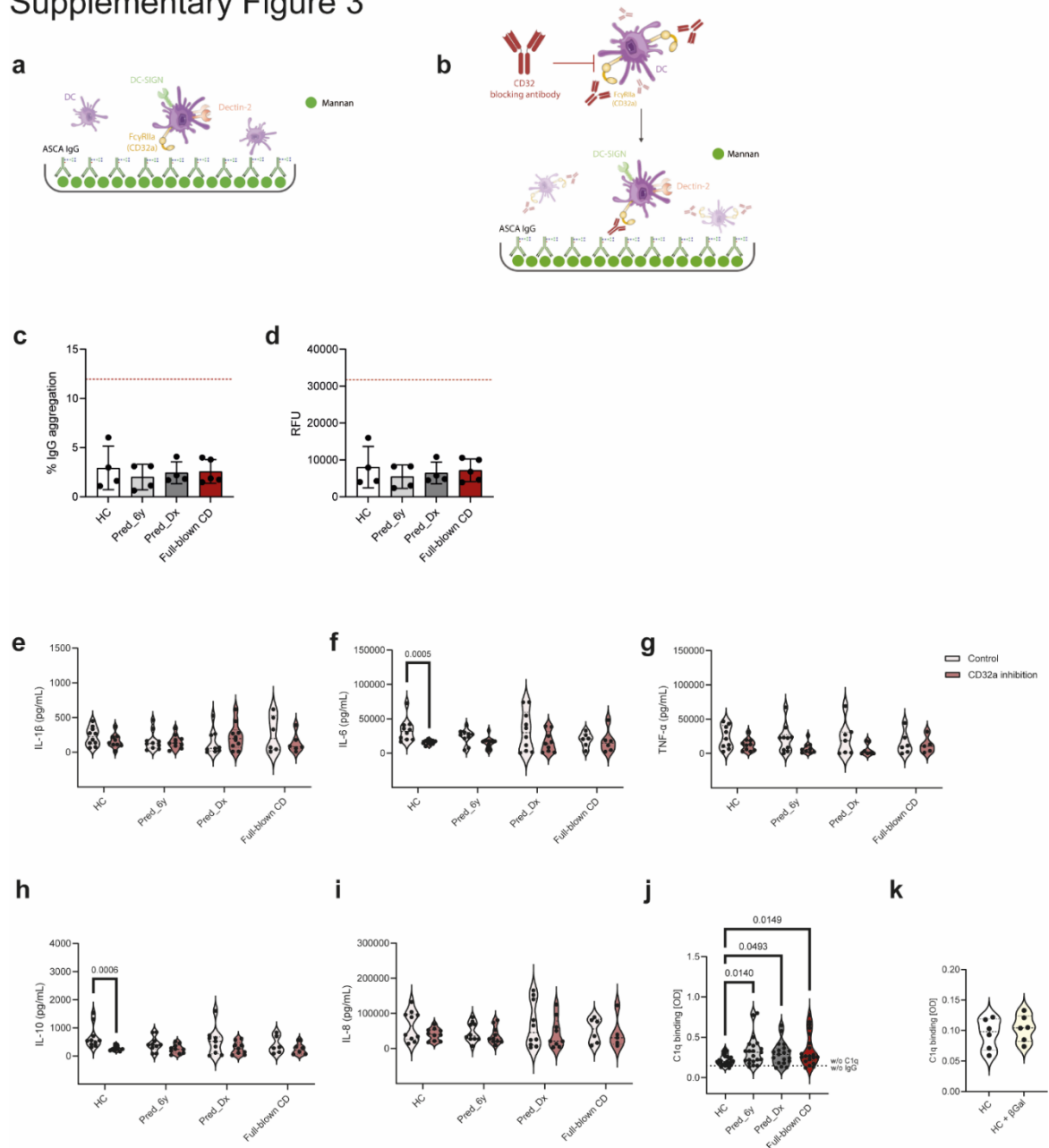
patients, at pre-clinical phase (-6 years; HC, n=7; CD, n=8) and at diagnosis (HC, n=8; CD, n=8); p-values from two-sided t-test are reported. **(d-e)** Quantification of ASCA IgG levels and titers in the serum from HC, preclinical and diagnosed CD, as well as in full-blown CD patients. In (d): HC, n=42; -6y, n=38; -2-4y, n=16; -1-2y, n=19; dx, n=40; full-blown CD, n=20. In (e), HC, n=5; Pred_6y, n=3; Pred_Dx, n=3; full-blown CD, n=3. (b: n=16-42/group; c: n=3-5/group). In (d) and (e), data presented were analyzed comparing each condition with the control (HC), by Kruskal-Wallis test with uncorrected Dunn's test and are shown as mean $\pm$ SD; p-values are shown in the graphs. Each data point represents the data from a single patient/subject (biological replicates).

Supplementary Figure 2



Supplementary Figure 2. Association between glycan traits measured at different time points and ulcerative colitis onset. Total IgGs from serum samples from n=249 UC patients at different time points (preclinical phase: 1-2, 4-6, and 6 years before diagnosis; and at diagnosis) were isolated and analyzed by mass spectrometry. The size of the bubble corresponds to p-value from two-sided t-test from logistic regression (-log10 scale); while the bubble color corresponds to the odds ratio. Only association significant at 10% FDR are reported.

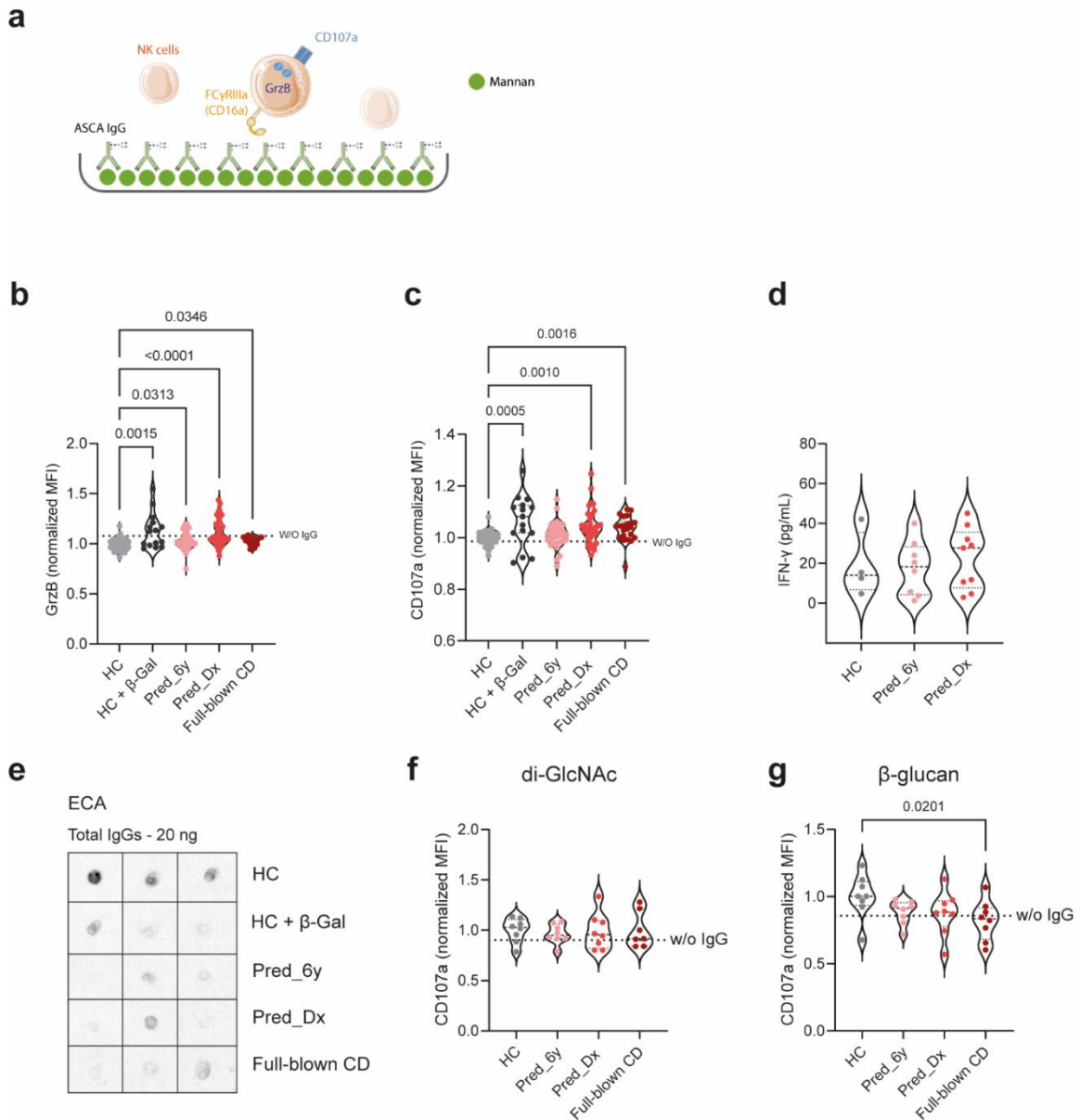
Supplementary Figure 3



Supplementary Figure 3. Characterization of ASCA IgGs and activation properties. **(a)** ASCA were selected from total IgGs through binding to mannan-coated plates and then incubated with dendritic cells (DCs). **(b)** DCs with blocked FcγRIIIa were also cultured with ASCA to evaluate the Fc-γR interaction. **(c,d)** Percentage of protein aggregation and relative fluorescence units (RFU) after IgG purification; red dashes correspond to aggregated form of a control IgG. HC (n=4), Pred_6y (n=4), Pred_Dx (n=4), Full-blown CD (established disease) (n=5). Data are presented as mean ± SD. **(e-i)** Cytokine production of DCs incubated with ASCA from preclinical and CD samples upon blockade of FcγRIIIa (CD32a). For IL-1β (e), control is composed by n=10 HC, n=10 Pred_6y, n=9

Pred_Dx and n=6 full-blown CD; CD32a inhibition condition is composed by n=9 HC, n=10 Pred_6y, n=10 Pred_Dx and n=6 full-blown CD. For IL-6 (f), control is composed by n=10 HC, n=10 Pred_6y, n=10 Pred_Dx and n=6 full-blown CD; CD32a inhibition condition is composed by n=10 HC, n=10 Pred_6y, n=9 Pred_Dx and n=6 full-blown CD. For TNF- α (g), control is composed by n=9 HC, n=10 Pred_6y, n=7 Pred_Dx and n=6 full-blown CD; CD32a inhibition condition is composed by n=10 HC, n=9 Pred_6y, n=7 Pred_Dx and n=5 full-blown CD. For IL-10 (h), control is composed by n=10 HC, n=10 Pred_6y, n=9 Pred_Dx and n=6 full-blown CD; CD32a inhibition condition is composed by n=9 HC, n=10 Pred_6y, n=9 Pred_Dx and n=6 full-blown CD. For IL-8 (i), control is composed by n=10 HC, n=10 Pred_6y, n=10 Pred_Dx and n=6 full-blown CD; CD32a inhibition condition is composed by n=10 HC, n=10 Pred_6y, n=9 Pred_Dx and n=5 full-blown CD. **(j)** C1q binding of ASCA IgGs from HC (n=25), preclinical (n=21) and CD patients (Pred_Dx, n=19; full-blown CD, n=20). **(i)** C1q binding of ASCA IgGs from HC individuals, with (n=6) or without treatment (n=6) with β -galactosidase for galactose ablation, analyzed by two-tailed Mann–Whitney t-test. Data in (c), (d) and (j) were analyzed comparing each condition with the control (HC), by Kruskal-Wallis with uncorrected Dunn's test; data in the remaining graphs were analyzed comparing treatments within each group by Mann–Whitney t-test; p-values are shown in the graphs. Each data point represents the data from a single patient/subject (biological replicates).

Supplementary Figure 4

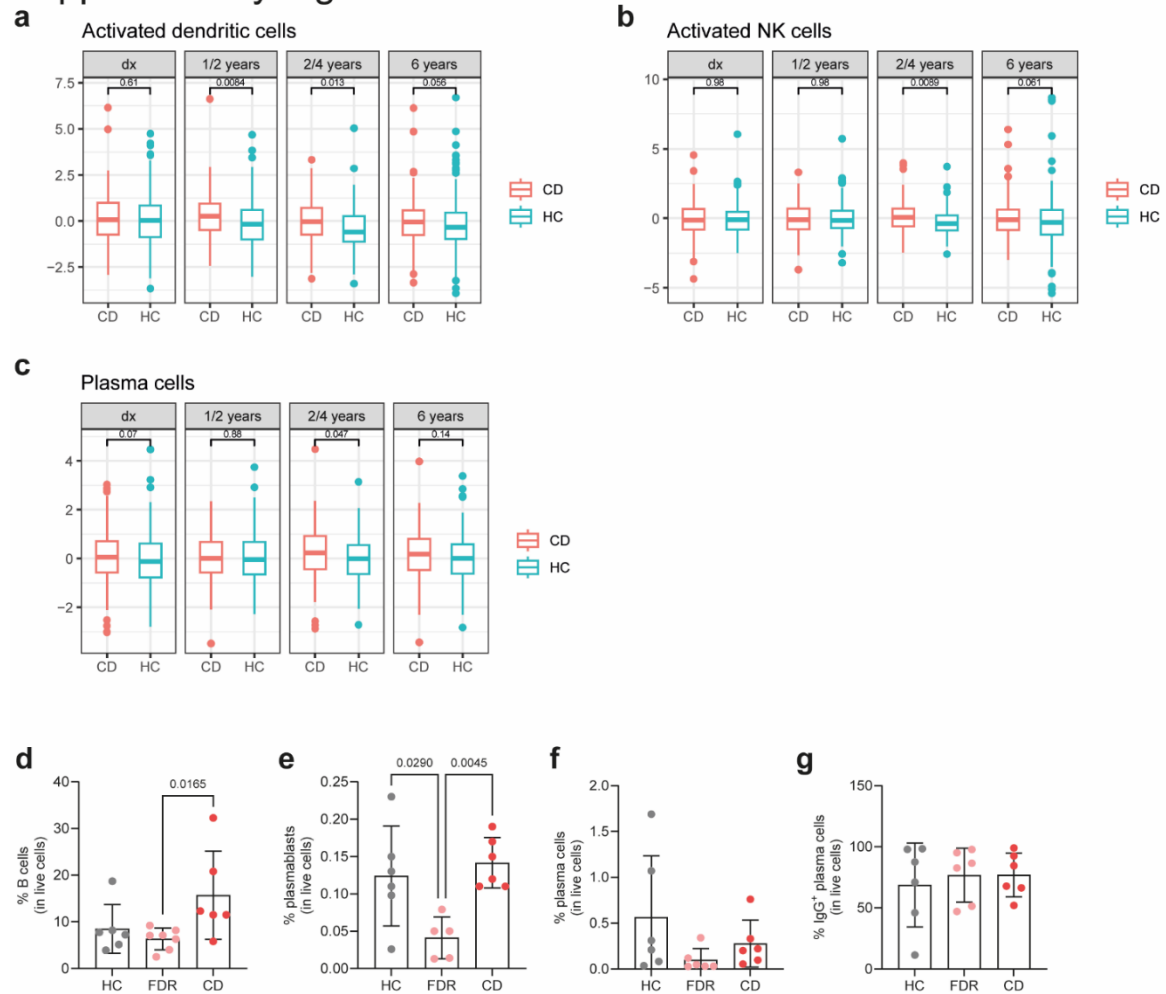


Supplementary Figure 4. NK cell activation and functional characterization upon IgG binding.

(a) ASCA were isolated from total IgGs through binding to mannan-coated plates and then incubated with natural killer (NK) cells. (b) NK cells incubated with ASCA from preclinical (n=42), diagnosed CD (Pred_Dx, n=44; full-blown CD, n=16), or agalactosylated HC (n=15) IgGs displayed increased granzyme B production when compared with HC (n=41). (c) A similar profile was also found for CD107a expression (HC, n=39; HC+βGal, n=15; Pred_6y, n=33; Pred_Dx, n=37; full-blown CD, n=22). (d) Interferon-gamma (IFN-γ) production of NK cells when incubated with ASCA (HC, n=4; Pred_6y, n=8; Pred_Dx, n=9). (e) Representative dot-blot showing that total IgGs from preclinical

and diagnosed CD patients have a decreased galactosylation when compared to HC. The decrease in galactosylation of IgGs from HC treated with β -galactosidase was also confirmed. **(f-g)** CD107a expression on DCs co-cultured with diGlcNAc-specific IgGs (f) or β -glucan-specific IgGs from HC and CD patients (g) (normalized to HC); for (f), HC, n=8; Pred_6y, n=8; Pred_Dx, n=8; full-blown CD, n=7. For (g), HC, n=8; Pred_6y, n=8; Pred_Dx, n=8; full-blown CD, n=8. Data in (b), (c) and (d) were analyzed comparing each condition with the control (HC), by Kruskal-Wallis with uncorrected Dunn's test; data in (f) and (g) were analyzed comparing each condition with the control (HC), by one-way ANOVA with uncorrected Fisher's LSD. Each data point represents the data from a single patient/subject (biological replicates).

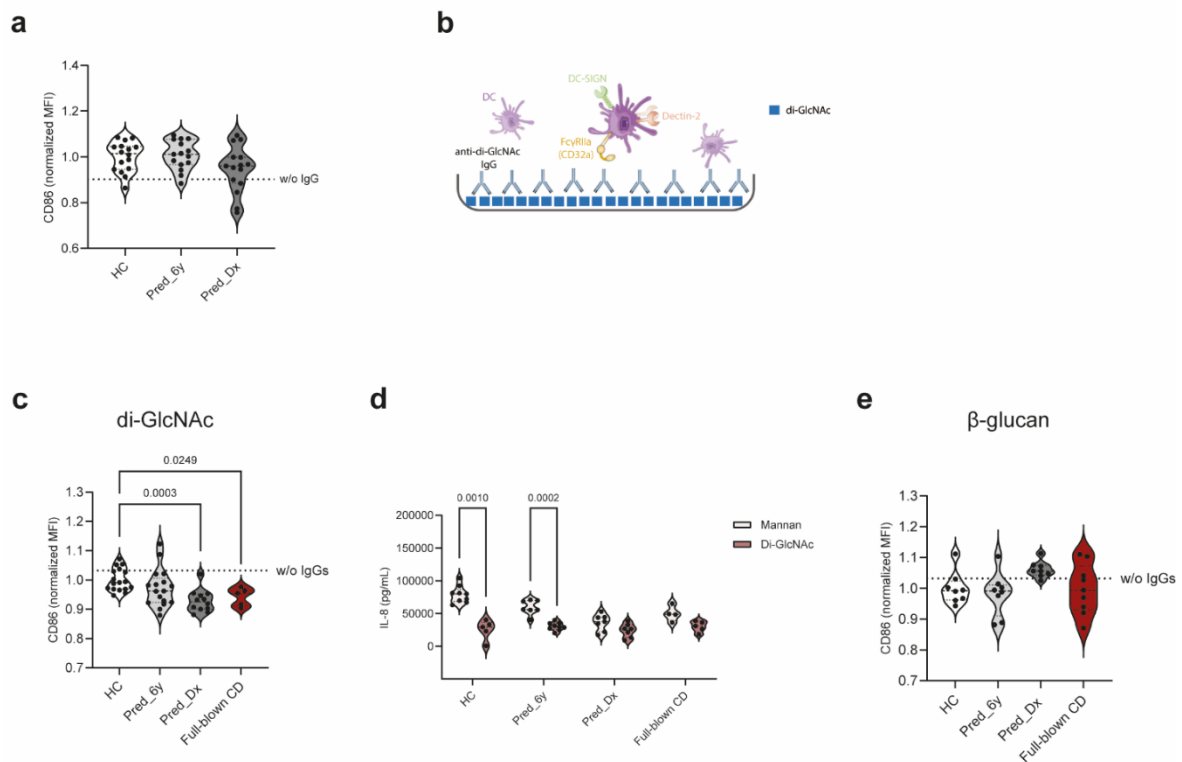
Supplementary Figure 5



Supplementary Figure 5. Activation and functional characterization of immune cells. (a-c)

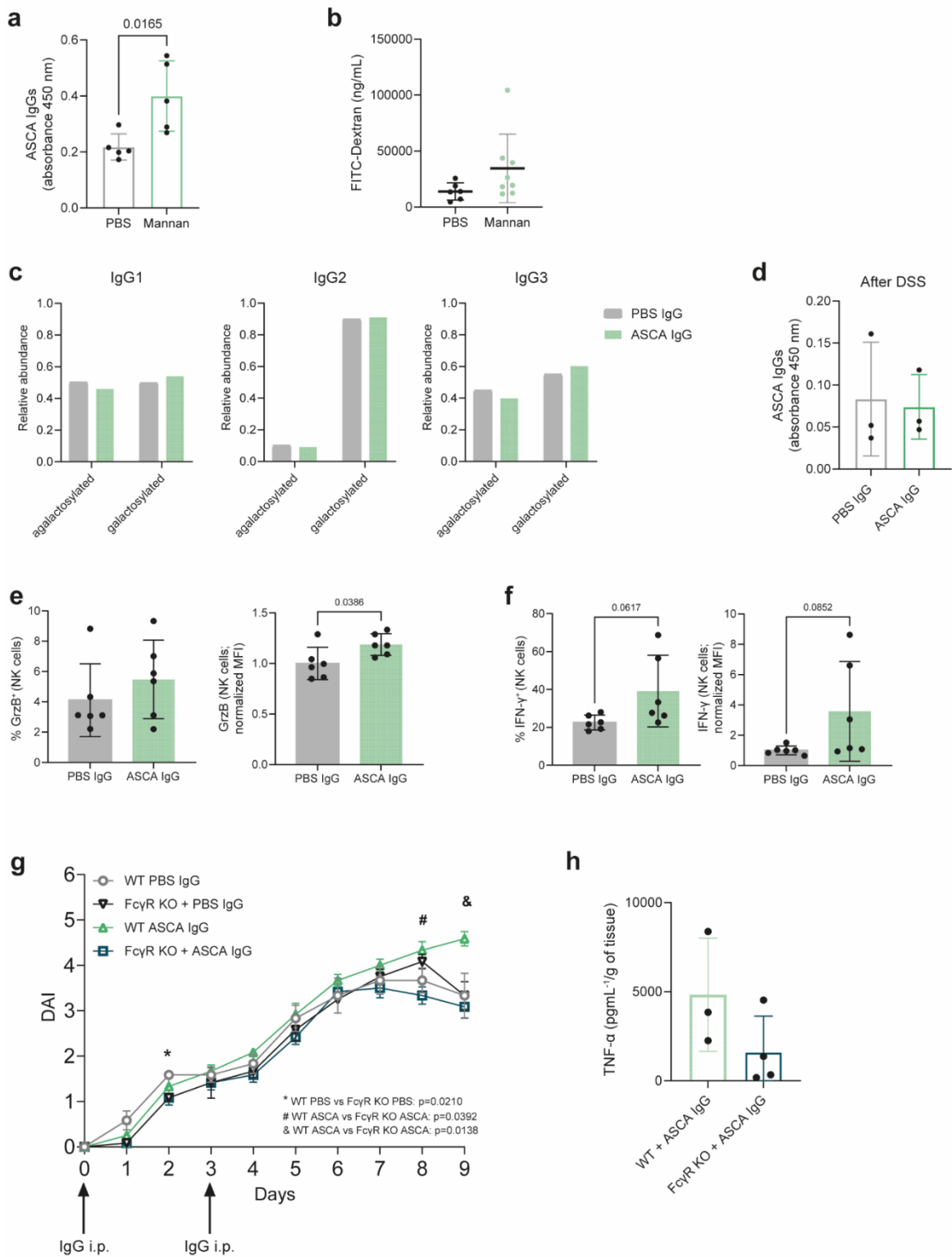
Relative frequency of immune profile of activated dendritic cells, activated NK cells, and plasma cells from Crohn's disease (CD) patients versus healthy controls (HC), according to SomaLogic analysis (n=200 CD, n=200 HC). **(d-g)** PBMCs isolated from blood of healthy controls (HC), first-degree relatives from CD patients (FDR), and CD patients with inaugural disease were analyzed for the frequency of B cells (n=6 HC, n=7 FDR, n=6 CD), plasmablasts (n=6 HC, n=5 FDR, n=6 CD), and plasma cells (total and IgG⁺; n=6 HC, n=5 FDR, n=6 CD). Data were analyzed comparing each group with FDR, by Kruskal-Wallis test with uncorrected Dunn's test, and presented as mean $\pm$ SD. Each data point represents the data from a single patient/subject (biological replicates).

Supplementary Figure 6



Supplementary Figure 6. Dendritic cell activation upon non-ASCA IgG incubation. **(a)** CD86 expression on DCs incubated with unbounded IgGs (not bound to mannan) (HC, n=16; Pred_6y, n=15; Pred_Dx, n=15). Data were analyzed by Kruskal-Wallis test. **(b)** Total IgGs were cultured on di-GlcNAc-coated plates to select anti-di-GlcNAc IgGs, and then incubated with DCs. **(c)** CD86 expression on DCs co-cultured with diGlcNAc-specific IgGs from HC and CD patients (normalized to HC); HC, n=17; Pred_6y, n=17; Pred_Dx, n=12; full-blown CD, n=5. Data were analyzed comparing each condition with the control (HC) by one-way ANOVA with uncorrected Fisher's LSD. **(d)** IL-8 production of DCs when incubated with di-GlcNAc-specific IgGs, compared to ASCA. Mannan condition is composed by n=9 HC, n=7 Pred_6y, n=7 Pred_Dx and n=4 full-blown CD; di-GlcNAc condition is composed by n=5 HC, n=9 Pred_6y, n=7 Pred_Dx and n=5 full-blown CD. Data analyzed comparing treatments within each group by Mann–Whitney t-test. **(e)** CD86 expression on DCs co-cultured with β-glucan-specific IgGs from HC and CD patients (normalized to HC); HC, n=8; Pred_6y, n=8; Pred_Dx, n=8; full-blown CD, n=9. Data were analyzed comparing each condition with the control (HC) by one-way ANOVA with uncorrected Fisher's LSD. Each data point in the figures represents the data from a single patient/subject (biological replicates).

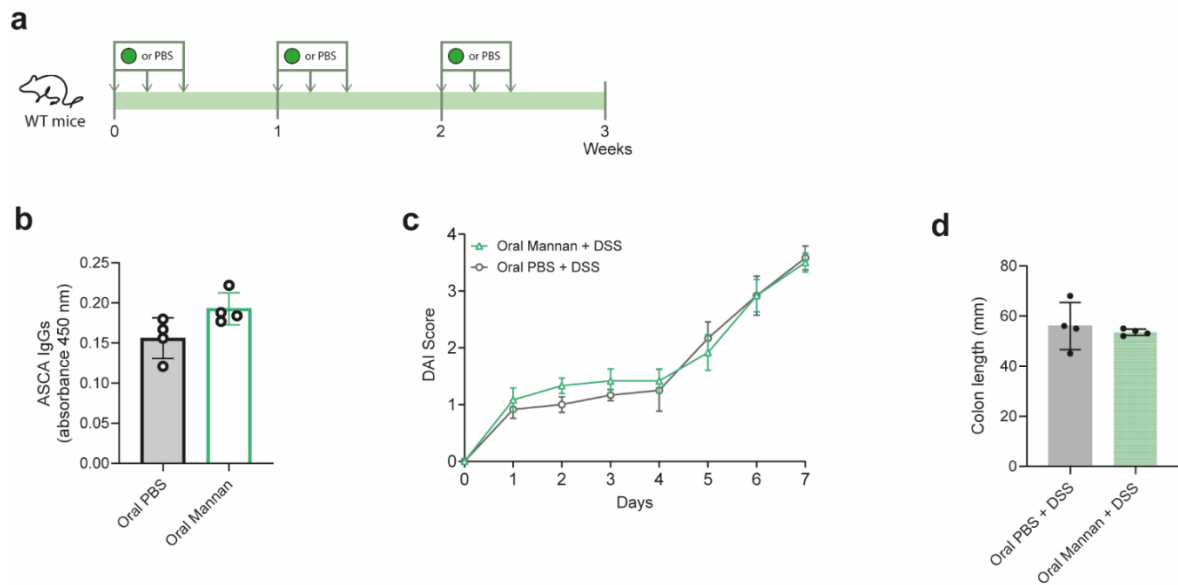
Supplementary Figure 7



Supplementary Figure 7. Characterization of murine ASCA IgGs and impact in intestinal permeability. (a) Mice were inoculated with 12 mg of mannan subcutaneously, every 3 days, during 5 weeks (n=5). Control group received PBS by the same route in similar timepoints (n=5). ASCA

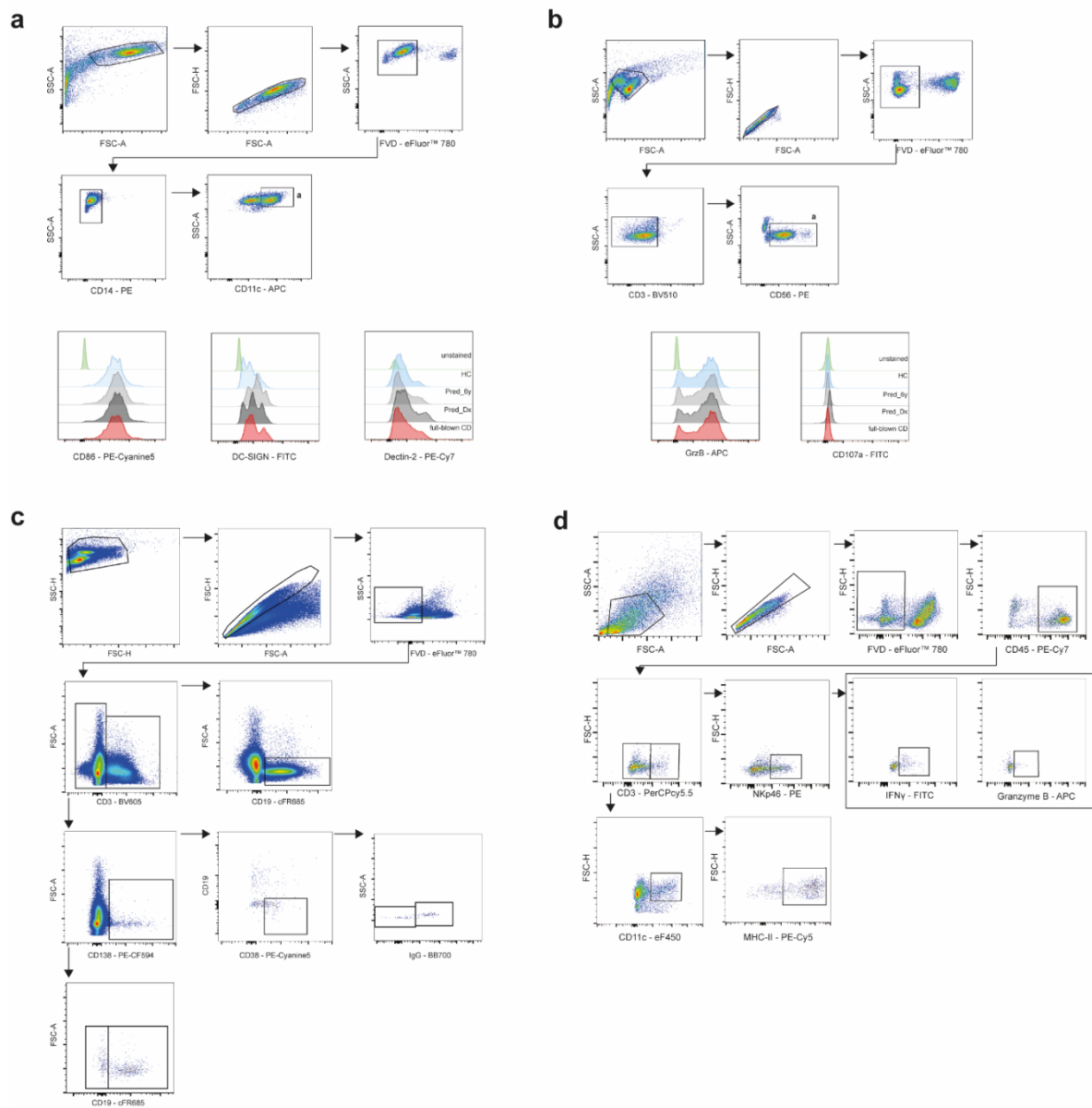
enrichment was assessed after 5-week treatment by ELISA. **(b)** Intestinal permeability was assessed by FITC-dextran assay (PBS, n=6; mannan, n=8). **(c)** Murine ASCA galactosylation levels were assessed for IgG1, IgG2 and IgG3 isoforms in a pool of IgGs collected from several mice immunized with mannan. The galactosylation proportion was also assessed in mice treated with the vehicle (PBS). **(d)** ASCA enrichment in mice treated with ASCA IgGs (n=3) or PBS IgGs (n=3) upon DSS-induced colitis. **(e-f)** Frequency and expression of Granzyme B (e) and IFN- γ (f) in NK cells in the colon of mice immunized with ASCA (n=6) or PBS IgGs (n=6). **(g)** WT and Fc γ R KO mice were inoculated twice with 100 μ g of total IgGs (ASCA-enriched or from PBS-injected mice - control) (n=4/group), while colitis was chemically induced by administration of 2% DSS in drinking water. WT mice inoculated with ASCA IgGs displayed increased susceptibility. * represents significant differences between WT PBS vs Fc γ R KO PBS (day 2 post-DSS); # and & represent significant differences between WT ASCA vs Fc γ R KO ASCA (day 8 and day 9 post-DSS, respectively). **(h)** TNF- α was quantified in supernatants from colonic extracts of WT ASCA IgG (n=3) and Fc γ R KO ASCA IgG (n=4). In (g), data were analyzed by two-way ANOVA with Tukey's multiple comparison test and are presented as mean $\pm$ SEM. Scatter dot plots are presented as mean $\pm$ SD. Frequency of Granzyme B in (e) and data in (h) were analyzed by two-tailed Mann-Whitney test. The remaining were analyzed by two-tailed unpaired t-test. Each data point in the figures represents the data from a single subject (biological replicates).

Supplementary Figure 8



Supplementary Figure 8. Anti-mannan IgGs derived from oral supplementation with mannan do not impact colitis susceptibility. (a) Wild-type (WT) mice were treated with mannan by oral gavage to promote the generation of anti-mannan antibodies (ASCA-like) (n=4). Control mice were treated with PBS (n=4). (b) Anti-mannan IgGs were quantified in the serum and collected from both groups (n=4/group). (c-d) Colitis was chemically induced by administration of 2% DSS in drinking water. No major alterations were found for colitis susceptibility, as shown by a similar DAI (c), and no alterations in colon length (d) (n=4/group). Data in (c) were analyzed by two-way ANOVA with Šídák post-test and are presented as mean $\pm$ SEM. Data in (b) and (c) were analyzed by two-tailed unpaired t-test and are presented as mean $\pm$ SD. Each data point in the figures represents the data from a single subject (biological replicates).

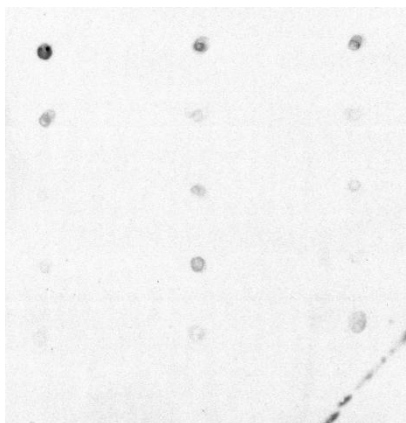
Supplementary Figure 9



Supplementary Figure 9. Gating strategies used in the flow cytometry analyses. (a) For the co-culture of human DCs with ASCA IgGs, cells were gated by CD14^{low}, CD11c⁺ cells, after exclusion of duplets and dead cells. The median fluorescence intensity (MFI) of CD86, DC-SIGN and dectin-2 was assessed in CD11c⁺ cells. **(b)** For the co-culture of human NK cells with ASCA IgGs, cells were identified as CD3⁻, CD56⁺ cells, after exclusion of duplets and dead cells. The median fluorescence intensity (MFI) of granzyme B and CD107a (LAMP-1) was assessed in CD3⁻CD56⁺ cells. **(c)** For the analysis of B cells, plasma cells and plasmablasts, duplets and dead cells were excluded and cell populations were defined as follows: B cells - CD3⁻CD19⁺; plasma cells - CD3⁻CD138⁺CD19⁻CD38⁺; plasmablasts - CD3⁻CD138⁺CD19⁺. **(d)** For the animal model, duplets and dead cells were

excluded, and DCs were identified as CD45+CD3-CD11c+ cells, while NK cells were selected as CD45+CD3-NKp46+ cells. IFN γ and granzyme B were assessed within NK cells, while MHC-II was analysed within DCs.

Figure 4e – unprocessed blot



Chapter 5

The role of glycans in health and disease:
Regulators of the interaction between gut
microbiota and host immune system



Review

The role of glycans in health and disease: Regulators of the interaction between gut microbiota and host immune system

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ARTICLE INFO

Keywords:

Glycans

Host

Human gut microbe

Immune sensing

ABSTRACT

The human gut microbiota is home to a diverse collection of microorganisms that has co-evolved with the host immune system in which host-microbiota interactions are essential to preserve health and homeostasis. Evidence suggests that the perturbation of this symbiotic host-microbiome relationship contributes to the onset of major diseases such as chronic inflammatory diseases including Inflammatory Bowel Disease. The host glycocalyx (repertoire of glycans/sugar-chains at the surface of gut mucosa) constitutes a major biological and physical interface between the intestinal mucosa and microorganisms, as well as with the host immune system. Glycans are an essential niche for microbiota colonization and thus an important modulator of host-microorganism interactions both in homeostasis and in disease. In this review, we discuss the role of gut mucosa glycome as an instrumental pathway that regulates host-microbiome interactions in homeostasis but also in health to inflammation transition. We also discuss the power of mucosa glycosylation remodelling as an attractive preventive and therapeutic strategy to preserve gut homeostasis.

1. Introduction

The human gut is home to a diverse collection of microorganisms – the intestinal microbiota – that has co-evolved with the host immune system, maintaining a symbiotic relationship essential for homeostasis [1]. The gut microbiota comprises a dense and diverse ecosystem that participates in many physiological functions but also in the pathogenesis of many diseases such as chronic inflammation, autoimmune diseases and cancer. Besides bacteria, that dominate the microbiome, other microorganisms, such as fungi, virus, phages, archaea and protists, are also part of the gut microbiome and alterations in these microorganisms are also linked with inflammatory processes.

The microbiota of a healthy individual is not static but rather can be highly dynamic, depending on the geography and environment but also in microbiota species diversity in a concept of “intra-host microbiota evolution”, in which microorganisms, belonging to different species, continuously divide inside the gut of both animals and humans [2]. The microbiota and microbiome-derived metabolites play an instrumental

role in the education of the host immune system in a process that occurs concomitantly with the adaptation of the host immune system with the dynamic evolution of the microbiota. Exposures early in life are instrumental in defining microbiome composition and immune system development with impact in defining the future risk for inflammatory diseases such as Inflammatory Bowel Disease (IBD). This optimal bidirectional interaction between microbiota and host assures a balance in terms of both stimulatory and regulatory signals needed to maintain homeostasis and immunological tolerance. Many factors can contribute to perturb the symbiotic relationship between microbes and the host immune system such as changes in diet, the overuse of antibiotics, exposure to xenobiotics (such as chemicals, microplastics), air pollution, infections, personal lifestyles, stress, and even climate changes [3,4]. These perturbations in host-microbiome relationship are now believed to contribute to the dramatic and rapid increase in chronic inflammatory and autoimmune diseases [5], including IBD [6,7].

The mucosa glycocalyx is the repertoire of glycans/sugar chains that covers the surface of gut mucosa. It is a major biological and physical

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interface between intestinal mucosa and microorganisms and therefore constitutes a fundamental modulator of host-microbe relationships, both in homeostasis and in disease. In fact, the gut mucosa glycome is composed of a variety of different glycans structures (carbohydrates/sugar chains) exposed at the mucosa surface. These host glycans are vital mediators of interactions between microorganisms and the immune system, and thus key targets in a disease context that remain unexplored. The human gut microbiota can degrade and use host glycans as a major source of nutrients and energy and as a niche for colonization. Microbial fermentation transforms glycans into short-chain fatty acids (SCFA) which serve as nutrients for colonocytes and for gut homeostasis. Microbial species can differ in their glycan preferences, based not only on the mucosa glycocalyx composition but also through different carbohydrate-active enzymes – CAZymes. Therefore, the repertoire of glycans structures expressed at the surface of the gut mucosa (gut glycome) constitutes a fundamental biological interface with the gut microbiota, influencing not only the composition but also, consequently, microbial-derived metabolism, including SCFA, bile acids and amino acids that impact homeostasis and inflammation development.

In this review, we focus on the prominent role of glycans, including N-glycans, mucins and O-glycans, that comprise the mucosal surface of the gastrointestinal tract (mucosa glycocalyx). We present its regulatory properties in host-microbiome relationships and discuss how this crosstalk is instrumental for homeostasis, however when perturbed, gives rise to the onset of major chronic inflammatory and autoimmune diseases such as IBD. In addition, we also discuss how several microorganisms devote a significant proportion of their genomes to glycan degradation through a vast arsenal of CAZymes and how this modulates host-microorganism relationship and immunity. Finally, we acknowledge how the gut mucosa glycome constitutes a promising target for reprogramming host-microbiome relationships with potential impact in prevention, treatment and therapeutic outcomes of inflammatory diseases.

2. Immune system sensing of mucosa glycome and microbial glycans

The gastrointestinal system is considered the largest immune organ in the body, embedding complex and multifaceted immune regulatory pathways. The immune system is divided in the innate and adaptive branches, with dendritic cells (DCs), macrophages and innate lymphoid cells (ILCs) as key players of the innate immune system, and B and T cells as central to the adaptive immune responses. The innate immune system is much more ancient than the adaptive, which is only found in jawed vertebrates.

The recognition of pathogen-associated molecular patterns (PAMPs) is mediated by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs). Many of the PAMPs detected for by PRRs are microbially-derived glycans and polysaccharides. These receptors stimulate a pro-inflammatory response in immune cells, characterized by the activation of signal transduction pathways, which are responsible for cytokine production. TLRs are a major line of defence against pathogens, driving cellular responses through the production of signalling molecules (such as MyD88) as well as activation and transcription of important cellular regulators, such as nuclear factor- κ B (NF- κ B), and upregulation of genes encoding for proinflammatory cytokine production [8].

NLRs comprise a myriad of proteins that play a pivotal role in the regulation of the gastrointestinal environment. Major signalling pathways can be activated downstream of NLRs, such as NF- κ B, mitogen-associated protein kinase (MAPK) signalling, and inflammasome activation [9]. Nucleotide-binding oligomerization domain (NOD) proteins, such as NOD1 and NOD2, and PYD-domain-containing proteins, such as NOD-like receptor family pyrin domain (NLRP) 6, are some of the most relevant examples with a role in the regulation of gut immunity [10]. NOD1 and NOD2 can be expressed by epithelial cells in the gut, being

able to identify fragments of peptidoglycans, such as muramyl dipeptide, present in several Gram-positive and Gram-negative bacteria. In turn, NLRP6 plays a regulatory role in host-microbiome interactions by downstream expression of antimicrobial peptides and mucus secretion by goblet cells [11,12]. Indeed, the absence of NLRP6 is described to drastically shape the composition of microbes in the gut and increase the susceptibility to intestinal inflammation [13], demonstrating the pivotal role in the balance of the intestinal milieu.

The immune response in the gut can also be regulated by microbial-derived metabolites. SCFAs, encompassing acetate, propionate, and butyrate, are produced by gut microbiota from the metabolism of fibres and other dietary components, shaping the gut mucosa environment by acting on intestinal epithelial and immune cells [14]. SCFAs can impact host physiology by different processes, such as by inhibition of histone deacetylase (HDAC) and by G-protein coupled receptor signalling (such as via GPR43). Both mechanisms are described as promoting anti-inflammatory responses, ultimately leading to gut homeostasis [15–17]. Butyrate is also known as a major primary substrate of colonic epithelial cell energy metabolism [18]. Accordingly, it is described that IBD patients display a decreased amount of SCFAs in faeces (such as propionate, butyrate and acetate), when compared to healthy individuals [19]. Secondary bile acids are also described to impose tolerogenic immune responses in the gut, via inhibition of Th1 and Th17 immune responses and promoting regulatory T cell development [20, 21]. As such, SCFAs and secondary bile acids are pivotal microbial-derived players that contribute for the regulation of the gut immunity.

Glycans and mucosa glycocalyx plays a major role in host-microbiome relationship and in gut immunity, influencing the bidirectional interactions between host immune response and microbes through glycans structures that can be exposed to both host cells and microorganisms. The gut mucosa surface is decorated with a variety of different glycans structures (the mucosa glycocalyx), forming the so called "self-associated molecular patterns" that store critical biological information [22]. Glycan-binding proteins (GBPs), such as galectins, siglecs, and C-type lectins, expressed or produced by immune cells, scan the biological information encoded by glycans expressed by both microbes and host cells. The recognition of specific carbohydrate structures by GBPs in the gut microenvironment defines the fate, the nature and the outcome of immune responses, resulting in either a pro-inflammatory or anti-inflammatory immune response.

There are several bacterial-derived symbiosis factors such as zwitterionic polysaccharides (ZPSs) identified from human commensals and have been shown to modulate T cells. The most prominent examples being from *Bacteroides fragilis*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*. *Bacteroides fragilis* is a common commensal but given the right conditions this species can become pathogenic. It has the potential to produce eight capsular polysaccharides, where 'A' is the most common (PSA) and one of the most studied in terms of immune system stimulation [23,24]. The structure of PSA is a repeating tetrasaccharide of α 1,3AADGal α 1,4GalNAc[β 1,3 Gal] β 1,3 Gal[4,6pyruvate], where AAD is acetamido-4-amino-2,4,6-trideoxy [25]. ZPSs are detected via the type II major histocompatibility complex (MHCII) in antigen-presenting cells [26]. *Streptococcus pneumoniae* type I ZPS structure is the repeating trisaccharide α 1,3AADGal α 1,4Gal α 1,3 Gal. *Staphylococcus aureus* CP5 and CP8 have the repeating trisaccharides β 1,4ManNAc α 1,4FucNAc β 1,3FucNAc and β 1,3ManNAc α 1,3FucNAc α 1,3FucNAc, respectively [27–29]. Vaccines using bacterially-derived polysaccharide and glycans are of increasing interest [30].

Microbe-derived CAZymes have also been shown to be major factors in modulating the immune system. For example, a study on how a polysaccharide lyase from Group B *Streptococcus* promotes bacterial invasion during pathogenesis during pregnancy, which induces pre-term labour in humans [31]. Notably, this CAZyme was demonstrated to be a crucial virulence factor in pregnant non-human primates and impaired neutrophil function. This demonstrates that CAZymes have evolved not

just to acquire nutrients for an organism, but as mechanisms for colonisation and invasion.

3. The mucosa glycocalyx and secreted glycoproteins

The glycocalyx is a dense gel-like layer composed of glycoproteins and glycolipids that coats the surface of epithelial cells in mucosal tissues. Within this microenvironment, *N*-linked and *O*-linked glycans are carbohydrate structures attached to proteins and lipids (Fig. 1). In addition, glycosaminoglycans (GAGs) and glycolipids are also important components of the glycocalyx. Mucosa glycosylation serves to protect the underlying protein/lipid from degradation and is the major molecular interface between the host and microorganisms. In the gastrointestinal tract, a layer of mucus overlies the glycocalyx that is largely composed of secreted gel-forming mucins. Approximately 80 % of the mucin structure is composed of glycans [32], both *O*-linked glycans (the majority), and *N*-linked glycans. Besides mucin *O*-linked glycans, *N*-glycans are common decorations on secreted proteins in the gastrointestinal tract mucosa. In the colon, the mucosal layer is renewed every couple of hours and a significant proportion of the human gut microbiota have evolved to access this glycoprotein as a nutrient source [33]. In addition, five grams of glycosylated mucosal IgA are secreted into the large intestine daily and microbes have also been shown to access these

as a carbon source [34]. Together, these components play a crucial role in maintaining mucosal barrier integrity, hydration, and immune defence.

3.1. Mucin *O*-glycans structures

Twenty-two mucin genes have been identified in the human genome, denoted MUC 1–22. Common to all these protein sequences are large sections rich in serine or threonine (PTS domains) that *O*-glycans can be added to post-translationally. Some of these are secreted gel-forming mucins while others are membrane-bound, and expression of the different MUC genes is associated with different tissues. For example, MUC2 is the secreted gel-forming mucin expressed in the large intestine and MUC5AC and MUC5B are the secreted gel-forming mucins expressed predominantly in the respiratory tract and stomach [35]. The *O*-glycosylation of the different mucin proteins is highly variable and is influenced by the location and genotype, for example. Despite their complexity, *O*-glycans are only comprised of five different monosaccharides, so heterogeneity is generated through the variety of ways in which these monosaccharides can be linked, as well as sulfation and acetylation. In general, *O*-glycans can be split into three parts: the core, the middle extension, and the capping structures. The core can have eight possible configurations but the common structure to all comprises

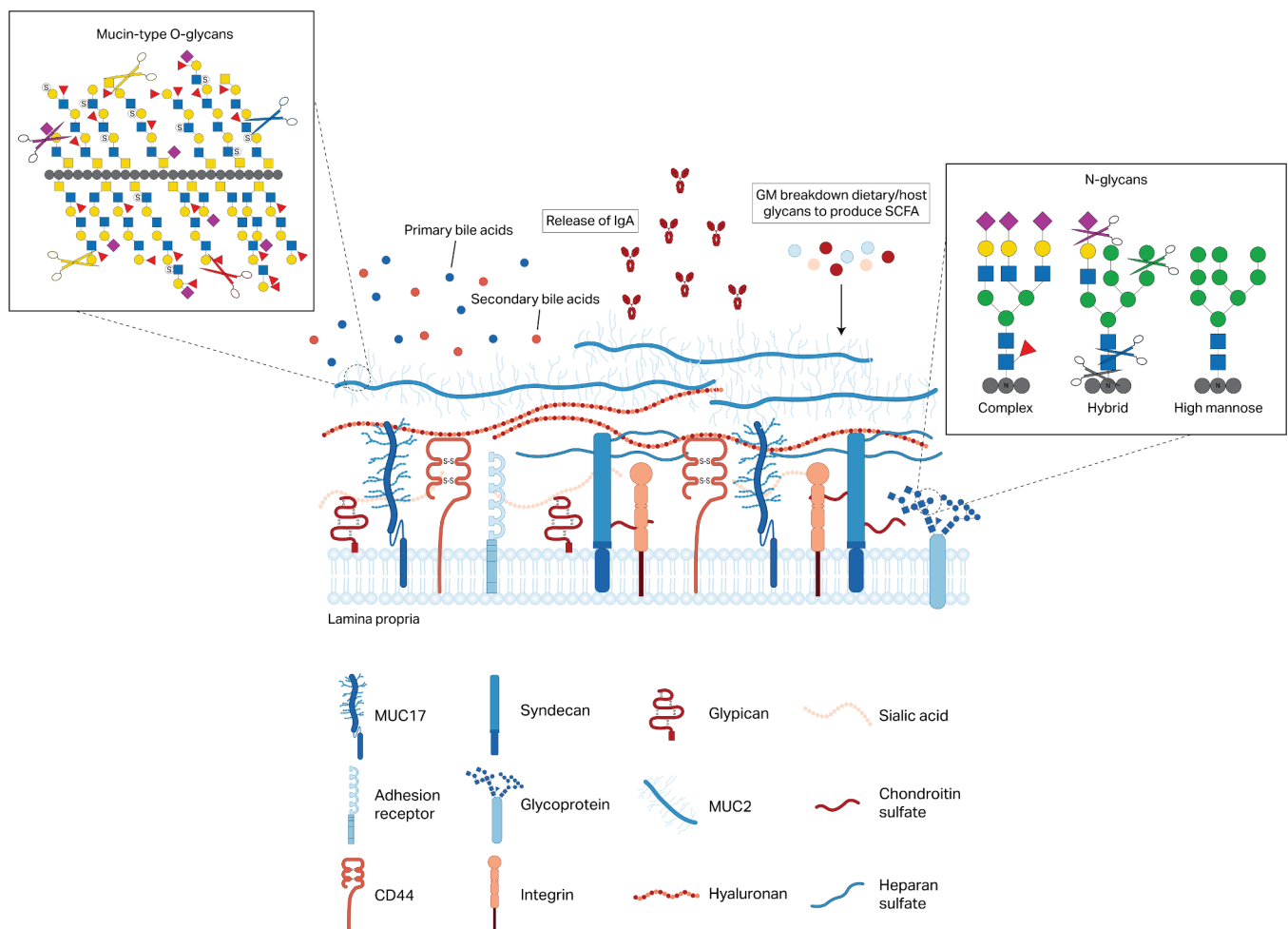


Fig. 1. A model of a healthy colonic human glycocalyx. Glycoproteins and glycolipids are abundant on the outside of colonocytes and together form a protecting coat for these epithelial cells. The types of glycans included in this layer are wide – mucins, *N*-glycans, and glycosaminoglycans. In the colon and above the glycocalyx there is a relatively thick mucin layer comprised mainly of secreted mucins, but these also have *N*-glycan decorations. In this diagram, the thickness of the mucin layer is underplayed to emphasise other aspects of this epithelial surface. Gut microbes produce a huge variety of different CAZymes to tackle host glycans (represented by different coloured scissors). They use the liberated sugars as a carbon source and a by-product of this process are SCFAs. In addition to the glycocalyx and thick mucus layer, the host produces large amounts of IgA that is secreted into the lumen of the colon.

a *N*-Acetylgalactosamine (GalNAc) monosaccharide linked to a serine/threonine. In the large intestine, for example, gel-forming MUC2 predominantly has core 3 and 4 structures. The middle extension for gel-forming secreted mucins is repeating *N*-Acetylglucosamine (GlcNAc) disaccharides linked via β 1,3 bonds. In humans, LacNAcs can also be linked via β 1,6 bonds and are dubbed 'branching' or 'I antigens'. Along the extensions, fucosylation and sulfation are common and generate heterogeneity. Capping the extensions are a variety of α -linked monosaccharides and these include Lewis antigens, blood group structures, and 'stomach epitope' (α 1,4-linked *N*-Acetylglucosamine (GlcNAc)). The composition of *O*-glycan in a mucin will depend on the enzyme expression and this will vary with location, genetics, time, and infection, for example. Greater than 100 different *O*-glycans were identified from MUC2 in one study [36].

In addition to secreted mucins, membrane-bound mucins are vital to the proper functioning of the glycocalyx of mucosal surfaces. In terms of the gastrointestinal tract, MUC17 is highly expressed in the small intestine and MUC1 is expressed in the stomach and respiratory system [35]. These membrane-bound mucins cover the surface of enterocytes and, although this is a relatively understudied area of glycobiology, existing studies indicate an important role in maintaining a barrier between host cells and microbes [37]. Notably, MUC17 specifically has been shown to be protective against enteroinvasive *Escherichia* (*E.*) *coli* [38]. In the stomach, MUC1 has been demonstrated to be shed from the epithelial surface to act as decoys during *Helicobacter pylori* infection [39].

3.2. *N*-glycans structures

N-glycosylation occurs in all eukaryotic cells, playing a crucial role in the processing and function of many proteins [40]. *N*-glycans have a core pentasaccharide structure of a chitobiose at the reducing end and three mannose residues – a core mannose linked via a β 1,4 to the chitobiose and two antenna mannose are linked via a α 1,3 and 6 to this core mannose. Further extensions on these two antenna mannose determine whether the final structure is classified as high-mannose, hybrid or complex *N*-glycan [40]. High-mannose *N*-glycans have a very strict composition in terms of linkages with up to nine mannose residues in total. Mammalian complex *N*-glycans are highly variable in composition, but as a generalisation there are up to two LacNAc disaccharides added to both arms, and these can be fucosylated and/or sialylated. Hybrid *N*-glycans have one arm of each of these types. The prominent expression of *N*-glycan structures in the human intestine places them as ideal molecular interfaces both with microorganisms and with the immune microenvironment. In fact, immune cells are equipped with a variety of *N*-glycan-recognizing receptors that can instruct either a pro-inflammatory or anti-inflammatory immune response [41]. Importantly, alterations in *N*-glycosylation are involved in different diseases including cancer [42] and chronic inflammation, of which the last is further dissected in this review.

3.3. Glycosaminoglycans (GAGs)

Another significant component of the glycocalyx are GAGs, and in humans there are hyaluronic acid, chondroitin sulfate, heparan sulfate, dermatan sulfate and keratan sulfate. These are a consistent nutrient source for gut microbes as they will be mixed in with the mucus as it moves away from the epithelial surface and is sloughed off dead cells. They have been demonstrated to be a high-priority substrate for the archetypal human gut microbe, *Bacteroides thetaiotaomicron*, which indicates their importance as a nutrient source to colonic microbes [43]. The molecular mechanisms of GAG breakdown have been extensively studied in this species, but beyond that, the fate of GAGs in the colon has not been further explored [44,45].

4. Carbohydrate-active enzymes (CAZymes) from human gut microbes

Central to the relationship between the host and its gut microbiota is the use of dietary and host-derived fibres by microbes as a nutrient source. In return, they produce short-chain fatty acids (SCFAs), which serve as nutrient source for colonocytes. SCFAs are currently understood to also have a number of additional functions contributing to host health [46]. The human gut microbiota is a very dense and competitive ecosystem and its exposure to many different dietary fibres throughout evolution has resulted in a vast collection of CAZymes. There are examples of species that are adept at accessing large numbers of different glycans, such as the archetypal *Bacteroides thetaiotaomicron*, while others have very narrow specificity for only a couple of substrates, such as *Akkermansia muciniphila* and its focus on mucin as a nutrient source. CAZymes that perform catabolic roles are split into glycoside hydrolases (GH), polysaccharide lyases (PL), carbohydrate esterases (CE), and auxiliary activity (AA) enzymes. There are also PNGases that are not currently classed as CAZymes and specifically remove *N*-glycans from proteins.

5. Gut glycome variations impact the microbiome composition: natural variations

There are natural variations in the composition of host glycans present in the gut that vary between different species, individuals from the same species, and for an individual over time. These natural variations are largely associated with the capping decorations on host glycans, including in *O*-glycans and *N*-glycans. These capping epitopes are also the sugars that are most exposed and available to the microbes in the gut, such as terminal sialic acid or fucose and several studies have highlighted correlations when looking at these capping epitopes and gut microbial compositions [47]. Furthermore, some pathogens have evolved the ability to bind to some capping structures, so an individual's glycome can influence their susceptibility to different diseases. As a consequence of natural variations resulting of evolution [48], there is the overlapping of some natural glycan epitopes between microorganism and the host, underlying the concept of "glycan mimicry", which puts glycans as essential molecular determinants in self versus non-self discrimination. The ability of microorganisms to engage in glycan mimicry is a way of hijacking host immune response and thus colonising the host [22,49].

Here, we cover examples of the influence of what can be classed as natural variations.

5.1. FUT2 - alpha 1-2 fucosyltransferase

In terms of the hosts' glycome, the different possible fucosylations influence the microbiota composition. In humans, the expression of fucosyltransferase enzymes responsible for producing these decorations vary between individuals and with age [50]. One of the most influential genes in this regard is FUT2, which is responsible for the addition of α 1,2-fucose on Lewis B epitopes and blood groups in secretions, such as mucin and human milk oligosaccharides. Humans who carry the FUT2 gene are known as 'secretors' [50]. There are now a relatively high number of studies that explore the relationship between 'secretors' and their gut microbiota composition. As such, the Dutch Microbiome project included nearly eight thousand people and highlighted a link between FUT2 expression and higher levels of *Bifidobacterium bifidum* [51]. A comparable link was observed in a study on eighty-two healthy Finnish people, with secretor status linked to several *Bifidobacterium* species [52]. Non-secretors are also associated with an increase susceptibility to some infections. For instance, studies with *Fut2*-deficient mice demonstrated a decrease in species diversity [53] and increase in susceptibility to infection with *Salmonella enterica* serovar Typhimurium, *Enterococcus faecalis* and *Citrobacter rodentium* [54,55]. In mice,

the FUT2 phenotype was found to be influential enough to alter the pattern of upregulation of CAZymes in the archetypal *Bacteroides thetaiotaomicron* [53]. Intriguingly, in this study, diet was found to be a stronger influence on microbial composition over the FUT2 status [53]. *Fut2*-deficiency is also associated with increased susceptibility to an array of pathogens [56] and Crohn's disease (CD) [57]. Conversely, secretors may be more susceptible than non-secretors to some infections, for example, *Helicobacter pylori* and norovirus have been shown to bind Lewis B and Blood Group H antigens [58,59]. One study found a 98 % protection for non-secretors against rotavirus gastroenteritis, which was better than the vaccine [60]. These studies highlight how influential a person's genetics can be in their gut microbiota composition and predisposition to disease.

5.2. Blood group carbohydrate antigens

Another example of natural variation of the glycome between individuals are the A and B blood groups, which is a GalNAc or galactose (Gal) linked via an $\alpha 1,3$ bond to the Gal of the H blood group, respectively. The presence of these epitopes varies globally and throughout different ethnicities [61]. These glyco-epitopes are present in many different tissues and secretions, so have the potential to be a contact point for all commensals and pathogens. Enzymes from gut-dwelling bacteria that specifically act on A or B blood group structures have been characterised in detail and represent a direct example of co-evolution between microbe and host. For instance, GH109 and GH110 are examples of families capable of removing the α -GalNAc and α -Gal from blood group structures, respectively [62]. It is currently unknown to what extent these epitopes influence the gut microbiota in general or their influence on predisposition to disease. However, blood groups are points of contact for malaria (*Plasmodium falciparum*), for example, so it is feasible that other bacteria will have similar mechanisms of interactions [63].

5.3. Human milk oligosaccharides and the impact in infant gut microbiota composition

One of the starkest examples of how host glycans influence the gut microbiota are studies monitoring the composition of microbes in infants fed with human milk or formula, and then also the introduction of plant dietary glycans during the later stage of weaning. Studies of the gut microbial composition of infants with different diets have demonstrated a relationship between human milk oligosaccharides and *Bifidobacterium longum subsp. infantis* dominating this ecosystem [64]. Furthermore, the gut microbial composition of formula-fed infants is much more similar to that of a weaning infant [65]. Formula-fed infants also lose out on a whole array of immune-active components compared to those with access to human milk. There is significant evidence to suggest that the infant gut is a markedly different environment compared to adults in terms of host glycans. For instance, those infants capable of expressing A or B blood groups only do this at about 50 % of that of an adult [47] and, in mice, at birth the gastrointestinal tract is heavily sialylated, but switches to fucosylation later on [66]. This may partly influence the types of pathogens that can thrive in infants compared to adults and diet will also be influential. Regarding *N*-glycan fucosylation, the FUT8 human enzyme adds the $\alpha 1,6$ -fucose to the core GlcNAc of complex *N*-glycans, characteristic of mammalian *N*-glycans [67]. In breast milk, many of the bioactive proteins produced are decorated with *N*-glycans, such as the immunoglobulins and lactoferrin, and three-quarters of the *N*-glycans are fucosylated [68]. Studies investigating the link between *N*-glycans in breast milk and the infant gut microbiota are rare, but one highlighted a correlation between $\alpha 1,6$ -fucose on *N*-glycans in the breast milk of Chinese mothers in promoting the growth of *Bifidobacterium* and *Lactobacillus* species in their infants [69]. In infant mice fed by a *Fut8*^{+/+} mother, there was a decrease in *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* species and an increase in *Lachnospiraceae* and *Akkermansia*

species compared to the infants fed by a *Fut8*^{+/+} mother. These different observations hint at the importance of *N*-glycosylation in human milk, although the precise details and molecular mechanisms are yet to be fully understood.

6. Gut glycome variations impact the microbiota composition: disease context

In addition to the natural variations, there have been over a hundred congenital disorders associated with altered glycosylation identified in humans so far [70].

6.1. FUT8 and ulcerative colitis

A complete absence of FUT8 production is a recognised congenital disorder with multiple impacts, including gastrointestinal and respiratory abnormalities [71]. Conversely, increased FUT8 levels in humans is associated with intestinal diseases, such as ulcerative colitis (UC), which is a type of IBD largely affecting the distal colon. In agreement with this, a *Fut8* knockout in mice was found to be protective against colitis [72]. However, the *Fut8* knockout in mice also shows abnormal development of the immune system, indicating this epitope is critical to the proper functioning of the immune system [73,74]. Therefore, there is an optimal expression of FUT8 required to produce a healthy phenotype overall and extremes in expression may predispose to intestinal or immunological diseases.

There is now evidence that the relationship between FUT8 and the development of UC is multifactorial. Firstly, the core fucose decorations produced by FUT8 have important roles in many cellular processes, but in terms of the intestine, a lack of FUT8 renders T cells much less responsive to inflammatory signals in mice [72]. Secondly, FUT8 influences the trafficking of both transmembrane and gel-forming mucins out of the goblet cells. The higher the levels of FUT8, the lower the secretion and therefore the thinner the mucus layer is. In healthy individuals, the levels of FUT8 are relatively low in the distal colon (secretion is high), but UC patients have relatively high levels of FUT8 in this location (secretion is low), resulting in a thinning of the mucus layer and disease. The mucus layer does have FUT8-fucosylations present and, therefore, must have some *N*-glycosylation, although what these are and how abundant is yet to be fully defined. Thirdly, the amount of fucose influences the physical properties of the mucus layer, where an increase in fucosylation leads to more adhesive mucin [75]. Therefore, in FUT8-high disease conditions, the mucins are much stickier and are not regenerated as rapidly as in healthy conditions.

6.2. N-glycosylation branching and IBD

Alterations in *N*-glycan structure have also been linked to intestinal diseases, such as IBD [76]. For instance, a decrease in the expression of MGAT5 glycogene, encoding N-acetylglucosaminyltransferase V (GnT-V enzyme), which is responsible for the addition of the $\beta 1,6$ GlcNAc branched *N*-glycans, is linked to the development of several chronic inflammatory and auto-immune diseases. The deficiency in the expression of complex branched *N*-glycans was shown to impact the functioning of mucosal T cells and plasma IgG in particular [77,78]. In line with this, UC patients were found to exhibit a deficiency in the expression of GnT-V-mediated branched *N*-glycans that was associated with a hyperactive T cell-mediated immune response and disease severity [78]. Accordingly, mice with a GnT-V deficiency have a remarkable susceptibility to severe forms of colitis, which was further repaired by treatment with glycans (GlcNAc), that led to control of intestinal T cell-mediated immune response and suppression of disease progression [79]. Recently, it was also demonstrated that alterations in glycome can even precede disease onset highlighting the relevance of glycans in health to intestinal inflammation transition. This altered glycome observed in the preclinical phase of IBD was correlated with increased

levels of anti-microbial antibodies (anti-*Saccharomyces cerevisiae* antibodies – ASCA), predicting disease development and initiating health to disease transition [80].

Paediatric ulcerative colitis is generally more aggressive than the adult version, although the causes are undefined, and there is a high risk of long-term consequences [81]. In this study, microbiota samples from paediatric UC contained higher levels of atypical *N*-glycans such as those with no antenna (paucimannosidic) and truncated *N*-glycan structures in the gut microbiota [82]. This may be a result of enzyme activities from gut microbes or the *N*-glycans not being assembled properly. This is in line with similar observations found in other autoimmune diseases revealing abnormal exposure of mannose-enriched *N*-glycans in epithelial surface associated with immune activation [83]. Observations like this provide promising suggestions for disease biomarkers [82].

7. How the gut microbiota shapes the host mucosa glycome

Some bacterial species have been described as able to promote epithelial fucosylation, as well as being capable of metabolizing fucose as an energy source or for cellular mechanisms to increase bacterial fitness [84,85]. Goto and colleagues described in a study in mice the interaction of glycans-microbiota-immune response [86]. The authors stated that type 3 innate lymphoid cells (ILC3) impose intestinal epithelial *Fut2* expression and fucosylation. This process requires the presence of IL-22 and lymphotoxin in a commensal bacteria-dependent and -independent manner. Particularly, commensal gut microbiota promoted the production of IL-22 by ILC3, which in turn induced the *FUT2* upregulation on small intestinal epithelial cells (IECs). They have also shown that impairment in intestinal fucosylation increased the susceptibility to infection by *S. Typhimurium* [86]. In another study conducted concurrently on the same topic, it was demonstrated that exposure to Toll-like receptor ligands leads to rapid α 1,2-fucosylation of the IECs in mice. This process is dependent on the sensing of TLR agonists, together with the production of IL-23 by DCs, activation of innate lymphoid cells and *Fut2* expression by IL-22-stimulated IECs [54]. Fucosylated proteins are then released into the lumen and fucose is metabolized by the microbiota, ultimately reducing the expression of bacterial virulence genes and improving the host tolerance to *C. rodentium*. Thus, this study proposed that rapid fucosylation of IECs may act as a protective mechanism by using the resources from the host to uphold vital host-microbial interactions during periods of stress induced by pathogens [54].

8. A changing microbial composition in disease

Alterations in gut microbiota composition and function, termed dysbiosis, is observed in several disease states. In IBD, fluctuations in microbial communities are described, although the precise triggers and mechanisms remain unclear. Several studies have focused on the characterization of the gut microbiota in disease, despite the challenge of reaching a specific signature due to inter-individual diversity. A decrease in representatives of the Firmicutes phylum is probably the clearest alteration associated with IBD [87]. Increase in representatives of the *Enterobacteriaceae* family was also reported [88]. Moreover, several bacterial species are known to be protective, such as *Faecalibacterium* and *Lactobacillus* [89–91].

Alongside mapping gut microbiota composition, the interactions and function of these components are key to understand the impact in health to disease transition. The Human Microbiome Project has characterized the metagenome and metatranscriptome of IBD patients, showing that there is predominant transcription of pathways by individual microbes, which reinforces the idea that the loss of a specific organism in disease may have extensive consequences than those suggested only by its genomic abundance [92]. Another study has pinpointed an increase in facultative anaerobes at the expense of obligate anaerobes, as well as alterations in microbial transcription, metabolism (such as SCFA and

bile acids), and levels of serum antibodies [93]. Furthermore, taxonomic and functional shifts were observed in periods of disease activity.

9. Pesky pathogens - hijacking and camouflage

Prominent human gut microbial species are able to breakdown host glycans for use as a nutrient source [34,94,95] and recent reviews outline this in more detail [96,97]. Concerning the sharing of substrates, one of the best studied is sialic acid, which is a common terminal sugar on mucins and *N*-glycans and therefore highly abundant along the gastrointestinal tract. While some species may produce enzymes to remove sialic acid from glycans, but not use it as a carbon source, others will use sialic acid while lacking the capability to remove it. Examples of this include the sharing between commensals such as 1) different *Bifidobacterium* species and 2) between *A. muciniphila* and *Roseburia* spp. or *Faecalibacterium* spp. [98,99]. The removal and sharing of sialic acid by colonic microbial species has been reviewed extensively recently [97]. Pathogens can hijack this part of the ecosystem, for example, sialic acid from the intestinal tissue microenvironment is critical for the outgrowth and colonization of *E. coli* and contributes to the exacerbation of intestinal inflammation in mice [100].

Interestingly, some species of microbes have also evolved to use sialic acid as a form of camouflage or ‘glycan mimicry’ by incorporating it onto their cell surfaces. A prime example of this glycan mimicry is carried out by the sexually transmitted disease *Neisseria gonorrhoeae*, which puts sialic acid from the host onto its lipooligosaccharides [101].

10. The power of diet

When considering how the human gut microbiota and human glycome exert their influence on each other in both healthy and diseased states, it is difficult to exclude the influence of diet. This section highlights some of the seminal pieces of research that feature the impact diet, host genetics, and gut microbial compositions have on each other. Generalisations have now been defined, but studies have also emphasised how differently our individual gut microbiota can respond to stimuli.

It is now established that a high and variable fibre diet generally promotes a high species diversity (alpha diversity) of the gut microbiota and that high alpha diversity provides greater protection against pathogens [102]. Response of the human gut microbiota composition to changes in diet has been shown to be very rapid (beta diversity), but this is reversible if a change in diet is implemented over a relatively short time period [103]. This is true for both bacterial and fungal populations in the colon [103]. Different plant fibres have been shown to increase particular bacterial genera, but there are often individual outliers whose gut microbiota will not respond in the same way as the majority, and this is likely due to the CAZymes encoded in their microbiome. For instance, when wheat bran arabinoxylans were given to a 21-person cohort there was a general increase in *Bifidobacteria* and this was more pronounced in individuals with no *Prevotella* species in the gut microbiotas [104]. This demonstrates that arabinoxylans are direct substrates for these genera and exemplifies the varied responses an individual’s gut microbiota may have to the same fibre. Another recent study, including over a thousand people from the PREDICT 1 trial (Personalised Responses to Dietary Composition Trial), found connections between specific foods and bacterial species. For instance, between full-fat yogurt and *Bifidobacterium animalis* and *Streptococcus thermophilus*, and coffee and *Lawsonibacter asaccharolyticus* [105]. However, the authors stress that direct relationships are difficult to elucidate confidently and the molecular mechanisms behind these relationships are yet to be described. The authors were able to observe associations between different diets and particular species, for example *Clostridium* species and an animal-based diet [105].

There are insufficient studies examining the variability of the human gut microbiota between different populations around the world to

provide generalisations, however, the studies that do exist indicate that there are still exciting observations to be made. For instance, in one study, the presence of a genus of bacteria that can degrade particularly complex plant carbohydrates was present in children from a rural African village in Burkina Faso, and absent in European children [106]. The different human populations also had different dominant genera, which is thought, at least in part, to be linked to the long-term influence of diet. Another study searched metagenomic data sets for CAZymes that degrade marine plant polysaccharides (agarose, carrageenan, and porphyrin) and found a correlation with human populations that historically consume these fibres [107]. The genes encoding these CAZymes were particularly prevalent in Japanese individuals' gut microbiomes, for example, but completely absent in the Hadza hunter-gathers of Tanzania who would have never eaten marine-derived plants. Analysis at the genomic level elucidated that these CAZymes had been transferred to the genomes of gut microbes in at least four separate events and the origin of these genes were likely bacterial epibionts on algae. This is an example of how diet has altered the CAZyme content of the human gut microbiota [107].

The host's ability to digest its diet has also been shown to influence the gut microbiota composition, so that an identical meal eaten by two people may not have the same characteristics by the time it reaches the large intestine. This has been well characterised for starch and the number of amylase (a CAZyme that humans are capable of producing) genes a person may have [108]. People with a relatively large number of genes will be able to access the glucose from all the soluble starch in their diet, leaving only "resistant starch" to reach the large intestine. People with relatively few amylase genes may not be able to use all the soluble starch, meaning that both types can arrive in the colon. Many gut microbial species can use soluble starch, but very few produce the specialist CAZymes for resistant starch. This therefore means that people with a relatively large number of genes encoding amylase, select specifically for those microbes capable of accessing the glucose in resistant starch when they have starch in their diet.

Mice models are also used in the context of synthetic human gut microbiotas. One study using fourteen different species analysed the effect of high- and low-fibre diets. Both diets produced relatively stable compositions over time, but these had notable differences [109]. Under a low-fibre diet, mucinophilic bacterial species (*A. muciniphila* and *B. caccae*) increased in proportion overall as mucin is the main carbon source available under these conditions. Notably, the colonic mucus layer was much thinner in these mice compared to the high-fibre diet, despite increased transcription of the MUC2 gene [109]. This thinner mucus layer means that the bacteria in the lumen are now closer to the epithelium and there is increased susceptibility to the pathogen *Citrobacter rodentium* [109]. In a subsequent study, the removal from *A. muciniphila* from this model during a low-fibre diet reduced the susceptibility to pathogens, but under high-fibre *A. muciniphila* was protective against infection [110]. These studies strongly emphasise that an effect a particular species exerts is context dependent and it remains to be seen if these observations hold true in humans.

The importance of diet is also exemplified by the relationship between human milk oligosaccharides and *Bifidobacterium longum* subsp. *Infantis* which was recently demonstrated in mice to eclipse any advantage of pioneering species [65]. The microbial species that colonise the infant gut come from the environment they are born into and those microbes that arrive first have an advantage in staying relative to the ones that arrive later – this is called priority effects. When human milk oligosaccharides were provided with *Bifidobacterium longum* subsp. *Infantis* this species could outcompete the pioneering species, but in the absence of human milk oligosaccharides this was no longer the case, and the same effect could not be repeated with other infant-associated *Bifidobacterium* species [65]. These studies highlight the impact diet can have in increasing or reducing the probability of diseases being triggered or progressing.

11. The power of GlycoMedicine: mucosa glycosylation remodelling as a potential remedy

Several potential approaches targeting intestinal glyco-microenvironment can be developed, including the remodelling mucosa glycome through glycan supplementation or by modifying glycosyl-transferases and glycosidases activity in the intestinal microenvironment.

11.1. Gut microbiome effects on immunotherapy response

Increasing evidence has pinpointed the role of gut microbiome in modulating the effectiveness of immunotherapies, including the therapeutic outcomes of immune checkpoint inhibitors [111–114].

In fact, the diversity and composition of the gut microbiota have been positively correlated with anti-PD-1 therapy responses, as observed in patients with melanoma [113]. Among microbiota community, *Faecalibacterium*, *Lachnospiraceae* and *Lactobacillus* (belonging to Firmicutes), *Prevotella* and *Parabacteroides* (belonging to Bacteroidetes), and *Bifidobacterium* (in Actinobacteria) have been associated with increased response to immunotherapy [115,116]. Conversely, certain species belonging to Bacteroidetes and Proteobacteria were associated with decreased immunotherapy response [117] alongside the presence of *Helicobacter pylori*, which demonstrated to reduce efficacy of anti-PD-1 therapy [118]. Taken together, this evidence highlights that gut microbiota variability between individuals is a potential contributor to either disease progression or response to immune checkpoint inhibitor treatment [113].

The mechanisms by which the microbiome affects immunotherapies are diverse, comprising direct influence by microbial signals and metabolites, microbial antigenicity and microbial effect through the modulation of immune response. For example, *F. nucleatum* increases PD-L1 expression due to stimulation of STING signalling pathway, promoting the efficacy of anti-PD-L1 antibodies [119]. Metabolites including inosine, SCFA, trimethylamine N-oxide (TMAO) and indole-like compounds display an important role in modulating immunotherapy response. For instance, enhanced levels of SCFAs in plasma and faeces were shown to improve the immunotherapy responsiveness being associated with favourable survival prognosis [113]. In contrast, SFCAs were also shown to limit anti-CTLA-4 activity, as increased levels of butyrate and propionate in the blood conferred resistance to the antitumour effect of CTLA-4 blockade by limiting the accumulation and activation of memory, CD4⁺ T cells and DCs [120]. Additionally, inosine produced by *A. muciniphila* and *Bifidobacterium pseudolongum* improved the effect of immune checkpoint inhibitors due to DC activation and further Th1 immune response [113,121,122]. Similarly, *Bacteroides fragilis* was shown to enhance the function of CTLA-4 blockade by triggering DC maturation and stimulating IL-12-dependent Th1 cell immune responses [113]. Conversely, as a result of dietary tryptophan metabolism by *Lactobacillus*, indole compounds were shown to interfere with inhibition of tumour-associated macrophage activity promoting tumour growth, and thereby enhancing immunotherapeutic efficacy. TMAO secreted by *Clostridium spp* was also shown to limit immunotherapy efficacy [113, 123].

Commensal microbiota can also affect immunotherapy, stimulating antitumor responses via T cell cross-reactivity with tumour antigens via T helper cells or cytotoxic T lymphocytes (CTLs) [124]. Remarkably, oral supplementation with *A. muciniphila* or *Bifidobacterium* successfully restored unfavourable gut microbiota, thereby enhancing the therapeutic efficacy of anti-PD-L1 therapy. This improvement was achieved by activating CCR9⁺CXCR3⁺CD4⁺ T lymphocytes and tumour-specific CD8⁺ T cell responses, respectively [113]. Adoptive T cell transfer (ACT) and CpG-oligodeoxynucleotide (CpG-ODN) immunotherapies have demonstrated to be also modulated by gut microbiota profile. The perturbation of microbiota composition by antibiotic exposure was found to eliminate the effectiveness of CpG-ODN and ACT therapies in

mice, by impairing key the production of key cytokines such as TNF α and IL-12 [113,125–127].

Notably, it is recognised that gut bacteria may also provide protection against the toxicity caused by immunotherapies. Studies in mouse models have shown that *Bifidobacteria*, *Bacteroides fragilis*, and *Burkholderia cepacia* can reduce intestinal immunopathology during anti-CTLA-4 treatment [122,128]. Conversely, some gut microbes, such as *Lachnospiraceae* spp. and *Streptococcus* spp, have been linked to an increased risk of immune checkpoint inhibitor-induced toxicity, particularly in melanoma patients undergoing anti-PD-1 therapy [113].

11.2. Supplementation with monosaccharides and glycans

The therapeutic properties of specific glycans as supplements have been explored. *Ex vivo* supplementation with *N*-acetylglucosamine (GlcNAc) of mucosal T cells from patients with active UC was demonstrated to increase branched *N*-glycosylation in the T cell receptor (TCR), hampering T cell hyperactivation and inhibiting Th1/Th17 immune response, ultimately leading to a decrease in pro-inflammatory cytokines [79]. As a proof-of-concept, *in vivo* treatment with GlcNAc significantly reduced disease severity and colitis progression by regulating T cell-mediated immune response in the intestinal mucosa [79]. Remarkably, a preliminary investigation into the oral administration of GlcNAc in pediatric IBD unveiled its potential as a potent therapeutic agent. Over half of the children receiving GlcNAc treatment experienced clinical remission along with histological improvement in terms of promoting intestinal lining of the mucosa [129]. In addition, oral gavage of mice with chito-oligosaccharides (COS), short chains of repeating units of *N*-GlcNAc, resulted in decreased colonic inflammation and reduced activation of inflammatory cells with lower levels of pro-inflammatory cytokines (TNF- α and IL-6) [130].

Similarly, diet supplemented with glucosamine demonstrated a positive impact on colitis-associated symptoms. In fact, glucosamine-supplemented diet promoted increased expression of tight junction proteins zonulin (ZO)-1 and occludin, and decreased the expression of pro-inflammatory markers, such as TNF- α and IL-1 β in the colonic mucosa [131]. Tolonen and colleagues demonstrated, in mouse models of intestinal damage, the therapeutic efficacy of synthetic glycans (SGs) which are metabolized by commensals but not by important pathogens [132]. Interestingly, SGs treatment significantly enhanced faecal microbiota associated with anti-inflammatory properties namely *Lachnospiraceae*, *Ruminiclostridium*, and *Parabacteroides* [132], commonly decreased in IBD patients [133]. Also, supplementation with SGs displayed therapeutic effect in colitis induced DSS and in context of *Clostridium difficile* infection by improving survival, impairing weight loss, diarrhoea and decreasing epithelial erosion, mucosal inflammation, or ulceration [132]. In parallel, administration of FOS (fructo-oligosaccharides) enhanced the expression of Muc2 mucin in rats treated with trinitrobenzenesulphonic acid (TNBS).

Human milk oligosaccharides (HMOs) have been shown to display several benefits such as protection against infections agents, enhancement of intestinal barrier functions, and multiple immunomodulatory functions. Fermentation of HMOs displays a bifidogenic effect, enhancing the *Bifidobacterium* genus and *Lactobacillus* bacteria [134]. Moreover, HMOs have been shown to inhibit the adhesion and infection of several intestinal pathogens including enteropathogenic *E. coli* serotype O119, *Vibrio cholerae*, *Salmonella fyris*, *Helicobacter pylori*, *Campylobacter* (*C.*) *jejuni* and *Listeria monocytogenes* [135–139]. Particularly, in both *in vitro* and mouse experiments, fucose-enriched HMOs demonstrated ability to prevent the binding of *C. jejuni* [140,141]. Likewise, increased 2'-fucosyllactose and 6'-sialyllactose in human milk displayed an anti-adhesive effect, impairing *E. coli* attachment to intestinal cells [142]. However, concentrations of HMOs in human milk have also been associated with the risk of mother-to-child HIV transmission [143]. Notably, higher levels of non-3'-sialyllactose HMOs were associated with protection against postnatal HIV transmission [143].

Besides the direct impacts to microbial adhesion and infection, HMOs have also demonstrated immunomodulatory properties. Sialylated HMOs have been shown to reduce neutrophil activation and leukocyte adhesion [144,145]. In addition, treatment with 2'-fucosyllactose was shown to decrease inflammatory cytokine production in the intestinal environment and induced the enhancement of the epithelial barrier [146]. A study using humanized mouse models found that fucosyl- α 1,3-GlcNAc (3FN) and lacto-N-biose (LNB) significantly altered the faecal microbiota. In particular, 3FN was shown to enhance butyrate and acetate levels and modulate inflammatory cytokines [147].

Like HMOs, mucins *O*-glycans have been demonstrated to act as a natural inhibitors of *Candida albicans* pathogenicity, reducing the virulence of this fungal pathogen [148]. Indeed, mucin-associated glycans can mimic host cell surface receptors, blocking pathogen attachment and colonization into mucosal surfaces. MUC2 protects against the adhesion and infection of *S. enterica* and enteropathogenic *E. coli* to colonic mucosa [149,150]. Additionally, administration of sialylated mucin in ST6GALNAC1-deficient mice improved the thickness of the mucus barrier and decreased the colitis-induced inflammation [151]. Mucin glycans *N*-acetyl-D-glucosamine (NAG) and fucose were known to reduce the biofilm formation of pathogenic bacteria associated with IBD such as adhesive-invasive *E. coli* (AIEC) strain LF82 and other pathogenic *E. coli* [152].

Furthermore, it is recognised that prebiotics, that are glycan-based substrates, can offer some beneficial effects in gastrointestinal health in human IBD patients, by remodelling intestinal microbiome composition, improving the intestinal barrier function, and ultimately contributing to a proper interplay between intestinal microbiome and host cells [153–155]. Regarding host-microbiota interaction, terminal sugars of prebiotics can interfere with the attachment of several potential pathogens to glycans expressed on intestinal epithelial barrier, preventing their adherence and colonization [153,154,156]. Typical prebiotics are glycan-based substrates such as galacto-oligosaccharide (GOS) rich in mannan oligosaccharides. GOS was shown to prevent colonization of *C. difficile* *in vitro* [157]. Furthermore, GOS-treated mice infected with *C. rodentium* exhibited a decreased colonic disease score [158]. In addition, glycomacropeptide (GMP), a glycoposphopeptide comprising extensively sialic acid, galactose, and galactosamine residues, has found clinical application in managing phenylketonuria (PKU), obesity and IBD. GMP diet applied to mice substantially reduced their Proteobacteria composition and increased caecal concentrations of SCFA [159]. In infants receiving prebiotic-supplementation formula with GOS and FOS, the levels of *Bifidobacteria* and *Lactobacilli* increased in a dose-dependent manner compared to those receiving non-supplemented formula [160]. Of note, *Lactobacillus* and *Bifidobacterium* have been widely used as probiotics to ameliorate the symptoms of IBD [161,162]. In parallel, administration of FOS in patients with active CD significantly increased the faecal bifidobacteria concentrations while decreased disease activity, with improved IL-10 expression in mucosal DCs [163]. Additionally, in rats with TNBS-induced colitis, prophylactic supplementation with lactulose impaired myeloperoxidase activity, decreased the levels of colonic TNF α and enhanced the concentration of *Lactobacilli* and *Bifidobacteria* in feces [164].

11.3. Adding inhibitors to alter the gut microbial ecosystem

Similar with other glycan alterations, intestinal inflammation is fostered by altered protein-glycan interactions that attracts inflammatory cells and mediates host-microbiota interactions. These interactions can be adjusted through glycome reprogramming or using glycan-recognizing receptor inhibitors as a therapeutic strategy to block inflammatory mechanisms. In this regard, over the last few years several preclinical and clinical studies have been developed to explore the potential therapeutic application of inhibitors of protein-glycan interactions. There are few therapies targeting Siglec currently being

explored in clinical trials [165]. Functionally, Siglecs are fundamental in maintaining immune homeostasis, mediating immune cell killing, pathogen clearance, and cytokine production [166]. In a mouse model, the administration of an anti-Siglec-F antibody resulted in a significant decrease of intestinal inflammation characterized by reduced levels of eosinophilic inflammation and Th2 cytokines, decreased intestinal permeability and IgE levels [167]. Additionally, several Siglec-8 agonists have been tested in clinical trials [165]. Monoclonal antibody Lirentelimab (AK002) demonstrated to successfully deplete eosinophils and inhibit mast cells showing a therapeutic efficacy for eosinophilic gastritis and duodenitis. In line, a Phase II clinical trial (NCT03664960) demonstrated that patients receiving AK002 exhibited improvement of clinical symptoms and reduced gastrointestinal eosinophils [168]. In parallel with Siglecs, Galectins-glycan interactions also play a fundamental role in the regulation of intestinal inflammation [169]. Paclik and colleagues demonstrated that the *in vivo* administration of

Galectin-2 was able to ameliorate acute and chronic murine colitis by reducing pro-inflammatory cytokine production and promoting the apoptosis of mucosal T cells [170]. Similarly, both prophylactic and therapeutic administration of Galectin-1 have been demonstrated to inhibit inflammatory cytokine production and induce apoptosis in mononuclear cells within colon tissue, resulting in a decreased disease severity in mice [171].

Recurrent infection with *Salmonella*, a major source of human foodborne illness, was associated with a progressive intestinal inflammation in mouse models, revealing that persistent infection with this microbe induces an endogenous neuraminidase activity. It was shown that the Neu3 neuraminidase expression in response to *Salmonella* infection led to intestinal alkaline phosphatase (IAP) desialylation and degradation, essential in imposing a proinflammatory environment associated with disease onset, which highlights the potential role of Neu3 inhibitors to prevent and treat colitis [172,173].

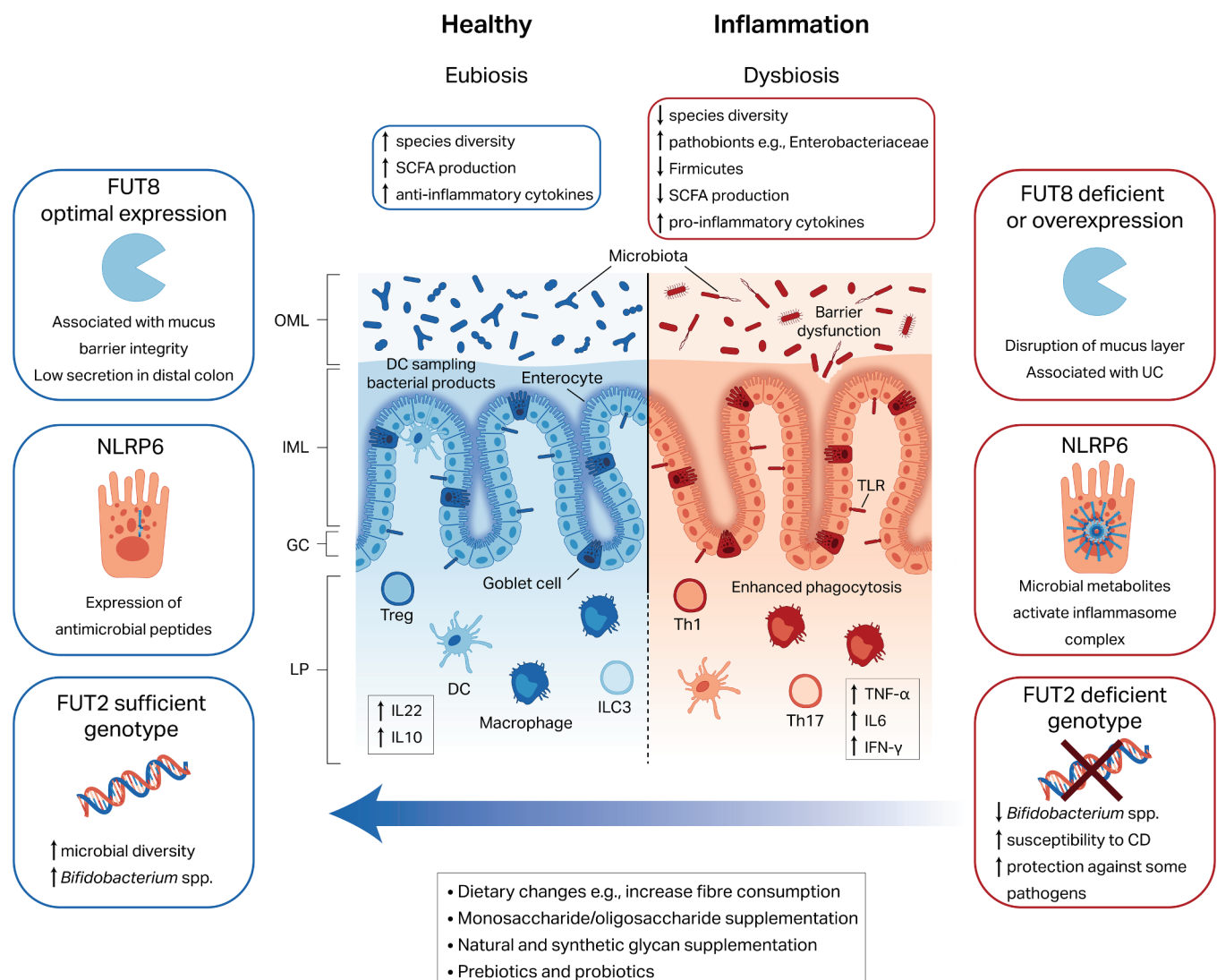


Fig. 2. Alterations in the intestinal environment – transitions from health to inflammation. A summary of relevant differences between healthy and inflamed states, focussing on the microbiota, mucus barrier, genotype contribution and immunology. The healthy state (depicted in blue) is often accompanied by higher diversity of microorganisms, concomitant with higher levels of SCFAs and anti-inflammatory mediators, such as IL-10 and IL-22. It is also characterized by competent expression of *FUT8* and *FUT2* (as examples), as well as *NLRP6*, which in turn positively impact epithelial barrier integrity. On the contrary, an inflammatory setting (depicted in red) is usually characterized by lower diversity of microbial species, promoting a niche for the growth of pathobionts. This environment is also characterized by lower levels of SCFAs and increased levels of proinflammatory cytokines, such as TNF-α, IL-6 and IFN-γ, as well as disruption of mucus layer, promoting a “leaky” intestinal barrier. This inflammatory state may be mitigated through dietary supplementation of fibres, mono/oligosaccharide and glycans, as well as pre/probiotics. OML – outer mucus layer; IML – inner mucus layer; GC – glycocalyx; LP – lamina propria; SCFA – short-chain fatty acids; UC – ulcerative colitis; CD – Crohn’s disease.

Remarkably, oral administration of a sialidase inhibitor and consequent low levels of intestinal $\alpha 2,3$ -linked sialic acid significantly impaired *E. coli* outgrowth and ameliorated colitis severity in mice [174].

Overall, the numerous approaches targeting glycosylation to enhance the homeostasis of the intestinal microenvironment are being considered. By modulating the availability of intestinal glycan content to achieve an optimal microbiota composition, providing protection against pathogens, and enhancing immune function, these strategies have boosted glycomedicine to the forefront of clinical setting as a promising field that may significantly contribute to overall gut health.

12. Conclusions

In this review, we have discussed the biological relevance of gut mucosa glycome as a key player in the relationship between the gut microbiota and the human immune system. This compelling body of evidence has been contributing to our understanding of the cellular and molecular mechanisms underpinning the impact of the mucosa glyco-calyx on host-microbiome relationships associated with homeostasis in healthy states, to inflammation transition and disease onset (Fig. 2). The studies described in this review, and others that could not be included here for brevity, have provided general understandings about how the human glycome and the microbiota interact and how this impacts gut immunity and disease. These studies also highlight how those interactions can change with pressure – environmental triggers, diet or disease, among others. Importantly, this collection of studies has highlighted that response to pressures is not always predictable. Further elucidation of the molecular mechanisms of these interactions in healthy individuals in the future will contribute to understanding different diseases associated with the gut and beyond.

The power of GlycoMedicine through mucosa glycosylation reprogramming holds promise for both prevention and treatment of major chronic intestinal diseases, including IBD and others. High-quality intervention studies are required to further elucidate how glycans *per se* or approaches targeting mucosal glycosylation can be used to promote gut homeostasis, prevent initiation of inflammation, and augment existing therapies to improve efficacy, treatment responses and patient outcomes.

CRedit authorship contribution statement

Leticia Tavares-Gomes: Writing – review & editing, Writing – original draft, Visualization. **Joana Gaifem:** Writing – review & editing, Writing – original draft, Visualization. **Cláudia S. Rodrigues:** Writing – review & editing, Writing – original draft, Visualization. **Cassie R. Bakshani:** Writing – review & editing, Writing – original draft, Visualization. **Salomé S. Pinho:** Writing – review & editing, Writing – original draft, Visualization. **Lucy I. Crouch:** Writing – review & editing, Writing – original draft, Visualization.

Acknowledgements

Co-funded by the European Union (GlycanTrigger, Grant Agreement No: 101093997). Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or the European Research Council Executive Agency. Neither the European Union nor the granting authority can be held responsible for them. Salomé S. Pinho acknowledges funding from European Crohn's and Colitis Organisation (ECCO) Pioneer Award 2021. Salomé S. Pinho also acknowledges the International Organization for the study of Inflammatory Bowel Disease (IOIBD) and the Portuguese Foundation for Science and Technology (FCT; EXPL/MED-ONC/0496/2021). Cláudia Rodrigues thanks FCT for funding (2020.08422.BD). Joana Gaifem acknowledges funding from European Crohn's and Colitis Organisation (ECCO Grant) and FCT (DOI 10.54499/2020.00088.CEECIND/CP1608/

CT0001). The Crouch Lab is funded by The Academy of Medical Sciences (SBF0061175) and the Wellcome Trust/Royal Society Henry Dale Fellowship (224240/Z/21/Z).

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Chapter 6

General discussion

IBD occurs from an inappropriate immune-mediated intestinal response that is driven by genetic susceptibility, intestinal microbiota and environmental factors. The prevalence of IBD has been increasing in the 21st century, becoming a major global disease [1]. Despite recent improvements on clinical management, IBD remains incurable, having a significant impact in patients' quality of life due to its chronic, relapsing and debilitating features [2].

The determination of the mechanisms underlying IBD represents a significant challenge, largely due to the complex interplay of multiple factors. The incomplete understanding of specific triggers often results in a persistent gap in treatment. Furthermore, inadequate early intervention, characterized by delayed diagnosis and prognosis, often result in inappropriate therapies, increasing the risk of more severe IBD and negatively affecting patients' quality of life [3, 4]. Additionally, while new diagnostic methods and therapies have contributed to improved patient outcomes, significant challenges remain. The proportion of patients achieving a complete response remains unfortunately low. The relapsing nature of IBD, as well as the high cost of newly introduced biologics, such as anti-TNF inhibitors, place a significant economic burden on both patients and healthcare systems [5]. Furthermore, the majority of existing IBD treatments are accompanied by considerable adverse side effects, and the optimisation of treatment strategies and accessibility continues to be a major challenge [6]. Addressing these issues through novel clinical interventions and greater affordability of treatments is essential to improving the overall quality of life for people with IBD.

There is growing evidence in IBD of the existence of a preclinical phase marked by immunological changes that precede the onset of symptoms by several years [7, 8]. Understanding this preclinical phase is crucial, since it presents a window of opportunity to detect early immunopathological abnormalities before clinical symptoms appear. Therefore, expanding our knowledge about these early stages could improve disease prediction and uncover novel targets for IBD prevention, potentially allowing earlier interventions that may slow or halt disease progression.

There is an unmet need to improve our understanding about the factors driving gut microbiota dysregulation and the mechanisms that influence host immunity associated with IBD. To address this gap of knowledge and investigate the initial changes contributing to IBD onset, we aimed to explore whether and how changes in the composition of mucosal *N*-glycans affect microbiota composition and function and, in turn, how these shape the regulation of gut immune responses in IBD.

Hence, in the study presented on chapter 3, we revealed that a deficiency in complex branched *N*-glycans at the gut mucosa led to an impaired intestinal environment, characterized by increased gut permeability and dysbiosis, promoting intestinal inflammation. This mucosal *N*-glycan switch was also associated with downregulation of

ILC3-IL-22 immune response, with the transition of ILC3 towards an ILC1 proinflammatory phenotype, with increased proinflammatory cytokine production (e.g TNF α , IL-6, IFN- γ) and decreased *Nod2* expression. Remarkably, we further demonstrated that the glycosylation remodelling at steady state through prophylactic supplementation with GlcNAc of colitis-susceptible glycoengineered mice was able to restore the expression of ILC3-IL-22 module, suppressing inflammation and preventing disease onset.

Among the different hallmarks of IBD, there is growing evidence to suggest that alterations in epithelial barrier properties have a substantial impact on the disruption of mucosal homeostasis [8, 9]. Intestinal barrier dysfunction, characterized by abnormal permeability, has been identified as a central feature of both CD and UC [10, 11]. For example, the severity of UC is linked to the level of damage in the intestinal epithelium [12-14]. Furthermore, alterations in the expression and subcellular distribution of tight junction proteins are commonly observed in IBD patients as mentioned in chapter 1 [15, 16]. The profile reported in IBD patients is consistent with the findings obtained in *Mgat5*^{-/-} mice, which exhibit increased intestinal permeability and disrupted tight junction expression, marked by elevated levels of claudin-1 and claudin-2, along with reduced expression of claudin-3 and claudin-4.

Increasing body of evidence have supported the hypothesis that loss of intestinal barrier functions can occur either abruptly or gradually, leading to chronic inflammatory diseases. In this line, the role of “leaky gut” with increased intestinal permeability has been explored in the pathogenesis of IBD [17, 18]. Interestingly, it was found that abnormal intestinal permeability is a potential biomarker to screen high-risk FDRs, since it was showed that increased intestinal permeability was associated with a significant risk factor for CD onset, preceding diagnosis by as many as 3 years [19]. Notwithstanding, the precise causes contributing to this increased permeability in IBD or in “pre-IBD” patients remain not well defined and further studies are needed to pinpoint the causative underlying mechanisms.

Based on the currently available data, it is known that gut microbiota alterations and epithelial damage impact intestinal permeability allowing the translocation of luminal content to the inner layers of the intestinal barrier, representing a dysfunctional host–microbial interface [9]. Moreover, studies in FDRs of patients with CD have shown that the genetic background has a limited effect on intestinal permeability, suggesting that other factors within the local intestinal environment may play a more dominant role in mediating increased permeability [8]. Along with these findings, healthy FDR of CD patients exhibit altered gut microbiome composition, characterized by reduced bacterial diversity, which is strongly associated impaired barrier function [20]. This is particularly noteworthy, as our findings reveal that in addition to impaired intestinal barrier function, the absence of complex *N*-glycans in *Mgat5*^{-/-} mice also imposes a dysbiotic microbiota composition. In this line, it

would be paramount to assess the microbiota composition in IBD patients or FDR harbouring risk alleles of *MGAT5* gene to determine whether this may contribute to the higher susceptibility to develop a dysbiotic state. It is relevant to note that increased epithelial permeability and altered microbiota were observed under homeostatic conditions. This indicates that the dysfunction of the epithelial barrier and the gut microbiota is already compromised prior to disease onset (mimic by DSS treatment). We can therefore postulate that an altered glycocalyx signature may act as a primary factor in enhancing intestinal permeability and impact intestinal tight junction expression, concomitantly with an altered microbiota. This may subsequently lead to increased antigen penetration, potentially triggering an amplified inflammatory response. It is also relevant to highlight that not only an altered epithelial barrier may influence microbiota dynamics, but the opposite is also valid, i.e., an altered intestinal microbiota may be linked to disruptions in the epithelial barrier. This was demonstrated in previous studies focusing on how alterations in microbiota composition can destabilise epithelial tight junctions [9].

It is possible to infer that the perturbation of the glycocalyx induced in our model, characterized by the pronounced expression of mannosylated glycans, interferes with the steady-state activities and interactions of intestinal microbiota. Indeed, changes in fermentable polysaccharides can lead to a loss of gut microbial species and/or functions, resulting in a significant shift in microbiota community dynamics, namely increased proteolytic activity and reduced colonization resistance favouring a harmful microbiota community [21]. In line with this hypothesis, we have demonstrated, in a cohousing setting, that *Mgat5*^{WT} displays a worse disease course upon sharing the microbiota with *Mgat5*^{-/-} mice. In contrast, the dysbiotic phenotype and elevated susceptibility of *Mgat5*^{-/-} mice to colitis was hampered upon cohousing with *Mgat5*^{WT}. These results suggest that the gut environment underprovided of complex *N*-glycans (in *Mgat5*^{-/-} mice) fostered the modulation of microbiota towards a detrimental microbial profile associated with intestinal inflammation. Indeed, the opposite effects observed after sharing the microbiota between *Mgat5*^{-/-} and *Mgat5*^{WT} mice, suggesting that alterations in the mucosal glycocalyx in *Mgat5*^{-/-} mice may downregulate the homeostatic levels of certain commensal bacteria, whereas allowing the overgrowth of specific pathobionts, leading to higher susceptibility to inflammation. Corroborating the potential role of these preclinical dysbiotic microbiota in IBD, microbial alterations, combined with elevated proteolytic activity were observed before the clinical onset of UC [8].

Considering all these observations, it is plausible that disruption of the glycocalyx composition, as observed in *Mgat5*^{-/-} mice, may result in an imbalance within the microbiota community. Specifically, bacteria belonging to the Firmicutes phylum, which are restricted in their ability to degrade host-derived polysaccharides [22, 23], may be adversely affected.

On the contrary, less prevalent species may undergo proliferation due to a reduction in competition and an increase in nutrient availability. Indeed, significant decreased in *Blautia*, *Ruminococcaceae* NK4A214 group and *Lachnospiraceae* genera (all belonging to Firmicutes phylum) were found in *Mgat5*^{-/-} mice comparing to wild-type. All these microbiota elements are important SCFAs producers and are particularly important in mediating anti-inflammatory responses and contributing to probiotic functions, thereby promoting the maintenance of gut homeostasis [24]. For instance, the *Blautia* genus, a commensal anaerobe within the *Lachnospiraceae* family, is also known for inhibiting pathogenic bacterial colonization through its antibacterial activity [24, 25]. *Blautia* stimulate mucus secretion through propionate-mediated GPR43 activation [26] and upregulate intestinal Treg cells [27]. Interestingly, glycan transport and metabolic machinery of *Blautia*, has been identified as relatively limited, suggesting a reduced capacity to metabolize carbohydrates [24]. This finding aligns with our observation of reduced abundance of these genera in *Mgat5*^{-/-} mice, which is associated with changes in the mucosal glycocalyx. Notwithstanding, *Blautia* species are considered core members of the intestinal microbiota due to the involvement in cross-feeding interactions with other microorganisms. These contribute to the regulation of key metabolic pathways and enhance the stability of the gut microbiome [24]. Comparative analyses of microbial communities between IBD patients and healthy individuals have shown that *Blautia* abundance is significantly reduced in patients with CD [24] and in active UC [28, 29]. Additionally, the *Ruminococcaceae* NK4A214 group is known for producing particularly butyrate, and by its beneficial role for gut health. Underrepresentation of *Ruminococcaceae* NK4A214 group has been observed in IBD patients, especially in active CD [30]. Similarly, *Lachnospiraceae*, another butyrate-producing genus from the *Lachnospiraceae* family, plays a key role in strengthening the intestinal barrier and reducing inflammation. In agreement with our observations, studies have shown a significant reduction in *Lachnospiraceae* abundance in UC patients compared to healthy individuals [31]. Notably, butyrate, is one of the main energy sources for colonocytes and enhances the expression of tight junctions, improving intestinal barrier integrity [32]. These evidence, illustrates the beneficial role of these species on epithelial barrier function through the action of SCFAs.

On the other hand, we have found that families from the Actinobacteriota and Proteobacteria phyla were enriched in *Mgat5*^{-/-} mice. The elevated prevalence of *Coriobacteriaceae* (belonging to the Actinobacteriota phylum) in our model aligns with previously reported findings in both CD and UC [31]. Similarly, increased abundance of *Paracoccus* (belonging to the Proteobacteria phylum) has been demonstrated to be positively correlated with inflammatory infiltrations and intestinal damage in a colitis disease mouse model. Moreover, this bacterial species is found overrepresented in non-responders'

patients to anti-TNF- α therapy [33]. Afterwards, we observed that upon sharing intestinal microbiota with glycan competent mice, *Mgat5*^{-/-} mice exhibited a pronounced enrichment in SCFAs-producing taxa such as *Ruminococcaceae*, which are associated with enhanced disease attenuation [34, 35].

Considering the microbial taxa affected in the absence of *Mgat5* and their association with disease outcome, the major groups of microbes affected are key producers of SCFAs. Indeed, we have found that the dysbiotic phenotype of *Mgat5*^{-/-} mice was accompanied by reduced SCFA levels, alongside a significant decreased expression of SCFA receptors (Gpr43, Gpr109a) and transporters (Mct1, Smct1). This suggests that the SCFA signalling pathways may be compromised in *Mgat5*^{-/-} mice. Given that the function of SCFAs in mediating gut homeostasis is almost entirely dependent on these receptors and transporters [36], it is therefore crucial to comprehend how these pathways are hampered and to determine the potential consequences of such defects for the increased pathology observed in mice lacking *MGAT5*. Two potential mechanisms may explain the downregulation of the SCFA-sensitive GPCRs, GPR109A and GPR43 in this context. Firstly, the expression of GPR43 and GPR109A in the intestinal mucosa is governed by the presence of bacterial metabolites in the colonic lumen, meaning that the levels of these receptors are highly dependent on the availability of their specific substrates [36, 37]. Thus, it can be inferred that the reduced expression of GPR109A (activated by butyrate) and GPR43 (activated by all three SCFAs, butyrate, propionate and acetate) may be a consequence derived of reduced SCFA levels due to the deficiency of certain gut bacteria. Alternatively, it is also important to consider that GPCRs contain sites of *N*-glycosylation [38, 39]. In particular, GP109A displays an *N*-terminal atypical motif hat has been described as essential for its proper surface trafficking and function [38]. Therefore, the absence of complex-branching *N*-glycans may directly interfere with the normal expression of these receptors, disrupting the beneficial signalling of SCFAs. The hampered signalling of GPR109A and GPR43 has been demonstrated to exert a central influence on the pathogenesis of intestinal inflammation. Indeed, mice lacking *Gpr43* and *Gpr109a* exhibited a marked susceptibility to DSS-induced colitis undergoing severe colonic inflammation [40, 41]. Regarding SCFA transporters, similar to GPCRs, the expression and function of MCT1 and SMCT1 are also influenced by the availability of SCFAs [42, 43]. For example, MCT1 is the major transporter for butyrate uptake in IECs. Its expression is upregulated by butyrate through positive feedback via the NF- κ B pathway involving the activation of GPR109A [44].

From a clinical context, alterations in the levels of SCFAs and the respective receptors and transporters have been well documented in the context of IBD. Patients with UC and CD have been observed to exhibit significantly lower levels of SCFAs, specifically a decline in butyrate concentrations, due to a reduction in the number of SCFA-producing bacteria

[36]. Furthermore, studies have demonstrated a significant decrease in the expression of MCT1 and SMCT1 in the colonic tissue from patients diagnosed with IBD [42]. The gene encoding *MCT1*, and its protein expression are found to be downregulated in the inflamed mucosa of patients with UC and CD [45-47]. Furthermore, butyrate uptake is diminished in patients with UC in comparison to healthy individuals [46]. Interestingly, the expression of GPR43 protein was found to be reduced in the ileum of patients with both active and remissive CD compared to healthy individuals. It is particularly relevant that this may indicate the existence of CD-specific factors, rather than the inflammation alone, that play a key role in the downregulation of this SCFA receptor [36]. This aligns with our earlier hypothesis that changes in mucosal glycosylation patterns, coupled with reduced SCFA levels, may exert a substantial influence on the downregulation of the receptors.

The data thus far have revealed that the absence of complex *N*-glycans in *Mgat5*^{-/-} mice leads to a reduction in microbial diversity and abundance, impairing intestinal barrier integrity and increased susceptibility to colitis. These findings suggest that an altered glycan profile may play a central role in triggering imbalances at the host-microbial interface, contributing to the immunopathogenesis of IBD. The disruption of mucosal barrier and subsequent enhance of intestinal permeability probably leads to translocation of luminal contents into the mucosal layer. This may trigger aberrant innate and adaptive immune responses which ultimately give rise to intestinal inflammation, promoting the breakdown of immune tolerance and the development of IBD [18].

It is paramount to uncover the key biologic pathways that drive initial immune dysregulation and subsequently facilitate the transition from health to mucosal damage and clinical manifestation of IBD. As discussed previously in this thesis, ILC3 are key players in intestinal immunity, widely recognised for their critical contribution to intestinal homeostasis, enabling epithelial cell proliferation and tight junction expression via IL-22 production [48]. Moreover, the recruitment and function of ILC3 are tightly regulated by signals from the commensal microbiota, while ILC3 also help to regulate its composition, underscoring the reciprocal relationship between these immune cells and gut microbes [49].

Our findings indicate, for the first time, that the mucosal *N*-glycan switch in *Mgat5*^{-/-} mice results in the downregulation of certain ILC3 subsets, accompanied by impaired IL-22 immune response. This is in line with our findings on the impact in epithelial barrier function, suggesting that the reduced IL-22-producing ILC3 populations may contribute to a compromised mucosal barrier while failing to mediate homeostatic gut immunity. Indeed, several studies have pinpointed that reduced ILC3 levels and the absence of IL-22 in mice results in an inability to control commensal bacteria, thereby exacerbating colitis-associated inflammation [50].

Besides the impaired ILC3/IL-22 immune response, the increase predominance of ILC1, ex-ILC3/ILC1-like cells alongside with higher colonic pro-inflammatory cytokine production in the intestine (TNF- α , IL-6 and IFN- γ from ILC1) may be a plausible explanation for the establishment of low-grade proinflammatory character of *Mgat5*^{-/-} mice. Under stimulation by IL-12 derived by DCs (which was considerably increased in *Mgat5*^{-/-} mice), ILC3 differentiate into IFN- γ -producing ILC1 losing the ability to produce IL-22 [51, 52]. Moreover, ILC1 is known to contribute to the development of excessive inflammation by releasing IFN- γ and TNF- α [53, 54]. Indeed, intestinal inflammation, characterized by higher TNF- α and IFN- γ levels have been demonstrated to reduce tight junction integrity enhancing intestinal permeability [9], besides the perturbation of SCFA transport, via downregulation of both MCT1 and SMCT1 [42, 45].

Additionally, given the central role of ILC3 in maintaining symbiosis between host and gut microbiota, the detection of reduced ILC3, together with increased transdifferentiation into ILC1, is likely to reflect an immune response designed to control some form of altered microbial niche, such as microbiota dysregulation, infection and/or invasion. In corroboration with this hypothesis, the abundance of ILC1 in the fetal intestinal tract is markedly low. Furthermore, in the absence of symbionts, the plasticity of ILC3s to ILC1s transformation is non-existent [49], thereby indicating that the presence and differentiation of ILC3 in ILC1 is dependent on commensal microorganisms. Together, these findings support the idea that the increase in ILC3-to-ILC1 transition observed in *Mgat5*^{-/-} mice may be strongly influenced by the dysbiotic gut microbiota. Studies in intestinal biopsies of IBD patients have demonstrated decreased frequencies of ILC3 in contrast to higher levels of ILC1 [55-57], which are aligned with our observations. Similarly, recent discoveries in the inflamed mucosa of CD patients have also highlighted the increased abundance of ILC1 accompanied by elevated levels of IFN- γ , alongside with enhanced differentiation of NCR⁺ILC3 into ILC1 [58]. Collectively, this evidence supports the detrimental role of the transition from ILC3 to ILC1-like phenotype in the progression of inflammation and disease development.

A remarkable finding in this study concerns to the inflammatory signals observed in *Mgat5*^{-/-} mice, specifically the elevated levels of IL-6, TNF- α , and impaired *Nod2* expression. Elevated levels of IL-6 and pro-inflammatory biomarkers such as CRP have been significantly associated with pre-IBD stages, showing a positive correlation with increased IBD risk [59]. Loss-of-function or null mutations in *NOD2* compromise the antimicrobial functions of IECs, leading to shifts in gut microbiota composition and promoting colitis inflammation [60]. Moreover, a bidirectional crosstalk between ILC3-IL-22 and NOD2 was described since NOD2 is able to enhance IL-22 production by ILC3, and IL-22 in turn, supports *NOD2* expression [61]. Therefore, our observations in *Mgat5*^{-/-} mice may indicate

that this signalling axis is disrupted, damaging effective microbial sensing. Nevertheless, further studies to dissect the contribution of *Nod2* in this setting are needed.

Within these integrated findings, we believe that alterations in branching *N*-glycans may act as an early triggering event, eliciting inflammatory biomarkers (such as IL-6) and immune "defects" (e.g. dysfunctional epithelial barrier, ex-ILC3, impaired IL-22 signalling and reduced *Nod2*). This comprises changes in host–microbiome interactions underlying the breakdown of the intestinal immune defence system, which potentially set the stage for preclinical inflammation. Therefore, shifts in mucosal glycosylation might increase the risk of developing IBD, representing a key mechanism in the transition from health to intestinal inflammation. The identification of such mechanisms has the potential to lead to therapeutic breakthroughs aimed at modulating initial pathogenic processes for early intervention in IBD, which underscores the novelty of these work. Indeed, our further objective is to explore the potential translational opportunity of remodelling of defective glycocalyx profile as new clinical tool acting in the “window of opportunity”, allowing disease interception or prevention.

To further support the potential applicability of glycan remodelling from a clinical perspective, our previous findings have indicated that patients with UC patients with specific SNPs in the *MGAT5* are genetically predisposed to express lower expression of *MGAT5* on both colonic and circulating T cells [62]. In the same line, the present study further corroborates the clinical evidence that IBD patients exhibit diminished *MGAT5* expression in IECs, concomitant with a reduction in ILC3 abundance. These observations provide compelling support for the premise that targeting glycocalyx modifications may represent a promising avenue for early intervention in IBD, particularly in patients with deficiencies in *MGAT5* expression or in the synthesis of complex *N*-glycans. Moreover, glycan-based therapies are an important field of interest for targeting gut microenvironment and for the clinical management of IBD, as discussed in more detail in chapter 5 (section 11. *The power of GlycoMedicine: mucosa glycosylation remodelling as a potential remedy*) and appendix 1 (section 3.6.2 *Glycan-based therapies*). Indeed, several evidence have been illustrated the immunomodulatory properties of glycans as a therapeutic approach, for instance in paediatric IBD patients, where oral GlcNAc administration resulted in a clear endoscopic improvement and clinical remission [63]. Additionally, dietary supplementation with glucosamine, synthetic glycans, chito-oligosaccharides (COS) or FOS (fructo-oligosaccharides) has shown positive effects in modulating the gut microbiota, mitigating intestinal inflammation and decreasing IBD activity.

Remarkably, glycosylation remodelling through prophylactic GlcNAc supplementation in colitis-susceptible mice yielded promising results, effectively preventing the onset of disease. Glycan administration at steady state conditions was found to be successful in

reversing low-grade inflammation in mice, mainly characterized by the restoration of protective ILC3-IL-22 signalling, the prevention of ILC3 differentiation into ILC1, the repair of *NOD2* expression and the recovery of colonic SCFA levels.

Although prophylactic GlcNAc supplementation did not fully resolve all intestinal barrier defects, the observed increase in claudin-4 expression and the recovery of *Smct1* expression levels indicate an important improvement in mucosal epithelial function, suggesting the promotion of a more robust barrier. MCT1 is expressed on both IEC and immune cell membranes, whereas SMCT1 is restricted to the apical membrane of IECs [36]. Taking this into consideration, the upregulation of *Smct1* expression following glycan supplementation may reflect the improvement of epithelial barrier integrity. Still, this rise in *Smct1* expression points towards a broader increase in SCFAs, including propionate and acetate, beyond butyrate alone, since MCT1, which selectively transports butyrate, did not demonstrate alterations upon glycan supplementation. Nevertheless, contrary to MCT1 [64, 65], SMCT1 is glycosylated [66] which may provide a further insight into its more favourable response to supplementation. This improvement may be attributed to the direct effects of GlcNAc or indirectly through enhanced overall SCFAs production.

Thus, GlcNAc prophylactic supplementation shows promise as a possible prophylactic strategy in IBD, that may embrace different mechanisms of action to tackle distinct mechanisms of disease. As such, the prophylactic GlcNAc intervention could be indeed beneficial for the prevention of intestinal inflammation in IBD, with putative candidates the FDR of IBD patients, individuals carrying genetic variants in glycogenes, or IBD patients in remission phase (as an approach to prevent flares and disease recurrence).

Altogether, our compelling evidence set the ground, for the first time, for the potential of branched *N*-glycans as a primary force that shape gut microbiota composition/function and host immune response, supporting the instrumental role of glycans in the early IBD immunopathogenesis. Remarkably, these findings corroborate previous evidence revealing changes in the glycosylation pathways as one of top markers altered in the pre-clinical phase of CD development [67]. In parallel, through a prospective cohort study, it was also found that altered gut microbiome composition is associated with future development of CD in healthy first-degree relatives (FDRs), indicating as well that this preclinical gut microbiome signature might be a potential contributor in the pathogenesis of the disease [68]. In the same line, it was detected an altered host immune reactivity to microbial antigens, including ASCA, in healthy FDRs of patients with CD. Increased antibody response toward commensal microbes were linked with risk of future onset of CD, independently of biomarkers of gut barrier dysfunction, subclinical inflammation, and CD-genetic susceptibility [69]. Indeed, previous studies have already highlighted the impact of the IgG glycosylation in the context of IBD [62, 70]. Therefore, in order to push forward the

knowledge on the role of complex *N*-glycans in the pre-clinical phase, we wanted to explore the glycome signature in the antigen-specific IgGs production observed in IBD.

In the study of Chapter 4, we found that preclinical serum samples, collected up to 6 years before IBD diagnosis, showed a distinctive glycosylation pattern on circulating IgG antibodies. This pattern, characterized by reduced galactosylation of the IgG2 Fc domain (H3N4F1 glycoform), remained stable until disease onset. This IgG2 glycosylation trait was linked to higher levels of ASCA, suggesting a specific glycome-ASCA connection that emerges years before CD diagnosis. We also uncovered for the first time the pathological role of this specific ASCA IgG glycoform at a mechanistic level, showing that this IgG promotes inflammation through FcγR-dependent activation of immune cells, such as DCs and NK cells, thereby triggering inflammatory signalling. The inflammatory effects of ASCA IgG were shown to be highly dependent on mannose recognition and agalactosylation signature. Importantly, *in vivo* validation demonstrated that transfer of these antibodies to mice increased susceptibility to intestinal inflammation, an effect that is absent in FcγR-deficient mice.

The detection of this distinctive glycosylation signature in IgG may serve as an early serum biomarker for the prediction of CD, providing a crucial opportunity to intervene in the initial stages of the disease, potentially reversing or preventing its progression. Moreover, the functional impact of this glycome-ASCA hub on pro-inflammatory pathways highlights its potential as a promising therapeutic target for preventing the onset of disease.

One of the key challenges moving forward will be determining how ASCA IgG antibodies with distinctive glycosylation patterns emerge, exploring the factors driving the development of the unique ASCA IgG glycome. Concerning the glycosylation character of IgGs, it is known that the differences underlying antibody glycosylation may reflect differential B cell priming [71]. As a classical example, different vaccinations have demonstrated to induce distinct patterns of antigen-specific IgG glycosylation, suggesting that antibody glycosylation can be influenced by the delivery of specific inflammatory stimuli during B-cell priming [72]. Indeed, IgG Fc glycosylation and B cells activity is highly dynamic and determined by several microenvironmental factors and signals. Such factors can alter the enzyme activity (e.g. of glycosyltransferase), can foster the expansion of B cell populations producing antibodies with specific glycosylation profiles or, for instance, may interfere with glycosylation efficiency of individual B-cells [73, 74].

Inflammatory signals are pointed out as one of the key potential factors regulating the expression of glycosylation traits of antibodies [75]. Mechanistically, previous evidence has shown that antigens, through the presence of pro-inflammatory co-stimulation by APCs, induce pro-inflammatory T-cell immunity and pro-inflammatory splenic plasma cells with low levels of α2,6-sialyltransferase expression, leading to the production of low-galactosylated

and low-sialylated IgG antibodies [76, 77]. In contrast, under tolerogenic conditions protein antigens induce Treg cells and plasma cells expressing high levels of $\alpha 2,6$ -sialyltransferase and producing mainly galactosylated and sialylated IgG antibodies [76, 78]. In the same context, microenvironmental factors stimulating the innate immune system (e.g. CpG oligodeoxynucleotide, that promotes TLR9 signalling) or T-cell derived cytokines, such as interleukin-21, were showed to modulate IgG1 Fc-glycosylation [73]. The enrichment of agalactosylated antibodies across diverse autoimmune diseases with different ethiology is also an important indicator that elevated agalactosylated antibodies may occur in the context of chronic inflammation [71, 79, 80]. This may be linked with the induction of distinctive B cell response in a context of persistently high antigen loads [71].

Therefore, although it is currently impossible to determine the exact sequence of events in the development of agalactosylated ASCA IgG, some hypotheses can be raised. One of them may be associated with gut dysbiosis and fungal overgrowth during the preclinical phase, driven by an unknown trigger. For instance, several studies have shown that *C. albicans*, which is covered by mannan residues, is abundant in patients with IBD, namely in CD patients [81-84]. Alternatively, it is possible that a cross-reactive response may occur in relation to alterations of mucosal glycocalyx patterns induced by external microenvironmental influences. Specifically, an altered intestinal epithelial glycocalyx, characterized by an increased exposure of mannose residues (as observed in *Mgat5*^{-/-} in the study of chapter 3), may develop a glycan mimicry with fungal elements, initiating intestinal inflammation and contributing to disease development. Indeed, ASCA are classically recognized as anti-glycan antibodies against the mannan residues of the cell wall of *Saccharomyces cerevisiae* yeast. In this line, we propose that ASCA recognition is not only limited to the mannan from *S. cerevisiae* but may also extend to other mannan and mannose residues, for example, those found in *C. albicans* or in the epithelial glycocalyx. This leads to the hypothesis that a future revision of the canonical ASCA definition may be necessary. Similarly, some of the several antibodies detected in serum of CD patients are glycan-specific antibodies, namely ALCA, ACCA, AMCA, anti-laminarin (anti-L) and anti-chitin (anti-C), besides ASCA [85]. It is noteworthy that the gut microbiota perturbation and the cross-reactive interaction with mucosal glycans may not be entirely independent, but rather interconnected. Consequently, it is possible that these disturbances may enhance the likelihood of an anti-microbial immune response. These may involve both innate (initiated e.g. by DCs and ILCs) and adaptive inflammatory immunity cascades, which may subsequently elicit plasma cells to produce increased agalactosylated antibodies. As a feedback positive loop, the continuous production of agalactosylation ASCA may potentially trigger subclinical gut inflammation which in turn feeds production of agalactosylation ASCA, ultimately contributing to the development of IBD. Although we only focused on

bacterial composition in the context of *Mgat5*^{-/-} dysbiotic scenario, several evidence highlight the role of ILC3 in regulating intestinal mycobiome, particularly in preventing translocation and systemic inflammation caused by *C. albicans* [49, 86]. IL-22, in turn, not only supports microbial diversity, but also protects against fungal colonization by *C. albicans*, safeguarding the mucosa from inflammation [87]. Consistently, mice lacking ILC3 exhibit elevated IgG levels and an increased CD4⁺ T cell response against commensal microbiota [88].

Bearing in mind this evidence, we may raise the hypothesis that, along with the alterations in the mucosal glycocalyx, in *Mgat5*^{-/-} we could be facing a mechanism that may underpin the development of low-grade inflammatory response associated with the production of antibodies against intrinsic components of the gut, such as ASCA. In this line, the altered mucosal glycosylation profile associated with the disruption of the microbiome may perhaps destabilise the homeostatic immune response through the ILC3 response. This subsequently results in the inability to regulate the symbiosis with the commensal microbiota, including the mycobiome. Together with a compromised epithelial barrier, this may lead to the translocation of contents from the lumen to the lamina propria, which subsequently initiates a low-grade inflammatory response with the activation of adaptive and humoral immunity pathways, with the production of antibodies against microorganisms, such as ASCA. Although we lack specific data on IgG levels in *Mgat5*^{-/-} mice, it is reasonable to conjecture that the altered mechanisms observed in these mice may provide insight into the increased ASCA levels in a preclinical inflammatory context, that needs to be explored in further experiments.

Collectively, the findings of this thesis shed light the critical function of complex *N*-glycans in maintaining the homeostasis of intestinal environment and emphasize how their pathological loss, is a primary driver in the IBD development. We elucidated the role of an altered glycocalyx in the microbial and immunological dysregulation underlying pathogenic processes occurring during the early stage of the disease, with the goal of disclose new targets and developing potential preventive interventions. Moreover, we extended our study by uncovering the glycosignature throughout the preclinical phase, exposing a promising “glyco-tool” that could potentially predict the risk of IBD development. Altogether, this knowledge opens new avenues for innovative strategies for IBD clinical management and prevention.

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Chapter 7

Concluding remarks & Future Perspectives

The compelling body of evidence presented in this thesis represents a significant contribution in the ongoing effort to understand the mechanisms underlying IBD pathogenesis, envisioning to predict and prevent the disease development, through the lens of glycobiology.

Herein, we demonstrated the critical role of complex branched *N*-glycans in regulating gut microbial composition and ILC3/IL-22 immunity, underscoring the ability of these glycans to maintain intestinal environment in a homeostatic and protective state. We further highlighted the immunomodulatory effect of GlcNAc supplementation in effectively tackle subclinical inflammation, mitigating the transition from health to intestinal disease. These findings illustrate the relevance of glycans as double-edge interface in the modulation of the microbiome and the gut regulatory immunity, paving the way for novel therapeutic interventions using glycan supplementation. Given the critical impact of *N*-glycosylation changes on the regulation of the host-microbial dynamics and its association with subclinical inflammation, we sought to deepen our understanding about the glycans as early drivers of inflammation by investigating the glycosylation signatures in the pre-clinical phase of IBD.

We have disclosed an altered glycosylation pattern in circulating anti-microbial antibodies, specifically ASCA IgGs, that arises in the preclinical phase of CD. We further discovered that this glycome-ASCA hub also contributes to the mounting of an inflammatory immune response through DCs and NK activation. The identification of the glycome-ASCA inflammatory pathway years before clinical diagnosis unveils its potential use as a predictive serum biomarker for stratifying individuals with the risk of CD onset, as well as a possible target for disease prevention. Although the role and functional implications of ASCA IgG remain elusive, we herein provide pioneering insights into the potential mechanisms by which these antibodies may contribute to the pathogenesis of CD.

The current understanding of the factors underlying preclinical immune dysregulation is of significant interest, particularly those related to gut microbiota-host regulatory pathways, which represent promising therapeutic targets for intestinal diseases. In this context, the work presented in this thesis adds a crucial piece to the puzzle by identifying two novel glycan-driven mechanisms involved in the crosstalk between the microbiome and intestinal immune response that contribute to intestinal inflammation. These findings can pave the way for strategies aimed at fine-tuning immune function and microbiota composition for more effective clinical prevention. Glycan-derived therapies, in this regard, seem to hold great potential for IBD prevention.

Nevertheless, this thesis leaves several outstanding questions that require further elucidation.

What are the precise mechanisms by which an epithelial glycome enriched in high mannose glycans promotes the growth of certain gut microbes? How does the intestinal microbiota reprogram its glycan-degrading machinery in response to alterations in the glycocalyx?

It is fundamental to understand the temporal sequence of preclinical events triggered by the “glycan switch” associated with the disease. Future research must focus on deepening our understanding of the connection between the two early glycan-driven events pinpointed in this thesis. In the context of altered glycosylation patterns, is dysregulated immunity the primary driver, leading to changes in gut permeability and a dysbiotic environment? Alternatively, could shifts in microbiota composition be the primary factor, with the subsequent gut barrier dysfunction responsible for instructing an aberrant immune response? It is thus essential to clarify the role of other immune cell populations in the interplay with ILCs in the susceptibility to colitis observed in *Mgat5*^{-/-} mice. For instance, how do DCs, primed by ASCA IgGs, interact with ILCs? In addition, it is also paramount to further dissect the precise mechanisms by which GlcNAc exerts its regulatory effects.

Our findings highlight the need for prospective studies that investigate the dynamics of glycan pathways in high-risk populations, which may offer important knowledge into IBD pathogenesis. Furthermore, future research should evaluate microbiota composition and glycoprotein expression in human patients to establish correlations between glycosylation, microbiome and immunity, potentially serving as biomarkers in the clinics.

Providing the answers to these questions will be crucial for fully characterizing the impact of glycosylation on the development of intestinal inflammation. This knowledge will not only boost our understanding of IBD immunopathology but may also be applied to other intestinal inflammatory disorders.

Appendix 1

3 *N*-glycans as fundamental immune-checkpoints at the frontiers of inflammation, autoimmunity and cancer

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DOI: 10.1039/b000000x [DO NOT ALTER/DELETE THIS TEXT]

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Glycans cover the surface of essentially all cells, giving rise to the so-called glycocalyx. Glycans, and particularly *N*-glycans are key players in defining the fate and the nature of the immune response, participating in both pro-inflammatory and anti-inflammatory pathways occurring in inflammation, autoimmunity and cancer. Glycans and glycan-binding proteins (GBPs) are instrumental molecules that integrate the immunological landscape in homeostasis, being involved in how the host immune system perceives the self and non-self. Perturbations in these immune regulatory circuits created by glycans and GBPs may result in the loss of immune tolerance associated with autoimmunity and inflammatory conditions, but also in malignant transformation. This chapter will discuss the regulatory role played by *N*-glycans as fundamental immune-checkpoints in the interplay between the immune cells and their surrounding microenvironment associated with homeostasis and disease. Furthermore, we will also explore how *N*-glycans can act as promising diagnostic and prognostic

biomarkers in disease, as well as appealing targets for new therapeutic approaches in inflammation, autoimmunity and cancer.

3.1 Introduction

Glycans, also recognized as monosaccharides or polysaccharides, are carbohydrate structures that can be found intracellularly and extracellularly at the cell surface of essentially all cells ^{1,2}. Protein glycosylation occurs in the Golgi and Endoplasmic reticulum and comprises two major classes of glycoproteins according to the glycan-protein linkage: *N*-glycans (sugar molecules attached to an asparagine residue of proteins via the nitrogen atom) and *O*-glycans (sugar moieties attached via the oxygen atom to a serine or threonine residue of a nascent protein). This posttranslational modification is fundamental for numerous cellular and molecular biological processes, such as cell interaction and adhesion, cell-fate decisions, cell migration, regulation of receptor activation and signal transduction, embryonic development, distinction between “self” and “non-self” and regulation of the immune system ^{3–5}. Furthermore, glycans can act as ligands for glycan-binding proteins (GBPs), including galectins (Gal), C-type lectins (CTLs), and selectins, highlighting their role as key components in immune-regulatory circuits and in the balance between immune activation and immune suppression. Therefore, changes in glycans expression have remarkable implications in the homeostatic environment associated with the pathogenesis of major diseases ⁶. These changes can be triggered by different factors, including altered glycan biosynthesis, changes in the expression of substrate proteins and altered activity of enzymes essential in the glycosylation pathway ⁷. Several studies reported that alterations in glycosylation are associated with immune dysregulation in

different pathological conditions, including inflammatory bowel disease (IBD)
 8–11, autoimmune disorders^{12–14}, infection^{15–17} and cancer^{3,18–21}. In this chapter,
 we will focus on the *N*-glycosylation pathway, in both immune and epithelial
 cells, addressing how *N*-glycans regulate immune response in health and in
 5 disease.

3.2 Glycans as Immune regulatory circuits in Homeostasis

Glycobiology has a long history initiated with the discovery of the nature of
 carbohydrate structures through research in the human ABO(H) and Lewis blood
 10 groups^{22,23}. Over the last decades, glycobiology has gained increased
 importance in the immunology field, with several evidence demonstrating the
 pivotal role of glycans and GBPs in regulating both molecular and cellular
 mechanisms that govern the immune homeostasis, including innate and adaptive
 immune responses^{5,24–27}.

15 Glycans play crucial roles in homeostasis of both T and B cells, even from the
 early stages of their development, as reviewed by us²⁸. Highlighting some of
 the findings regarding *N*-glycosylation, complex *N*-glycans are paramount for T
 cell receptor (TCR) selection, by inhibiting death by neglect and by enhancing
 TCR sensitivity to low-affinity peptide major histocompatibility complex
 20 (MHC)²⁹. Recently, our group disclosed stage-specific glycome compositions
 during T cell development of both human and mouse thymocytes. The
 expression of complex *N*-glycans was shown to impact β -selection and TCR
 repertoire diversity. Moreover, deletion of complex *N*-glycans favored $\gamma\delta$ T cell
 development and impaired regulatory T cell (Tregs) expansion³⁰.

Besides T cell development, *N*-glycans also modulate T cell activity and function ³¹. Barbosa and colleagues demonstrated that blockage of *N*-linked glycans on human leukocyte antigen (HLA) class I (homolog of MHC-I in mice) led to protein intracellular denaturation, resulting in decreased levels of the protein on cell surface ³². Moreover, antigen-presenting cells (APCs) lacking complex *N*-glycans were shown to exhibit decreased MHC-II binding and impaired antigen presentation to T cells ³³. Glycans are also important in TCR signaling. In fact, Demetriou and colleagues showed that β 1,6- *N*-Acetylglucosamine (GlcNAc)-branched *N*-glycans catalyzed by *N*-acetylglucosaminyltransferase-V (GnT-V) (encoded by the *MGAT5* gene) regulate the TCR signaling and activation. These carbohydrates can be further elongated to form poly-*N*-acetylglucosamine (poly-LacNAc) structures, acting as ligands for Gal-3, creating a molecular lattice, that modulates TCR signaling and impacts the threshold for T cell activation ³⁴. Similarly, mice lacking the β 1,3-*N*-acetylglucosaminyltransferase (B3GnT2), resulting in less poly-LacNAc structures on *N*-glycans, displayed an increased TCR clustering, as well as an hyperactivation and proliferation of both T and B cells ³⁵. Glycans represent an important layer of information for the recognition by environmental players, namely by GBPs, which are crucial players in the immune-mediated response ³⁶. Particularly in the context of T cell activity and function, Gal-1, Gal-3 and Gal-9 present immunosuppressive effects on T cells. In particular, Gal-3 presents a dual effect on T cells, depending if its expression is intra- or extracellular. The former leads to blockage of apoptosis, while the latter, through binding to glycosylated receptors on the T cell surface, leads to inhibition of activated T cells ^{37–40}. Gal-1 and Gal-3 bind preferentially to poly-LacNAc

motifs and this assembly leads to the inhibition of transendothelial migration and induces apoptosis of T cells ⁴¹.

Glycosylation of the surface protein CD28 can also regulate T cell adhesion and stimulation. Mutations in the *N*-glycosylation sites of CD28 resulted in increased binding to CD80 ⁴². Additionally, a reduction in branched *N*-glycans led to a decrease in the surface expression and retention of CD25 and hampered IL-2 binding, ultimately preventing Treg activation and promoting T helper (Th) 17 differentiation ⁴³. In fact, cytokines regulate T cell *N*-glycome composition. In resting T cells, IL-2 signaling decreased branching by upregulating *Mgat1* and downregulating *Mgat5*. Since *Mgat1*-encoded enzyme (GnT-I) has higher affinity to UDP-GlcNAc, the limitation of this substrate resulted in reduced branching. Conversely, in activated T cells, TCR signaling elevated *Mgat5* expression resulting in the promotion of branching *N*-glycosylation ^{44,45}. The opposite effects in resting versus activated T cells suggest a mechanism of regulating activation and self-tolerance mediated by complex *N*-glycans. Opposite effects on T cell dynamics are described for other glycosylation alterations. For instance, core fucosylation, which is catalyzed by α 1,6-fucosyltransferase (Fut8) enzyme (encoded by *FUT8* gene), was also shown to impact T cell activation ^{46,47}. On the other hand, sialylated antigens induced immune-suppression by inhibiting effector T cell proliferation ^{48,49}.

B cell development is also highly regulated by *N*-glycans ²⁸, despite remaining so far underexplored. As example, core fucosylation impacts pre-B cell development by interfering with pre-B cell receptor (BCR) assembly affecting B cell intracellular signaling and proliferation ⁵⁰. Additionally, B cell-specific deletion of *Mgat1* gene resulted in decreased surface expression of the BCR,

hampering positive selection. Consistently, the deficiency in complex *N*-glycans on B cells was reported to be essential for the survival of T2 transitional peripheral B cells, while not affecting mature B cells. These evidences suggest complex *N*-glycans as fundamental players in maintaining the homeostatic threshold of pre-BCR/BCR signaling, allowing positive selection⁵¹. Moreover, extrinsic sialylation by α 2,6-sialyltransferase 1 (ST6GAL1) enzyme was found to be a significant pro-survival factor during B cell development, whereas B cell intrinsic ST6GAL1 was shown to be required for marginal zone (MZ) B cell development⁵².

B cell activity and function can also be modulated by *N*-glycans. Core fucosylation was described to affect the humoral response, with *Fut8*^{-/-} mice showing reduced levels of IgG subclasses (but not IgM) due to low cytokine levels, such as interleukin (IL)-4, IL-5, IL-6, interferon (IFN) γ , and transforming growth factor (TGF) secreted by Th cells⁵³. Upon B cell activation and differentiation, changes in the *N*-glycosylation patterns occur. Naive and memory B cells were reported to express GnT-V-mediated branched *N*-glycans at the surface, which contain poly-LacNAc motifs, serving as high-affinity binding sites for many galectins^{54,55}. On the other hand, germinal center (GC) B cells were described to be modified with β 1,6-GlcNAc- and galactose-containing disaccharides, precluding Gal-9 binding. Interestingly, Gal-9 was described to regulate BCR signaling, antagonizing naive B cell activation^{54,55}. Additionally, the BCR can form a complex with immunoregulatory glycoproteins, including the sialic acid-binding immunoglobulin-type lectins (Siglec-2 or CD22), an inhibitory receptor mainly found on B cells, which modulate the BCR activation threshold. Notably, *ST6Gal1* deficient B cells

showed suppressed BCR signaling, whereas mice deficient in both CD22 and its ligand (*Cd22^{-/-}St6gal1^{-/-}*) presented restored BCR signaling, demonstrating the importance of α 2,6 sialic acids/Siglec axis in immunomodulation of BCR signaling ⁵⁶.

Furthermore, *N*-glycosylation is crucial for the effector functions of immunoglobulins (Ig). IgG, a glycoprotein secreted by antibody-secreting B cells, consists of a crystallizable fragment (Fc), responsible for triggering effector functions, and antigen-binding fragment (Fab) arms, responsible for antigen binding. The Fc region of IgG is covalently attached to a single bi-
 antennary *N*-glycan which plays a key structural role in stabilizing the conformation of the Fc region⁵⁷. The Fc-linked *N*-glycan can comprise different glycoforms, generally combining the presence or absence of fucose, galactose, sialic acid and bisecting GlcNAc ⁵⁸. Although heterogeneous, the profile of IgG glycosylation is relatively constant, displaying high proportion of core
 fucosylation (96%), low bisecting (8%), sialylation (4%), and intermediate galactosylation (40%) ^{59,60}. Changes in the composition of these glycans significantly affect the effector function of IgG, as observed by the loss of function in non-glycosylated IgGs ⁶¹. Interestingly, galactosylation and sialylation levels decreased with age and displayed a significant gender
 dependence⁵⁹. Moreover, cytokines in the microenvironment can regulate IgG glycan patterns by regulating intracellular glycosyltransferases in human B cells. Increased levels of IgG galactose were observed in the presence of IFN γ , whereas increased levels of IgG sialylation were observed upon IL-21 and IL-17A stimulation ⁶².

3.3 Sweet mediators of Inflammation: changes in *N*-glycans as potential triggers of intestinal inflammation and disease progression

Increasing evidence indicates that changes in gut glycosylation may occur before the onset of disease, during the transition from health to inflammatory conditions^{63–65}. However, the mechanisms governing these glycosylation changes are still poorly explored. Intestinal inflammation can be triggered by the disruption of the intestinal barrier and/or the mucosal immunity, leading to chronic inflammatory conditions. IBD is a chronic inflammatory disorder of the gastrointestinal tract that encompasses Crohn's disease (CD) and ulcerative colitis (UC)⁶⁶. The causes contributing to the development of IBD are complex, being associated with a dysregulated immunologic response, alterations in the intestinal microbiome, genetic and environmental factors. Recently, the aberrant expression of glycans has emerged as a novel mechanism in the pathogenicity of IBD⁶⁷. Herein, we will describe the most common *N*-glycosylation changes contributing to inflammation onset and disease progression, focusing on IBD (see Figure 1A).

3.3.1 Sweet molecules, bitter consequences: Intestinal *N*-glycans as shapers of Chronic Inflammation

Intestinal mucosa is the major physical and biological barrier that integrates different stimuli, supporting host-microbe interactions and immune responses. Intestinal epithelial cells (IECs) express a diverse repertoire of *O*- and *N*-glycans that maintain mucosal homeostasis by shaping intestinal inflammation in the host⁶⁸. Interestingly, in homeostatic conditions, the bottom of the intestinal crypts in the small intestine (SI) is characterized by an enriched stem cell zone

abundant in mannose-enriched glycoproteins ⁶⁹. As we move towards the top of the crypts, not only cells become more differentiated, but also the complexity of *N*-glycans expressed by these cells increases, with a predominance of complex-type *N*-glycans bearing terminal LacNAc ⁷⁰. This LacNAc expression was
5 shown to be crucial for the control of stem cell behavior and proliferation ⁶⁹, possibly affecting the epithelial recovery after chronic inflammation of the tissue.

In chronic intestinal inflammation, the most well-described glycosylation alteration is the increased expression of immature and truncated glycans ⁷¹.
10 These alterations promote inflammation onset that perpetuates upon failure of tissue repair, contributing to disease pathogenesis. Additionally, in the intestinal epithelium, FUT1 and FUT2 mediate the expression of the major fucosylation class in humans and mice - the α 1,2-linked fucose ^{68,72,73}. Among the genetic and epigenetic factors that dysregulate *N*-glycans expression during inflammation,
15 *FUT1* and *FUT2* polymorphisms were associated with CD susceptibility ^{74,75}, with *FUT2* mutations also contributing to colitis-associated microbial dysbiosis in CD patients ⁷⁶. Moreover, in IBD patients, the expression of FUT2 and α 1,2-linked fucose were decreased ⁷⁷. Additionally, *Fut2*-deficient mice showed increased susceptibility to pathogenic inflammation and severe colitis upon
20 dextran sodium sulfate (DSS) treatment ⁷⁸. After fucose metabolic supplementation of these mice, an attenuated inflammation was observed upon DSS-induced colitis ⁷⁹. Of note, *FUT2* polymorphisms are also associated with other acute and chronic inflammatory disorders, including type I diabetes and psoriasis ^{80,81}. Together, these evidences strongly corroborate the seminal role

of epithelial fucosylation in controlling intestinal homeostasis by regulating host defense against pathogenic infection.

Despite being highly *O*-glycosylated, mucins are also decorated with *N*-glycans, important for the function of these glycoproteins. Interestingly, mass spectrometry-based *N*-glycan of mouse colonic Muc2 demonstrated that the binding of dendritic cells (DC)-associated C-type lectin (dectin)-2 to mucins is mediated by the high mannose *N*-glycans present on mucins⁸². This highlights the importance of mucin *N*-glycosylation profile in modulating host innate immunity and resolving inflammation.

Therefore, genetics, environmental, and immune factors converge to regulate the expression of *N*-glycans that, in turn, fine-tune gut immune responses associated with both homeostasis and to inflammation onset in the context of several diseases.

3.3.2 Intestinal *N*-glycans as shapers of Gut Microbiome and Immune response

In the last years, several studies have emphasized the impact of intestinal glycocalyx composition in regulating homeostatic immune response and in pathological host–microbial interactions^{83,84}. While variations in intestinal *O*-linked glycans have been widely recognized as a key contributor factor^{85–90}, our focus is to explore the impact of variations in *N*-glycosylation on symbiotic host–microbe communication.

The physical barrier provided by the epithelium is enhanced by a complex and diverse layer of *N*-glycans that are attached to the cell surface – the mucosa glycocalyx⁸⁴. One of the major aspects of intestinal *N*-glycans is their dual role:

acting as an additional shield that protects host cells from intestinal pathogens and driving the composition of the gut microbiota ⁹¹.

Regarding its protective features, epithelial α 1,2-fucose is described as an essential player in defending against several pathogenic and opportunistic
 5 microorganisms, by disrupting their attachment to the epithelium ^{92–94}. Indeed, *Fut2*-deficient mice display increased susceptibility to infection by *S. typhimurium* and *Candida albicans*, underscoring the protective role of epithelial α 1,2-fucose against pathogens ^{95,96}. Oppositely, recent studies identified GlcNAc-containing glycans as potential targets for pathogenic *S.*
 10 *typhimurium* during invasion and colonization of the SI ⁹⁷. This demonstrates the divergent role of glycans in the interaction with intestinal microbes. The expression of epithelial α 1,2-fucose was also shown to promote the growth of commensal microbes such as *Porphyromonadaceae*, *Ruminococcaceae*, and *Bacteroides* species which, in turn, confers resistance to pathogenic and
 15 opportunistic microorganisms ^{98,99}. Additionally, *Fut2* mutations have been associated with a dysbiotic gut microbiota, characterized by a reduction of *Bifidobacteria* and an expansion of the Lachnospiraceae family⁹⁵. It is noteworthy that dysbiosis has been tightly associated with several diseases, including IBD and colon cancer ^{100–104}. Together, *FUT2*-mediated fucosylation
 20 seems to be essential for the fine-tuning of microorganism regulation and survival.

N-glycans are used as a source of energy and nutrients by the gut microbiota via a glycan-utilization machinery, known as polysaccharide utilization loci (PULs), comprising GBPs, transporters, and glycan-degrading enzymes (CAZymes),
 25 enabling the recognition, import and degradation of different glycans ¹⁰⁵. The

intestinal glycan degradation by commensal bacteria results in the production of short-chain fatty acids (SCFAs), such as butyrate, propionate, and acetate. SCFAs play diverse roles, being widely recognized for regulating the function of the immune compartment reinforcing the pivotal role of glycans in the symbiotic crosstalk between host and microbiota. Particularly, butyrate is an important energy source of colonocytes, exhibiting both anti-inflammatory and anti-tumor properties^{106–108}. Butyrate strongly induces Treg differentiation and promotes IL-22 release from CD4⁺ T cells and innate lymphoid cells (ILCs)^{109,110}. Additionally, this microbial metabolite also exhibits an antimicrobial effect against *Acinetobacter baumannii*, *E. coli*, and *Staphylococcus pseudintermedius*, inducing the depolarization of the bacterial membrane and acidification of the cytosol^{111,112}. Furthermore, butyrate has also been associated with suppressed growth and increased apoptosis in cancer cells¹¹³. Of note, different studies demonstrated that butyrate sensing and signaling are altered in both IBD and colorectal cancer (CRC) patients^{114,115}.

Gut microorganisms differ in the number and type of glycans that they can utilize¹¹⁶. While certain microbes, such as *Bacteroidota*, are considered “generalists”, i.e., degrading different types of glycans, other species, including members of the *Firmicutes* phylum, are considered more “specialist” being restricted to one or few glycans^{116,117}. Some *Bifidobacterium* species were shown to have the ability to remove *N*-glycans from glycoproteins via β -mannosidases, allowing its rapid and selective growth¹¹⁸. Importantly, *Phascolarctobacterium* spp., a specific population of the gut commensal bacteria, utilize host-derived GlcNAc for their own growth¹¹⁹. The abundance of *Phascolarctobacterium* spp. is particularly relevant since it restricts the

opportunistic growth of *C. difficile*, which is responsible for one of the most common clinical complications in IBD patients ¹²⁰. Indeed, the degradation of GlcNAc impacts the virulence and pathogenesis of several human opportunistic pathogens, including *C. albicans*, *E. coli*, *Vibrio cholerae*, *Leishmania*
5 *donovani*, and *Mycobacterium* ¹²¹.

Bacteroides thetaiotaomicron, a predominant bacterial species in the ileum and colon of humans and mice, is equipped with a particular PUL to access mammalian high-mannose *N*-glycan, yeast α -mannan and complex *N*-glycan from human Ig ¹²². In particular, *B. thetaiotaomicron* metabolized complex *N*-
10 glycans via cleavage of GlcNAc-b(1,2)-mannose linkages ¹²³, and utilized epithelial α 1,2-fucose as a nutrient source ¹²⁴. The degradation of fucose residues controlled the expression of several bacterial genes, determining the fitness and behavior of bacteria ¹²⁵. Furthermore, upon incorporation and metabolization of epithelial fucoses, *Bacteroides fragilis* synthesized fucosylated capsular
15 polysaccharides, which are components of bacterial outer membrane ¹²⁴. The incorporation of host glycans by luminal microbes in their own glycoconjugates is an important strategy of colonization, acting as a mask to avoid the detection by immune cells ¹²³. This type of phenomenon calls for a seminal concept named as molecular mimicry ^{126,127}. Moreover, molecular mimicry concerns as well,
20 when alterations in *N*-glycans confounds the immune system and are recognized as non-self-glycan structures, ultimately promoting the loss of immunological self-tolerance.

Recent evidence reported the ability of gut microbiota to reprogram intestinal epithelial glycosylation ⁶⁸. Unlike conventionally housed mice, germ-free mice
25 revealed impaired expression of α 1,2-fucose in the ileum. Accordingly, the

colonization of germ-free mice with commensal *B. thetaiotaomicron* restored the expression of *Fut2* and α -1,2-fucosylation^{98,99}. Likewise, *B. fragilis* and segmented filamentous bacteria (SFB) were reported as inducers of epithelial α 1,2-fucose expression⁷⁸. In fact, SFB-deficient mice showed impaired ileal
5 epithelial fucosylation, suggesting that SFB is a major microbial stimulus inducing epithelial α 1,2-fucosylation in homeostasis¹²⁵. Importantly, commensal bacteria also promote intestinal epithelial fucosylation by an indirect mechanism involving mucosal immune cells. Goto and colleagues have shown that commensal gut microbiota interacts with ILCs, specifically ILC3,
10 stimulating the production of IL-22, which in turn induces the upregulation of *Fut2* expression on IECs. In addition, the same group reported that constitutively production of lymphotoxin by ILC3s plays a similar role in *Fut2* induction^{78,92}. Collectively, due to their crucial role in integrating microbial and immunological cues, *N*-glycans emerged as a key driving factor of the pathologic events, leading
15 to development of immune-mediated diseases.

3.3.3 Glycans as modulators of Immune-mediated responses in Chronic Inflammation

Together with inflammation-induced alterations in epithelial glycocalyx and its
20 role in gut microbiota, modulation of *N*-glycans have a direct role on immune cells. Herein, we will highlight how changes in *N*-glycans expression shape the immune-mediated response during inflammatory conditions.

T cell hyperactivation is central to the pathology of chronic inflammation and IBD. As abovementioned, glycans can mediate T cell function, activity, survival,
25 migration and cytokine production through the regulation of different key

receptors ²⁶. Our group demonstrated that UC patients present a deficiency in β 1,6-GlcNAc-branched *N*-glycans on colonic CD4⁺ T cells, associated with T cell hyperactivation and disease severity ⁸. At the genetic level, we revealed that UC patients display a *MGAT5* single nucleotide polymorphisms (SNPs),
 5 associated with reduced expression of *MGAT5*-coded enzyme (GnT-V) ¹¹. We further demonstrated that the hyperactivation of colonic T cells in UC patients can be controlled through the *ex vivo* metabolic supplementation with GlcNAc. Additionally, we showed that *Mgat5* knockout (KO) mice present higher disease severity to DSS-induced colitis, which was attenuated when supplemented with
 10 GlcNAc ⁹. Interestingly, mucosal T cells from inflamed tissue of IBD patients, as well as from mice under colitis, revealed increased levels of core fucosylation. Indeed, *Fut8* KO mice produced lower levels of Th1 and Th2 cytokines, leading to the development of milder colitis symptoms. Consistently, core fucosylation of the TCR is required for T cell signaling and production of inflammatory
 15 cytokines ⁴⁷. Furthermore, differential glycosylation of Th1, Th2 and Tregs can modulate their fate through binding to galectins. Th2 and Treg cells express higher levels of α 2,6-sialylation compared to Th1 cells, which protects them from Gal-1-mediated apoptosis ¹²⁸. Notably, it has been reported that UC patients display elevated levels of α 2,6-sialylation in colonic tissue and that the
 20 deletion of *ST6GAL1* gene inhibits the proliferation and activation of CD4⁺ T cells, leading to a reduction in pro-inflammatory cytokines ¹²⁹. Morosi and colleagues described that the upregulation of *ST6GAL1*, detected in colonic biopsies of IBD patients, masks T cells from Gal-1, inhibiting its binding and thereby inducing inflammation ¹³⁰. Particularly, Gal-1 expression was found to
 25 be upregulated in inflamed tissue of IBD patients compared to uninflamed tissue

and healthy controls ¹³¹. Conversely, Gal-2, Gal-3, Gal-4 and Gal-9 expression were downregulated in inflamed areas ^{36,132}. Interestingly, the expression pattern of galectin-encoding genes of IBD patients in remission was similar to the one observed in healthy controls ¹³¹, suggesting that the resolution of inflammation leads to the recovery of normal galectin gene expression ⁷¹.

Even though B cells play a pivotal role in gut immunity, there is still a gap of knowledge regarding the impact of B cell *N*-glycosylation in the regulation of homeostasis and inflammation. Host intestinal mucosa contains the largest population of plasma cells, displaying 80% of all antibody-secreting B cells ¹³³. Alterations in the glycosylation of IgG have demonstrated to impact the biological functions and structure of IgG, affecting their anti-inflammatory and pro-inflammatory phenotype ^{11,134–137}. In fact, it was described that patients with IBD exhibit lower levels of IgG galactosylation, as well as sialylation, which was associated with increased severity of the clinical outcomes ^{134,135,138}. Terminal sialic acids were shown to display an anti-inflammatory function on IgG, by increasing expression of the inhibitory FcγRIIB and reducing affinity to activating FcγRIII/IV ^{139,140}, which suggests that sialic acids in Fc promote a conformational change in IgG. Additionally, terminal galactose was shown to increase affinity of IgG to inhibitory FcγRIIB and C-type lectin-like receptor dectin-1, promoting an anti-inflammatory phenotype ¹⁴¹. In IBD patients, agalactosylated IgG with terminal GlcNAc was shown to enhance antibody-dependent phagocytosis ¹⁴². The effector function of agalactosylated IgGs might be explained either by the bind of mannan-binding lectin (MBL) selectively activating the lectin complement pathway ¹⁴³, or by the dependence on activating Fc receptors ¹⁴⁴. This decrease in IgG galactose can be related to the

downregulation of the $\beta 4GalT$ gene (that encodes for β -1,4-galactosyltransferase ($\beta 4GALT1$) enzyme, which adds galactose on top of GlcNAc residues) in B cells from CD patients ¹³⁴. Additionally, specific *MGAT5* gene SNPs observed in UC patients were found to be associated with agalactosylation of IgGs ¹¹. UC patients also display decreased fucosylation and increased bisecting GlcNAc on IgGs ¹³⁵. Both core fucosylation and bisecting of the Fc region are key determinants of antibody-dependent cell-mediated cytotoxicity (ADCC) ^{145–147}. In fact, nonfucosylated IgG1 showed a 100-fold increase of ADCC compared to its fucosylated counterpart ¹⁴⁶.

While most of the studies on the role of glycans in chronic inflammation and IBD have focused on adaptive immunity, it is important to note that innate immune cells also rely on *N*-glycans to modulate their function and intercellular communication. For instance, in macrophages, core fucosylation of CD14 is essential for Toll-like receptor (TLR)4 and TLR2 signaling. Despite the wealth of knowledge on the *N*-glycosylation role in adaptative immunity, *N*-glycans significance in innate immune cells during chronic inflammation remains largely unexplored, opening a new avenue of research to explore in the field.

These findings collectively underscore the critical role of immune cell *N*-glycosylation in regulating the immune response during chronic inflammation, acting as a key player in both health and disease.

3.4 Cellular Glycosylation in the breakdown of self-tolerance in Autoimmunity

Glycosylation is a tissue and cell specific process fundamental to defining the identity of a cell or an organism. However, changes in the *N*-glycosylation

profile can lead to abnormal recognition of “self” and “non-self” antigens associated with autoreactive immune responses. This concept known as “glycan mimicry” is at the basis of the loss of self-tolerance associated with autoimmunity¹⁴⁸.

5 Autoimmune diseases are chronic and debilitating diseases characterized by the dysregulation of the immune system, resulting in the uncontrolled immune response targeting its own tissues within the body and resulting in the damage of different organs. In this section, we will describe how changes in glycocalyx composition contribute to the immunopathogenesis of several autoimmune
10 diseases (see Figure 1B).

3.4.1 Impact of altered Glycocalyx in Autoimmune Diseases

A classic prototype of autoimmune disease is **Systemic Lupus Erythematosus (SLE)**, a multisystemic disease characterized by autoreactive immune cells and
15 abnormal autoantibody production against self-antigens which, together, lead to the deposition of immune complexes and organ damage. One of the most severe complications of this disease is lupus nephritis (LN), which involves renal damage that ultimately can lead to organ dysfunction and the need for kidney transplantation. In this disease, a full glycome characterization of LN tissue
20 revealed a unique glycan repertoire of renal epithelial cells¹³. In fact, imaging mass spectrometry analysis disclosed an accumulation of mannose-enriched *N*-glycans in renal epithelial cells from LN patients that were not observed in healthy kidney or other kidney diseases' samples. The accumulation of less complex *N*-glycans was related to the downregulation of Mannosidase Alpha
25 Class 2A Member 1 (*MAN2A1*) gene (encoding α -mannosidase-II enzyme) and

upregulation of Protein O-Mannosyltransferase 1 (*POMT1*) gene (encoding mannosyltransferase enzyme) expression. This was associated with increased infiltration of a subset of immune cells¹² that express DC-SIGN, which recognizes mannosylated glycans. Importantly, this specific glycosignature has shown predictive value, as it stratifies patients according to the risk of developing chronic kidney disease (with a specificity of 93%), being a potential diagnostic and prognostic glyco-biomarker to be used in the clinic. In accordance, a case-report study on two sisters with α -mannosidosis diseases showed an association with a truncated *N*-glycome (accumulation of mannose-enriched *N*-glycans) and the development of SLE disorder¹⁴⁹. Mice studies have also pinpointed glycans as important modulators of tissue homeostasis. The *MRL-lpr* lupus mouse model demonstrated an increased accumulation of less-complex *N*-glycans in kidney proteins, including paucimannose and mannosylated-type *N*-glycans¹⁵⁰. The *in vivo* deficiency of α -mannosidase-II (enzyme that hydrolyzes a mannose residue) resulted in the accumulation of mannose- and hybrid-type glycans. This resulted in the development of a disorder that resembles SLE disease, with glomerulonephritis, accumulation of autoantibodies and C3 complement deposition, highlighting the importance of less-complex *N*-glycans in the immunopathogenesis of this disease^{24,151}. Similarly, glycoengineered *Mgat5*-KO mice showed increased severity of colitis and experimental autoimmune encephalomyelitis (EAE), as well as spontaneous lupus-like syndrome with aging^{9,12}. In **rheumatoid arthritis (RA)** the pro-inflammatory milieu around the joints contributes to the destruction of bone and cartilage, leading to severe pain and joint swelling. Notably, in inflamed joints of RA patients, it was observed a downregulation of *ST6Gal1* expression of

synovial fibroblasts in tumor necrosis factor (TNF)-dependent manner, which consequently reduced the content of α 2,6-sialylated glycans and potentiated the inflammation and disease ¹⁵². Moreover, this inflammatory environment promoted the expression of β 1,4-galactosyltransferase-I in synovial tissue, suggesting a pathogenic role of inflammation and stromal *N*-glycans in RA immunopathogenesis ¹⁵³. Additionally, FUT1 enzyme was found to be upregulated in synovial fibroblasts of RA patients, resulting in increased production of fucosylated glycans, associated with fibroblast proliferation and adhesion, contributing to the tissue damage and inflammation of RA disease ¹⁵⁴.

Similar alteration was observed in **Psoriasis (PsO)**, an inflammatory autoimmune skin disease characterized by hyperproliferation of epidermal keratinocytes, where some patients may also develop joint damage known as **psoriatic arthritis (PsA)**. The *FUT8* gene was found to be overexpressed on skin biopsies of psoriatic patients and associated with disease severity. This glycosylation alteration led to increased activation of EGFR protein kinase and was related to increased proliferation of epidermal keratinocyte cells, emphasizing the impact of altered glycans during the development of psoriasis ¹⁵⁵.

Idiopathic inflammatory myopathies (IIM) are a group of rare heterogeneous disorders that affect the muscles and skin, leading to muscle weakness, atrophy, and ultimately imposing a poor quality of life on patients. A recent study characterized the glycosylation profile of muscle biopsies from IIM patients and found increased levels of high-mannose *N*-glycans due to decreased *MGAT1* and increased *MAN2A1* gene expression ¹⁴. This unique tissue glycome was associated with poor prognosis of IIM patients. Another subgroup of myopathies, the GNE autosomal genetic myopathy (caused by mutations in the

GNE gene), resulted in the loss of sialic acid synthesis in muscle cells and in misfolding of some glycoproteins¹⁵⁶. Interestingly, evidence suggested that oral glycan supplementation promoted branching *N*-glycosylation by glycoreprogramming the muscle environment, recovering the homeostatic *N*-glycans^{14,157}. **Type-1 diabetes mellitus (T1DM)** is a subgroup of metabolic disorders characterized by autoimmune pancreatic β -cell destruction resulting in low insulin levels and hyperglycemia. Non-secretors individuals with a non-functional *FUT2* allele and unable to express blood group antigens in secretions and on mucosal surfaces are associated with increased susceptibility to T1DM and resistance to certain host infections⁸¹.

Altogether, these evidences suggest that cellular glycome, and specifically changes in *N*-glycans may act as potential danger signals (DAMPs) for the immune system, contributing to the immunopathogenesis of autoimmune diseases.

3.4.2 Immunomodulatory role of Glycans in Autoimmunity: Glycans as shapers of Cellular and Humoral Immune responses

Abnormal glycosylation of the immune counterparts is also widely associated with a pathogenic autoreactive phenotype and the hyperactivation of the immune system.

Alterations in the *Mgat5* gene expression are well described in the context of T cell regulation and autoimmunity^{8,158}. Demetriou and colleagues have reported that the absence of β 1,6-complex *N*-glycans affects the cell surface expression of immune checkpoint receptors (programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4)) and results in TCR clustering, T cell

hyperactivation, reduced galectin–glycoprotein lattice and susceptibility to autoimmunity¹⁵⁸. In accordance, in **multiple sclerosis (MS)**, a chronic autoimmune disease characterized by demyelination and neurodegeneration of the central nervous system, the peripheral blood mononuclear cells (PBMCs) from these patients present a downregulation of the GnT-V enzyme activity¹⁵⁹. Consistently, the conditional Lck-Cre⁺Mgat2^{fl/fl} mice model, where T cells lack tri- and tetra-antennary branched *N*-glycans, developed an increased susceptibility to EAE a model that mimics MS. In fact, this susceptibility was potentiated in mice treated with kifunensine, promoting the accumulation of mannosylated glycans and inhibiting the compensatory poly-LacNAc structures¹⁶⁰. Moreover, the lack of Gal-3 was shown to attenuate EAE disease severity, where Gal-3 KO DCs produced IL-10 and inhibited the release of pro-inflammatory cytokines by T cells¹⁶¹. On the other hand, the absence of Gal-1 in MS-like disease resulted in Th1 and Th17 pro-inflammatory responses and microglial activation. Notably, the administration of Gal-1 suppressed this phenotype and attenuated EAE disease¹⁶². In addition, the absence of fucose attached to the *N*-linked glycans in *Fut8*-KO mice resulted in less CD4⁺ T cell activation, controlled T and B cell interaction, attenuating EAE syndrome⁴⁶. Moreover, specific *MGAT1* and *MGAT5* SNPs, together with CTLA-4, IL-2 receptor- α (IL2RA) and IL-7 receptor- α (IL7RA) SNPs, were correlated with an increased risk and severity for developing MS and T1DM^{163–165}. Genome-wide analysis of **PsO** patients revealed that specific *MGAT5* SNPs were also associated with altered *N*-glycosylation profile of activated T cells from these patients¹⁶⁶. Consistently, the conditional *Rag1*^{Cre}*Mgat1*^{fl/fl} glycoengineered mice model, harboring high-mannose *N*-glycans only in lymphocytes,

developed spontaneous inflammation in colon and kidney tissues, as well as imiquimod-induced psoriasis³⁰. In SLE, resting T cells from patients exhibited an activated-like phenotype according to the glycosylation status as well as reduced levels of Gal-1 binding¹⁶⁷. In fact, it was a consequence of increased expression of ST6GAL1/NEU1 and ST3GAL6/NEU1 ratios by the cells, resulting in the escape of autoreactive T cells by the Gal-1 apoptotic effect. Interestingly, serological levels of Gal-9 were significantly increased in patients with SLE and correlated with disease activity. This association was particularly pronounced in patients with renal damage, suggesting that Gal-9 could serve as a serologic marker of SLE pathogenesis¹⁶⁸. In aged *Mgat5*-null mice and α -mannosidase-II deficient mice (both develop SLE-like disease spontaneously), it was described, respectively, the infiltration of DC-SIGN-expressing $\gamma\delta$ T cells and the macrophage mannose receptor (MMR) protein expression on mesangial cells and macrophages. This unlocks a new axis of glycans and glycan-binding proteins in SLE immunopathogenesis^{12,24}. Evidence have shown that DC-SIGN is overexpressed in the podocytes of LN patients, and mice treated with anti-DC-SIGN antibody hampered the autoimmune signs by reducing proteinuria levels, T cell proliferation, proinflammatory cytokines, and tissue damage¹⁶⁹. In some autoimmune diseases, the *FUT8* gene is described to affect T cell activity and response, particularly for SLE patients in which increased levels of core fucosylation of CD4⁺ T cells are associated with disease severity⁴⁶. **Myasthenia Gravis (MG)** is a neuromuscular autoimmune disorder characterized by the weakness of the muscles and impaired neuromuscular transmission. In thymic epithelial cells from MG patients, was described that increased expression of Gal-1 mediates the interaction of thymic epithelium with T cells, leading to

abnormal thymus morphology and disease ¹⁷⁰. In addition, aged mice lacking Gal-1 (*Lgals1*^{-/-}) developed a spontaneous inflammation of salivary glands, similar to **Sjögren's syndrome (SjS)**, an autoimmune disease that affects exocrine glands ¹⁷¹. Likewise, this phenotype was also observed in other mice-
5 induced autoimmune-like diseases and linked with tissue damage ^{128,172}. Similarly, in **IIM**, the GNE activity promoted the poly-LacNAc extension, affecting the binding of Gal-1, which in turn might disturb T cell signaling and activity ¹⁵⁶. In IIM patients, it was also reported that the absence of branched *N*-glycans in CD4⁺ T cells was associated with an increased pro-inflammatory
10 phenotype and the release of IL-6 cytokine ¹⁴. Opposingly, Gal-3 KO mice showed attenuated symptoms of antigen-induced arthritis, a mouse model mimicking **RA** disease, with less joint inflammation and decreased levels of autoantibodies and proinflammatory cytokines ¹⁷³.

The role of B cells and IgGs cannot be left behind in the context of
15 autoimmunity, where autoantibodies are one of the hallmarks of this group of diseases. Changes in B cells and IgG glycome have been described in different disease contexts, from autoimmune and inflammatory diseases to infections and cancer ¹⁷⁴. A recent study described a dose-dependent and dual role of *N*-glycan branching in B cells function. Here, lack of *N*-glycan branching on B cells
20 resulted in reduced CD19 and BCR surface retention and promoted TLR signaling, generating a pro-inflammatory environment, that together exacerbated inflammatory demyelination in EAE mice model ¹⁷⁵. Furthermore, the interplay of Gal-9 with BCR controls its signaling transduction. Consistently, lower BCR thresholds and an accelerated rate of spontaneous
25 autoimmunity with renal damage and autoantibody production were observed in

aged Gal-9-deficient mice ¹⁷⁶. In **MS** patients, the analysis of IgG Fc glycosylation in the cerebrospinal fluid revealed reduced afucosylation and galactosylation, concomitantly with elevated bisecting GlcNAc in *N*-glycans associated with increased IgG effector functions. Moreover, the plasma IgG of MS patients displayed lower core fucose and increased content of high-mannose Fc *N*-glycans, which was also associated with their pro-inflammatory potential. In **SLE**, the Fc IgGs presented decreased galactosylation, sialylation, fucosylation along with increased bisecting GlcNAc, suggesting decreased immunosuppressive potential of circulating IgGs ^{177,178}. Lower levels of IgG Fc galactosylation were also described in **MG**, **PsA** and **IIM** patients ^{178–180}. Similarly, in **RA** disease, the first report of altered IgG-Fc glycome in patients described elevated agalactosylated glycans content compared with healthy individuals, which was further validated by others and associated with RA disease activity ^{181,182}. Moreover, in RA-specific anti-glycan antibodies against citrullinated peptides (ACPA-IgGs), the ACPA-IgG1-Fc glycosylation of synovial fluid differs from total serum IgG1, with reduced sialylation and elevated agalactosylation of ACPA-Fc on synovial fluid ¹⁸³. In addition, the lack of galactose in ACPA-IgG-Fc is associated with RA disease progression and inflammation ⁶⁴. Interestingly, the human IgG Fab regions can also carry *N*-glycosylation consensus sequences, and the Fab component of RA patients displays high levels of bisecting GlcNAc, galactose and disialylation, which may also be reflected in antigen-binding capacity ¹⁸⁴. Importantly, the presence of *N*-glycosylation sites in ACPA-IgG Fab affects B cell antigen binding and BCR signaling, contributing to the selection of autoreactive B cell clones, that might be involved in the RA pathogenesis ^{185,186}. In **MG** disease, specific

autoantibodies targeting crucial molecules at the neuromuscular transmission system, such as the nicotinic acetylcholine receptor (AChR) and muscle-specific kinase (MuSK), display elevated *N*-linked glycosylation motifs in the variable domains of AChR and MuSK autoantibodies associated with MG disease when
5 compared with healthy controls ^{178,187}.

Despite the differences in IgG glycome (Fc- and Fab-dependent) associated with pro-inflammatory responses in autoimmunity, the cellular and molecular mechanisms underlying this response remain to be elucidated, and further studies are needed to fully characterize these mechanisms.

10

3.5 *N*-Glycosylation in the Tumor Microenvironment

Changes in cellular glycosylation have been considered a hallmark of cancer, mediating critical events of tumor progression. In fact, glycans play a pivotal role in each pathophysiological step of malignant transformation and
15 particularly in cancer immunoediting ⁶.

Here, we will discuss the multiple roles of *N*-glycans in the tumor microenvironment (TME), either as major regulatory molecules of cancer cell behavior but also as master regulators of immune cell functions in the TME, such as innate immune cells and tumor infiltrating T cells (see Figure 1C).

20

3.5.1 Tumor cell Glycosylation alterations: the enemy at the dynamic crosstalk between cancer cells and immune cells

Tumor cells acquire a variety of mechanisms to evade the immune system, and one of them is the expression of aberrant *N*-glycosylation at the cell surface. In

the present section, we will describe some mechanisms related to *N*-glycosylation that tumors use to hijack the immune system.

The most remarkable alteration described in tumor cells is the abnormal expression of sialic acids. This coat of sialoglycans works as a shield, protecting tumors from being recognized and eliminated by immune cells, particularly through Siglecs¹⁸⁸. In hepatocellular carcinoma (HCC) tumor tissues, a strong inflammatory response was observed with lower expression of 2,6-sialic acid¹⁸⁹. In accordance, in an HCC cell line, it was described that overexpression of *ST6GalI* decreased CD8⁺ T cell infiltration in TME, as well as their cytokine production, namely IFN γ and TNF α ¹⁹⁰. Additionally, sialic acids present on tumors inhibited NK cells activation by engaging with Siglec-7 and -9^{191,192}. In line with these findings, in fibrosarcoma cell lines, tumor cell desialylation led to the induction of NK cell cytotoxicity¹⁹³. Moreover, a decrease in Siglec-9 ligands on tumor cells significantly reduced immunosuppression by enhancing T cell effector functions and their anti-tumor capacity^{194,195}. The key impact of sialylation is also demonstrated by the role of CD24. CD24 is a heavily sialylated glycoprotein and tumors expressing CD24 were shown to preclude phagocytosis by tumor-associated macrophages (TAM) through Siglec-10 binding¹⁹⁶. On the other hand, Gal-1 interacts with glycans in the TME, mediating malignant processes. In fact, α 2,6-sialic acids, highly expressed on tumor cells, prevented Gal-1 binding, which protected them from Gal-1-induced death^{197,198}. However, Gal-1 expression by tumors promoted the presence of TAMs harboring immune checkpoints, such as PD-L1 in the TME¹⁹⁸. Gal-1 role in the TME and particularly on T cells will be further discussed in more detail in the section *T cell surface glycosylation dynamics during tumor progression*.

The aberrant overexpression of β 1,6-GlcNAc branched *N*-glycans structures has been widely described on tumor cells. In fact, overexpression of complex branched *N*-glycans was described to induce pro-malignant and pro-invasive phenotypes, associated with poor prognosis of gastrointestinal cancer patients^{199–202}. Recently, our group had shown that the aberrant expression of β 1,6-GlcNAc branched *N*-glycans in CRC acts as an important immune checkpoint in cancer immunosurveillance and immunoediting. These glycan structures dampened the release of cytokines such as IFN γ and TNF α and correlated with increased Treg infiltration in the TME. Besides, the inhibition of β 1,6-GlcNAc branched *N*-glycans enhanced immune recognition of cancer cells¹⁸. In CRC tissues, a downregulation of bisecting *N*-glycans was observed to be associated with IFN γ resistance of tumor cells. Additionally, *MGAT3* overexpression was able to restore IFN γ sensitivity of tumor cells²⁰³. More recently, the expression of B3GNT2, involved in the biosynthesis of poly-*N*-acetylactosamine chains on tumors was described to decrease T cell activation by disrupting tumor-T cell interactions. Moreover, treating tumor cells overexpressing B3GNT2 with kifunensine was able to restore T cell function, increasing IFN γ production²⁰⁴. Altogether, these highlighted the impact of different *N*-glycan structures in tumor immune evasion.

One of the best described immunosuppressive pathways in cancer is the PD-1/programmed cell death 1 ligand 1 (PD-L1) axis. In this axis, the glycosylation of PD-L1 plays a major role in determining how it binds to its receptor, PD-1^{205–207}. In fact, β -1,3-*N*-acetylglucosaminyl transferase (B3GNT3) expression was described to be essential for the interaction of PD-L1 with PD-1.

Additionally, downregulation of B3GNT3 led to improved cytotoxic anti-tumor T cell responses ²⁰⁸.

Tumor cells produce antigens that can trigger an immune response. These tumor antigens can also be *N*-glycosylated, interfering with their recognition by the immune system. In fact, when the tumor antigen tyrosinase has its *N*-glycan chain at Asn317 site mutated, its presentation by MHC-I is increased ²⁰⁹. Interestingly, sialylated antigens bind to Siglec-E, mouse homolog of Siglec-9 in humans, on mouse DCs, which favored the differentiation of antigen-specific Tregs and reduced effector T cell numbers ⁴⁹.

Overall, these evidences promptly suggest the importance of altered *N*-glycosylation in the defense of cancer cells against anti-tumor immune attack, giving tumors an advantage to develop and proliferate.

3.5.2 GBP interactions as regulators of Innate immune responses in Tumor

Microenvironment

As previously stated in this chapter, GBPs are an essential part of the immune response, being commonly expressed by innate immune cells. In this section, we are going to focus on CTLs and their role in cancer upon *N*-glycan recognition. MBL can bind Le^A and Le^B in *N*-glycans expressed by CRC cell lines, specifically in the structures that decorate CD26 ^{210,211}. Indeed, MBL-mediated cellular cytotoxicity suppressed tumor growth in athymic mice injected subcutaneously with a human CRC cell line ²¹². The gastric cancer cell line MKN45 displayed high DC-SIGN binding motifs expression, and the inhibition of DC-SIGN and MR in monocyte-derived DCs (moDCs) led to lower CD86 levels when co-cultured with MKN45 cells ¹⁸. In addition, several human tumor

cell lines and cancer tissue samples displayed paucimannose and high-mannose *N*-glycans that can bind MR²¹³. Dectin-1 was also capable of binding *N*-linked saccharides in the B16 melanoma cell line, culminating in the activation of NK cells and induction of an anti-tumoral response²¹⁴.

5 On the flipside, DC-SIGN had been implicated in DC immunosuppression. In CRC cell lines, Carcinoembryonic Antigen (CEA) displayed high levels of fucosylated *N*-glycans²¹⁵, in particular, increased Le^X and Le^Y epitopes²¹⁶, which can be recognized by DC-SIGN expressed by immature DCs, potentially facilitating immune evasion²¹⁶. Another study had reported that DC-SIGN
10 detected elevated Le^A/Le^B antigens in CEA from the SW1116 CRC cell line, inducing the secretion of immunosuppressive cytokines, such as IL-10²¹⁷. Mac-2 Binding Protein (Mac-2BP) was also found to be expressed in CRC cell lines and exhibited aberrant Lewis antigen expression that was bound by DC-SIGN, an effect that was lost after PNGase F treatment, which cleaves *N*-linked glycans
15 from glycoproteins, indicating a dependency on *N*-glycans²¹⁸. This interaction impaired DC maturation, potentially limiting anti-tumoral activity²¹⁸. Cancer Antigen 125 (CA-125) is a glycoprotein frequently associated with Ovarian Cancer (OC) that presented significant differences in *N*-glycosylation sites in OC patients²¹⁹. Interestingly, the MR expressed by TAM can bind CA-125,
20 inducing the secretion of IL-10²²⁰.

All things considered, CTLs have shown a dual role in the shaping of the TME, making them an interesting target for the development of cancer therapies.

3.5.3 T cell surface Glycosylation dynamics during tumor progression

T cells have the capacity to specifically recognize and respond to tumor antigens, being crucial mediators of anti-tumor immune response. However, T cells are not always effective to eliminate tumor cells. Alterations to normal T cell *N*-glycosylation patterns may lead to the loss of function of these cells within the TME²¹. Here, we will depict the T cell *N*-glycosylation alterations that lead to the formation of immunosuppressive pathways in the TME, precluding an effective anti-tumor immune response.

As described in detail in the section “*Glycans as immune regulatory circuits in homeostasis*”, a wide variety of T cell receptors are glycosylated²⁶, and alterations in the glycosylation pattern of this subset of cells might impact the translocation, retention and signaling of these receptors on T cell surface, but also influencing its binding. Additionally, these alterations can also impact the binding to GBPs, such as galectins and Siglecs²²¹. It is now established that PD-1 requires *N*-glycosylation to be retained at the T cell surface, being its *N*-glycosylation pattern altered after T cell activation towards higher expression of poly-LacNAc and core fucose. On the other hand, deficient *N*-glycosylation led to PD-1 degradation²²². PD-1 *N*-glycosylation was also described to be essential to its binding to PD-L1, as well as for immunotherapeutic antibodies, such as nivolumab and camrelizumab^{222–224}. In fact, core-fucose at the asparagine N58 residue appeared to be crucial for the action of anti-PD-1 blockade antibodies^{223,225,226}. Interestingly, inhibition of Fut8 reduced PD-1 expression and improved T cell activation²²⁷.

Early in T cell activation, two signals are required: the first being the recognition of an antigen through MHC on APCs by the TCR and the second being a co-stimulatory signal, by molecules such as CD28²²⁸. Contrary to CD28, CTLA-4,

also expressed on T cells, induces a co-inhibitory signal on T cells by binding with higher affinity to CD80/CD86, downregulating T cell immune response²²⁹. CTLA-4 is known to be regulated by glycosylation^{230,231}. In fact, upon TCR activation, an increase of branched *N*-glycans in CTLA-4 occurred, leading to T cell suppression^{45,232–234}. In this line, *N*-acetylglucosamine supplementation was shown to increase CTLA-4 at the T cell surface, resulting in inhibition of T cell activation²³¹. Additionally, *Mgat5* KO T cells under low TCR stimulation, showed decreased CTLA-4 levels on T cell surface²³¹.

Other important receptors in tumor immunosuppression were also described to be *N*-glycosylated, namely the T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) and the Lymphocyte activation gene-3 (LAG-3)^{235,236}. In fact, it was shown that TIGIT PNGase F treatment prevented the binding to its receptor²³⁵. Curiously, LAG-3 was described to interact with Gal-3, promoting CD8⁺ T cell dysfunction²³⁶. Altogether, these observations demonstrate how *N*-glycosylation of T cells receptors are a key point in the immunosuppressive processes within the TME, thus emerging as a promising target for further studies.

Galectins play an important role in the TME, mediating T cell activation²³⁷. For instance, Gal-1 is involved in various T cell steps, playing a role in survival, differentiation, activation and function²³⁸, while in cancer, it has an immunosuppressive function^{237,239}. In fact, Gal-1 was correlated with decreased numbers of tumor infiltrating lymphocytes (TILs)^{240,241} and increased Tregs in the TME²⁴², promoting resistance to immunotherapy with immune checkpoint inhibitors²⁴⁰. Increased expression of α 2,6-sialic acids, due to overexpression of *ST6Gal1*, on CD45 receptor was shown to prevent Gal-1 binding and

consequently T cell induced death ¹⁹⁷. Interestingly, secretion of Gal-1 by $\gamma\delta$ T cells promotes malignant progression, dampening anti-tumor immune response ²⁴³. In the TME, Gal-3 abolished cytotoxic T cell function and IFN- γ secretion by establishing molecular lattices with glycans structures on the TCR and CD8 ²⁴⁴. Further, the use of LacNAc, a galectin ligand, to inhibit Gal-3 binding to cellular glycans, reverted anergy on human TILs ²⁴⁴. Gal-9 is also known to play a part in tumor immunosuppression. In fact, T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) is a receptor expressed on exhausted T cells and exerted its immunosuppressive function upon binding to Gal-9 ²⁴⁵. Gal-9 binds to glycans present on Tim-3, promoting apoptosis of tumor infiltrating CD8⁺ T cells ²⁴⁶ and treatment with PNGase F, led to an impairment of Tim-3/Gal-9 binding ²⁴⁵. Interestingly, it was recently demonstrated by Yang and colleagues that in exhausted T cells co-expressing PD-1 and Tim-3, PD-1 interacts with Tim-3 and Gal-9, attenuating Tim-3/Gal-9 induced T cell death, leading to the survival of exhausted T cells ²⁴⁷. Notably, Tim-3/Gal-9 interactions on TILs were described to play a role in cancer patients' resistance to anti-PD-1 immunotherapy ²⁴⁸.

Another class of GBPs that mediates immunosuppression are Siglecs, due to the aberrant expression of sialic acids on tumor cells ²⁴⁹. Siglec expression on normal/resting T cells is very low, however, on TILs their expression can be significantly increased. Siglec-9 was found to be highly expressed on TILs, specifically on PD-1^{high} T cells. Additionally, these T cells presented an effector memory phenotype that expresses inhibitory receptors, such as PD-1, CTLA-4, Lag-3 and Tim-3 ^{194,195}. Siglec-9 was found to inhibit T cell effector functions and its anti-tumor capacity ^{194,195}. In parallel, in mice, Siglec-E was also

observed to be upregulated on TILs ¹⁹⁵. Furthermore, disrupting the siglec-sialoglycan interaction leads to an increase of CD8⁺ T cells in the TME of a CRC mouse model ¹⁹⁵. Siglec-7 is expressed on non-exhausted effector memory CD8⁺ T cells with increased metabolic fitness, cytotoxic capacity and cytokine production. However, upon binding to sialoglycans, Siglec-7 acts as an immune checkpoint preventing T cell function ²⁵⁰. Additionally, Siglec-5 and Siglec-10 expression on T cells was also described and associated with suppression of T cell activity ^{251,252}.

Focusing on Treg *N*-glycosylation, it was demonstrated that the removal of *N*-glycans from the cell surface by PNGase F treatment results in decreased suppressive function, through a contact-dependent mechanism. Additionally, tri- and tetra-antennary *N*-glycans were described to be correlated with the expression of receptors involved in Treg suppression, such as CD39, CD73, inducible T-cell Co-stimulator (ICOS), Glucocorticoid-Induced TNFR Related (GITR), PD-1, PD-L1 and CTLA-4 ²⁵³. Recently, Pinioti and colleagues showed that targeting fucosylation on Tregs negatively impacts their proliferation and suppressive function, precluding tumor progression ²⁵⁴. However, the full mechanism by which *N*-glycosylation changes Treg suppressive functions remains to be elucidated.

Lastly, on TILs, exhausted CD8⁺ T cells were found to downregulate the oligosaccharyltransferase (OST) complex, which hampers IFN γ production ²⁵⁵. Moreover, restoring OST complex decreased CD8⁺ T cells exhaustion and increased the production of IFN γ , ultimately leading to the decrease of tumor growth ²⁵⁵. Suggesting *N*-glycosylation as an important player in T cell exhaustion.

Altogether, changes in *N*-glycosylation pattern on T cells and its receptors influence T cell function within TME, highlighting the importance of T cell *N*-glycosylation in the regulation of anti-tumor immune response within TME.

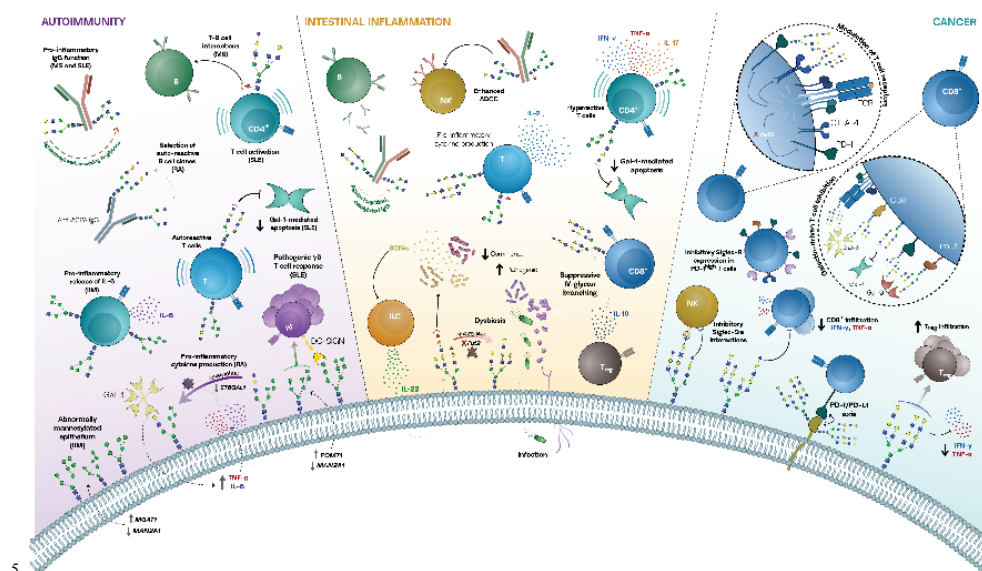


Figure 1: Glycans and glycan binding proteins as central regulators in the immunological networks contributing to inflammation (A), autoimmunity (B) and cancer (C). Cellular *N*-glycosylation undergoes dynamic alterations during the transition from health to pathological conditions, being crucial molecules involved in the regulation of both pro-inflammatory/autoreactive immune responses and immunosuppressive networks associated with inflammation/autoimmunity and progression to malignancy, respectively. *N*-glycans structures engage with glycan binding proteins (GBPs) to regulate immune response contributing to homeostasis. Changes in glycosylation occur as a result of genetic or environmental stimuli, triggering pathological conditions such as inflammation, autoimmunity and cancer. Less complex *N*-glycans such as mannose-enriched structures observed in both epithelial and immune compartments play a key role in the breakdown of self-tolerance during intestinal inflammation and autoimmunity. Abnormalities in cellular glycosylation patterns and their interplay with GBPs impact both cellular and humoral immune responses. Moreover, changes in mucosa glycosylation also perturb the symbiotic relationship between host and microbiome culminating in dysbiosis associated with intestinal inflammation and increased susceptibility to infections. In cancer, overexpression of branched *N*-glycans display a dual-edged sword, orchestrating tumor immune evasion. Branched *N*-glycans not only impose invasive and pro-malignant features to cancer cells but also serve as camouflage, shielding tumor cells from immune surveillance, while simultaneously disrupting immune effector functions. Concomitantly, altered *N*-glycans on T cells surface regulate anti-tumor immune response through

interaction with GBPs (such as galectins and siglecs) dampening their immune function and perpetuating immunosuppression within tumor microenvironment.

3.6 Leveraging *N*-glycosylation in GlycoMedicine

5 With glycans emerging as master regulators in the pathogenesis of major diseases such as chronic inflammatory and autoimmune diseases as well as cancer, new therapeutic perspectives are rising for these sugars as potential clinical targets ^{31,256,257}. Glycan-based biomarkers (see Table 1) and therapies (see Table 2) have been a remarkable field of interest for the clinical
10 management of diseases.

3.6.1 Glyco-biomarkers

Several studies have elucidated the relevance of **tissue-specific glycome** in various pathologies, specifically as potential glyco-biomarkers. Notably, lower
15 levels of branched *N*-glycans in colon biopsies of IBD patients at the time of diagnosis predict non-response to standard therapy with 75% accuracy. In contrast, high levels of branched *N*-glycans in UC patients were associated with a positive therapeutic and disease outcome. Furthermore, it was shown that low levels of complex *N*-glycans combined with high C-reactive protein (CRP)
20 levels, a marker of inflammation, dictates a poor prognosis in terms of non-response to therapy. This glyco-biomarker combined with CRP levels constitutes a powerful tool to improve prognosis and clinical-decision making process in IBD ¹⁰. Similarly, abnormal mannosylated *N*-glycans on kidney biopsies of LN patients predict the development of chronic kidney diseases
25 (CKD) with 93% specificity ¹². In context of cancer, certain molecular patterns

associated with tumor *N*-glycome have been used as a tool for disease monitoring^{258–260}.

Serum glycome has also been explored as important biomarkers of diseases. In fact, patients with active UC showed higher serological levels of L-selectin, Gal-1 and Gal-3 compared with healthy individuals^{261–263}. Specifically, Gal-1 has discriminated UC patients from healthy individuals with 87% of specificity and similar sensitivity²⁶². In SLE patients, a clinical trial is currently ongoing to evaluate the potential of Gal-9 as a biomarker of disease (NCT04558814)¹⁶⁸. In cancer, circulating Gal-1 is associated with higher recurrence and lower survival²⁶⁴ in melanoma patients treated with the anti-VEGF antibody bevacizumab²⁶⁴. Additionally, higher levels of α 2,6-sialylated and branched structures identified in serum glycome of pancreatic cancer patients estimate a poorer prognosis^{258,259}. CRC patients are enriched in multi-antennary and terminally sialylated or fucosylated, whose alterations follow the disease progression²⁶⁵. Moreover, the features of serum glycome could distinguish early non-small cell lung cancer patients from healthy individuals²⁶⁶.

Serum antibodies are also a fundamental piece in the pathogenesis of inflammatory and autoimmune diseases being an important tool to improve disease treatment and diagnosis. Firstly, antibodies against glycans or antibodies with altered glycosylation have been used in IBD diagnosis and to differentiate UC from CD patients. In fact, ASCA (anti-*Saccharomyces cerevisiae* antibodies), antibodies that target mannose-enriched glycans, are the most accurate biomarker of CD being widely associated with the severity of the disease^{267,268}. Anti-glycoprotein 2 and anti-mannobioside carbohydrate IgGs^{269,270} are also used for IBD diagnosis. Moreover, patients with CD exhibited

increased agalactosylation IgGs levels, which positively correlates with levels of CRP, conferring a bad prognosis characterized by extensive and progressive disease^{136,271}. Furthermore, in RA, glycan alterations of IgGs are present years before the onset of the disease²⁷². Specifically, the ACPA-IgGs variable domain glycosylation (VDGs), enriched in bisecting and sialylated *N*-glycans structures, predicted the development of RA²⁷³. Interestingly, at the time of RA diagnosis, the lower abundance of ACPA VDGs was associated with drug-free remission²⁷⁴. In SLE and MS diseases, altered IgG *N*-glycome are also potential biomarkers of disease development^{275,276}.

All these applications demonstrate how *N*-glycome assemble valuable information for the early detection and monitoring of inflammatory-, autoimmune-mediated diseases and malignancy.

Table 1. Glycan-based biomarkers in the realms of chronic inflammation, autoimmunity and cancer

	Biomarkers	Source
Chronic Inflammation (IBD)	Low expression of complex branched <i>N</i> -glycans (correlated with high calprotectin (CRP) levels) ¹⁰	Tissue (colon mucosa)
	Increased L-selectin, Galectin-1 and Galectin-3 levels ^{261–263}	Serum
	Increased levels of anti-Saccharomyces cerevisiae (ASCA), Anti-glycoprotein 2 and anti-mannobioside carbohydrate IgGs ^{269,270}	Serum
	Increased agalactosylated IgGs; Decreased IgG fucosylation (UC) and increased IgG fucosylation (CD); Higher IgG bisection (CD) ^{135–137,271}	Serum
Autoimmunity	High expression of Mannosylated <i>N</i> -glycans ^{12,14} – SLE and IIM	Tissue (Kidney and Muscle)
	Increased Galectin-9 ¹⁶⁸ – SLE	Serum
	Antibodies against citrullinated peptides (ACPA) IgGs variable domain glycosylation enriched in bisecting and sialylated <i>N</i> -glycan structures ²⁷³ - RA	Serum
Cancer	Increased Galectin-1 ²⁶⁴	Serum
	Increased levels of circulating α 2,6 sialylated and branched structures in plasma proteins ²⁵⁹	Serum
	Enrichment in multi-antennary and terminally sialylated/ fucosylated glycans ^{260,265}	Tissue (colon)

3.6.2 Glycan-based therapies

Despite the recent advances in the therapeutic approaches for cancer, inflammatory and autoimmune diseases, considerable subset of patients remains refractory to conventional treatment or do not achieve sustained remission^{277–}
⁵ ²⁸². Remarkably, the possibility of reprogramming cellular glycosylation as a way of achieving homeostasis is highly appealing. In fact, GlcNAc supplementation has a positive effect on altered mucosal T cell response from UC patients, suggesting the potential immunomodulatory properties of GlcNAc as a therapeutic approach for patients with UC^{8,9}. In line, a pilot study using
¹⁰ GlcNAc, has been developed in pediatric IBD²⁸³. Here, oral GlcNAc administration was reported to improve intestinal mucosal lining associated with clinical remission²⁸⁴. Moreover, *ex vivo* supplementation of GlcNAc in kidney biopsies recovered the homeostatic glycome, resulting in decreased infiltration of $\gamma\delta$ T cells, a lower content of inflammatory cytokines, such as IL-17a, IFN- γ ,
¹⁵ TNF- α and up-regulation of IL-10¹². Additionally, in MS and in IBD, *in vivo* GlcNAc administration was able to restore the branched *N*-glycans in TCR leading to a reduced T cell hyperactivation, avoiding inflammatory demyelination and disease severity^{8,9,44}. In IIM patients, the *ex vivo* GlcNAc treatment of fresh muscle biopsies also attenuated the inflammatory response
²⁰ promoting a higher content of branched *N*-glycans and IL-4, and reducing the levels of IL-6¹⁴. In GNE myopathy patients, an open-label phase 2 study (NCT02346461) had showed that oral administration of ManNac was safe and effective in enhancing sialylation in these patients, where a clinical trial is currently active to test the effectiveness of this approach (NCT04231266)¹⁵⁷.
²⁵ All these evidence suggests glycosylation reprogramming through glycan

supplementation as a promising therapeutic approach in chronic inflammation and autoimmunity, where two clinical trials are being assessed: a phase II/III trial (NCT01893606) and a phase III trial (NCT02504060) ²⁸³.

From an IgGs therapeutic perspective, *in vivo* administration of sialylated IgGs prevented the development of LN and arthritis by hampering the inflammatory responses mediated by pathogenic Th17 cells and autoantibodies ²⁸⁵. Moreover, in mouse models of LN and RA, the administration of solubilized glycosyltransferases, namely B4GALT1 or ST6GAL1, aiming to recover the levels of galactose and sialic acid respectively, was able to convert the pathogenic IgG in an anti-inflammatory phenotype controlling the inflammatory response ²⁸⁵. Immune complexes in SLE and joint swelling, as well as immune cell recruitment observed in RA were substantially improved through the treatment of autoantibodies with Endoglycosidase S, leading to the removal of *N*-glycans in IgGs ^{286,287}. Additionally, the depletion of autoreactive B cells was also considered a potential therapy mainly in autoimmunity, taking advantage of anti-CD20 monoclonal antibodies like Rituximab, ocrelizumab, and ofatumumab that are currently used for RA, MS and SLE patients ^{288–292}. Interestingly, a novel anti-CD20 monoclonal antibody with a distinct glycosylation profile of Fc-domain (G2 glycosylation pattern without fucose) was described as an optimized version of Rituximab, displaying an increased binding affinity for FcγRIIIa and a higher antibody-dependent cellular cytotoxicity in B cell lymphoma, being more effective in eliminating autoreactive B cells ^{293,294}. In SLE, treatment with neuraminidase has restored the Gal-1 binding to T cells, promoting an anti-inflammatory environment ¹⁶⁷. In a mice model of RA, the administration of Gal-1 resulted in clinical

suppression due to anti-inflammatory responses mediated by Th2²⁹⁵. On the other hand, the depletion of Gal-3 resulted in fewer clinical manifestations and lower levels of inflammation in RA¹⁷³ and MS¹⁶¹ mice models.

The therapeutic potential of galectins and siglecs in TME has also been studied. In fact, targeting Gal-1 binding improved CD8⁺ T cell infiltration and its cytotoxic capacity, enhancing tumor killing^{296–298}. Similarly, the blocking of Gal-3 with galactomannan, GM-CT-01, in patient-derived TILs was shown to be effective in improving CD8⁺ T cell anti-tumor response²⁹⁹. Additionally, Gal-3 inhibitor, GR-MD-02, was also tested in combination with immune blockade antibodies in solid tumors clinical trials³⁰⁰. Regarding siglecs, monoclonal antibodies were tested against Siglec-7, -9 and -15^{191,195,301–303}. Moreover, CAR-T cells engineered to express Siglec-7 and -9 demonstrated enhanced anti-tumor capacity, through improved recognition of sialoglycans on tumors³⁰⁴. In terms of GBP interactions, the most widely approach considered is the targeting, either with antibodies or binding peptides, of tumor antigens to GBP on innate immune cells to generate an enhanced immune response^{305–308}. The use of GBP agonists has also been explored as an anti-tumor therapy. For instance, the use of an agonist of Dectin-1, β -Glucan, showed promising results in combination with chemotherapy, improving long term survival of OC patients³⁰⁹.

Given the prominent role of glycans in the treatment of chronic inflammation and autoimmune diseases, also, in cancer, aberrant *N*-glycosylation poses a promising target in cancer treatment and precision medicine. One notable case is the conjugation of the anti-HER2 monoclonal antibody trastuzumab with a sialidase, which specifically targets HER2⁺ cells for desialylation. This

glycoreprogramming will favor CD8⁺ T cells and NK cell-mediated cytotoxicity by precluding Siglec engagement ^{310,311}. Furthermore, fluorinated analogues of sialic acid, which inhibit sialylation, abrogated *in vitro* migration and *in vivo* tumor growth ^{312,313}. *N*-glycan branching is another relevant feature of tumors with therapeutic potential. Indeed, treatment with 2-deoxyglucose (2DG), a glucose analogue that is converted in D-mannose entering *N*-glycosylation pathway, sensitize pancreatic tumor cells to CAR-T cell killing *in vitro* and *in vivo* ³¹⁴. Thus, glycan reprogramming may assist in treating solid tumors that are unresponsive to therapy such as CAR-T cell therapy. Other aberrant patterns of *N*-glycosylation, such as enhanced fucosylation have also been explored with the same goal ³¹⁵. Moreover, and as mentioned previously, T cells are highly regulated by *N*-glycosylation, which makes them a potential target to reinvigorate T cell function against tumors. To alter the *N*-glycosylation profile of T cells, Sasawatari and colleagues treated human T cells with 2DG showing an improved T cell anti-tumor immune response, characterized by increased cytotoxicity, IL-2R surface retention and inhibition of Gal-3 binding ³¹⁶. In the same line, Toyofuku and colleagues treated CAR-T cells with 2DG and observed that these cells had less binding affinity to immune-inhibitory receptors, presenting an increased cytotoxicity ³¹⁷. In particular, *N*-glycosylation of T cell receptors can also be target, as for example *N*-glycosylation of PD-1 and CTLA-4. For instance, therapeutic antibodies (STM418) to target the N58-glycan presented on PD-1 were developed and showed an enhanced induction of anti-tumor responses ²²². Furthermore, by editing the coding sequence of the N74 residue of *PDCD1* gene to remove PD-1 *N*-glycosylation reduced its expression on cell surface, improving anti-tumor activity of CAR-T cells ³¹⁸. Likewise,

therapeutic antibodies against the *N*-glycosylated forms of CTLA-4 (mbAb146) were also developed to increase T cell efficacy against tumors ³¹⁹.

Overall, and building upon the master regulatory properties of *N*-glycans in the immunopathogenesis of major diseases, their role as promising diagnostic and prognostic biomarkers, as well as potential therapeutic targets hold the promise of revolutionizing the glycomedicine field in a near future.

Table 2. *N*-glycosylation as potential targets for therapies in chronic inflammation, autoimmunity and cancer

	Glycotherapy	Target	Therapeutic effect
Chronic Inflammation (IBD)	GlcNAc supplementation ^{8,9}	T cells	Promotes <i>N</i> -glycan branching and a controlled immune response
Autoimmunity	GlcNAc supplementation ^{12,14,44,157} – SLE, MS, IIM	Tissue and T cells	Promotes tissue homeostasis by glycome reprogramming and controlled immune response
	Treatment with glycosidases ^{167,285–287} – SLE, RA	IgGs and T cells	Potentiates anti-inflammatory functions of IgGs and controlled immune response
	Administration of soluble glycosyltransferases ³²⁰ – SLE, RA	IgGs	Converts pathogenic IgGs into an anti-inflammatory phenotype
	Administration of Galectin-1 ^{162,295} – RA, MS	T cells	Potentiates Th2-mediated anti-inflammatory responses
Cancer	Blocking Galectin-1 binding ^{296–298}	T cells	Increased infiltration of CD8 ⁺ T cells and enhanced tumor killing
	Blocking Galectin-3 binding (GM-CT-01; GR-MD-02) ^{299,300}	T cells	Enhances anti-tumor CD8 ⁺ T cell response
	Siglec-7/9 expressing CAR-T cells ³⁰⁴	Tumor cells	Promotes the recognition of tumor sialylated glycans and potentiates CAR-T-mediated anti-tumor immunity
	Treatment with β -Glucan agonist ³⁰⁹	Dectin-1 in NK cells	Improved long term survival of ovarian cancer patients
	Combination of trastuzumab and sialidases ^{310,311}	HER2 ⁺ cancer cells	Desialylation of HER2 ⁺ cancer cells and enhanced CD8 ⁺ T cells and NK cell-mediated cytotoxicity
	Fluorinated analogues of sialic acid ^{312,313}	Sialylation in tumor cells	Precludes sialylation in cancer cells and causes the impairment of cell adhesion, migration, and tumor growth
	Treatment with 2-deoxyglucose (2DG) ^{314,316,317}	Tumor cells	Impairs <i>N</i> -glycan branching and improves CAR-T-mediated tumor killing and cytotoxicity
		T cells	Improves T cell anti-tumor response by increasing IL-2R retention and hampering Gal-3 binding
	Therapeutic antibodies (STM418) ²²²	PD-1 ⁺ T cells	Target glycans specifically on site N58 to increase anti-tumor responses and avoiding T-cell exhaustion
	Editing of N74 residue of PDCD1 gene ³¹⁸	PD-1 ⁺ T cells	Removes PD-1 <i>N</i> -glycosylation and improves anti-tumor function of CAR-T cells
	Therapeutic antibodies (mbAb146) ³¹⁹	CTLA-4 ⁺ T cells	Target CTLA-4 glycans to enhance T cell immune response

3.7 Conclusion

The interaction between glycans and GBPs constitutes a fundamental hub that tightly modulates host immune cell response both in homeostasis and in disease context. In this chapter, we comprehensively discussed how glycans and GBPs

integrates the canonical circuits that regulate both innate and adaptive immune responses. We discussed the main *N*-glycosylation alterations that occur in the transition from health to disease contexts, specifically in intestinal inflammation, autoimmunity, and malignant transformation, as summarized in Figure 1. Additionally, we addressed the potential clinical applications of *N*-glycans and GBPs as promising clinical glyco-biomarkers and novel targets for the development of new glycan-based therapies for improving personalized medicine, as described in Table 1 and Table 2.

10 Acknowledgement

This work was co-funded by the European Union (ERC, GlycanSwitch, Grant Agreement N° 101071386) and by the (GlycanTrigger, Grant Agreement N°: 101093997). Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or the European Research Council Executive Agency. Neither the European Union nor the granting authority can be held responsible for them. SSP acknowledges funding by the US Department of Defense, US Army Medical Research Acquisition Activity, FY18 Peer Reviewed Medical Research Program Investigator-Initiated Research Award (award number W81XWH1920053). SSP also acknowledges funding from European Crohn's and Colitis Organisation (ECCO) Pioneer Award 2021; the International Organization for the study of Inflammatory Bowel Disease (IOIBD) and the Portuguese Foundation for Science and Technology – FCT (EXPL/MED-ONC/0496/2021). SSP acknowledges 2022 Lupus Research Alliance (LRA Lupus Innovation Award) and Liga Portuguesa Contra o Cancro LPCC and MULTICARE for funding (C 3035006C17). JG

acknowledges European Society of Clinical Microbiology and Infectious Diseases (ESCMID Research Grant 2022) and European Crohn's and Colitis Organization for funding (ECCO Grant 2023). IA acknowledges funding from Ordem dos Médicos and BIAL Foundation (Maria de Sousa Award 2023), from
 5 Group of Studies for Autoimmune Diseases and from European Society for Clinical Investigation (ESCI Exploratory Research Grant 2024). AF acknowledges Portuguese Foundation for Science and Technology (FCT) for funding (2022.01422.PTDC). CMA [BD/07357/2021]; BSP [UI/BD/151550/2021]; ELG [UI/BD/152866/2022]; CSR [2020.08422.BD]; JG
 10 [2020.00088.CEECIND] and IA [2022.00337.CEECIND] received funding from the FCT of the Portuguese Ministry of Science, Technology and Higher Education.

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