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IMMUNOBIOLOGICAL STUDIES ON TWO HUMAN PATHOGENS: GROUP B *STREPTOCOCCUS* AND *ESCHERICHIA COLI*

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A safe and stable neonatal vaccine targeting GAPDH confers protection against GBS infections in adult susceptible mice. Joana Alves, Pedro Madureira, Maria Teresa Baltazar, Leandro Barros, Liliana Oliveira, Ricardo Jorge Dinis-Oliveira, Elva Bonifácio Andrade, Adília Ribeiro, Luís Mira Vieira, Patrick Trieu-Cuot, José Alberto Duarte, Félix Carvalho, Paula Ferreira. PLoS One. 2015 Dec 16;10(12):e0144196. doi: 10.1371/journal.pone.0144196.

An unexpected increase of the susceptibility to *Escherichia coli* infection in offspring of mother vaccinated with GAPDH from this pathogen. Joana Alves, Inês Lopes, Elisabete Teixeira, Adília Ribeiro, Patrick Trieu-Cuot, Paula Ferreira. *In preparation*

Using a neonatal mice model of *Escherichia coli* oral infection to decipher the innate immune response against this pathogen. Joana Alves, Helena Pinheiro, Leandro Barros, Inês Lopes, Elisabete Teixeira, Pedro Melo, Adília Ribeiro, Patrick Trieu-Cuot, Paula Ferreira. *In preparation*

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SUMMARY

Group B *Streptococcus* (GBS), a common designation for *Streptococcus agalactiae*, is the leading cause of morbidity and mortality in newborns and a rising agent of serious invasive diseases in immunocompromised non-pregnant adults. The *intrapartum* antibiotic prophylaxis to GBS colonized women has help in reducing the incidence of early onset GBS disease to the actual values. However, the limitation of these programs, the risk associated with antibiotic overuse, and the increasing rates of infection in adults, highlight the need for an effective GBS vaccine.

We have demonstrated that the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a GBS virulence factor and is an effective vaccine in a neonatal mice model of GBS lethal infection. Having in mind the emergence of GBS infection in non-pregnant adults, and the intent to use this vaccine in clinical practice, the first aim of this thesis was to evaluate the safety of the GAPDH vaccine and its efficacy against GBS infection in an adult susceptible and in a diabetic mouse model. For the purpose of testing the safety of the vaccine formulations, a comprehensive series of toxicological and clinical parameters were evaluated in repeated-dose immunized animals and related controls. To test its efficacy, sham- or rGAPDH-immunized adult susceptible mice were infected with GBS serotype Ia and V strains, the two serotypes most associated with the invasive disease in adults. Sham and vaccinated mice were also rendered diabetic by streptozotocin administration and infected with the serotype V GBS strain. The obtained results showed that GBS GAPDH vaccine is safe and confers protection against the infections caused by GBS in susceptible and diabetic adult mice.

GAPDH-based vaccines have been proposed against several bacterial and parasitic diseases due to its ability to bind to host extracellular matrices and/or modulate the host immune responses. The second aim of this thesis was to test whether this vaccination strategy could also be used against another neonatal pathogen, the *Escherichia coli*. In the last decades, this Gram-negative bacterium rise in importance as a cause of neonatal sepsis and meningitis, affecting mainly preterms and very low birth weight infants. Due to the increase on antibiotic-resistant *E. coli* strains and to the improvement of the survival of preterm infants, it is believed that the rates of *E. coli* neonatal infections will continue to grow in the next years.

Therefore, the development of efficient therapies against *E. coli* neonatal infections is urgent. The evaluation of the cross-reactivity showed that antibodies raised against the recombinant GBS GAPDH were not able to recognize *E. coli's* GAPDH, and *vice versa*, ruling out the possibility of cross-protection. For the vaccination study, female mice were immunized with *E. coli* GAPDH and the progeny was orally infected with the pathogen. Unexpectedly, maternal vaccination with the *E. coli* GAPDH vaccine not only did not confer protection to pups against oral infection, but increased the mortality of infected animals. These results show that GAPDH cannot be used as a target antigen for vaccination against *E. coli* infections, and there must be caution in the development of GAPDH-based vaccines.

The reason why newborns, especially preterm, are so susceptible to *E. coli*-causing sepsis and meningitis strains (mostly represented by *E. coli* K1) is not clear. Therefore, the third aim of this thesis was to characterize the innate immune response to *E. coli* K1 using a neonatal mouse model of infection that address the pathogenesis of vertical transmission that occurs in humans. We adapted a model of *E. coli* K1 oral infection, used in neonatal rats, to the mouse model. Thus, less than 24 or 48 h old Balb/c pups were orally infected with $2x10^6$ colony-forming units (CFUs) of *E. coli* K1 and sacrificed at specific timepoints. Similar to what happens in humans, an age-dependency for the systemic *E. coli* infection was observed, where only mice with less than 24 h are susceptible to this pathogen. To determine which immune mediators were produced first in response to *E. coli* K1, we started by a kinetics study of the bacterial colonization in blood, lung, liver, spleen and brain, during the first 48 h of infection. As soon as 30 min post-infection, *E. coli* K1 was detected in the pup's blood, lung and liver. However, the inflammatory response to this bacteraemia, evaluated by the production of pro-inflammatory cytokines and chemokines, and phagocyte recruitment to infected organs, was only observed 6 h post-infection. This delay in the development of an immune response was not due to IL-10, since antibody blocking the signalling of this cytokine did not influence the outcome of infection. On the other hand, anti-IL-1R treatment increased the susceptibility to *E. coli*, providing evidence that the pro-inflammatory cytokine IL-1β, has a protective role during *E. coli* K1 neonatal infection. These findings indicate that *E. coli* K1 is able to rapidly colonize the neonatal mice but the infected host does not promptly develop an immune response. Understanding the reason for the

delay of the immune response activation could allow the discovery of therapeutic targets against *E. coli* neonatal infections.

In conclusion, we have, in this thesis, determined the safety and efficacy of the GAPDH-vaccine against GBS adult infections and *E. coli* neonatal infections and characterized a murine model of *E. coli* K1 oral infection, uncovering the kinetics of immune response to this bacterium.

RESUMO

A bactéria estreptococos do grupo B (EGB) ou *Streptococcus agalactiae* é a principal causa de mortalidade e morbidade em recém-nascidos e um agente patogénico emergente de doença invasiva em adultos imunocompometidos. A profilaxia antibiótica *intrapartum* a mulheres colonizadas com esta bactéria permitiu reduzir a incidência das doenças neonatais de início precoce causadas por EGB para os níveis actuais. Contudo, as limitações desta profilaxia, o risco associado ao uso abusivo de antibióticos, bem como o aumento das infecções em adultos, realçam a necessidade do desenvolvimento de uma vacina eficaz contra as infecções por EGB.

O nosso grupo mostrou que a enzima glicolítica gliceraldeído-3-fosfato desidrogenase (GAPDH) é um factor de virulência do EGB e, quando usada como vacina, confere protecção contra infecções letais por esta bactéria em ratinhos recém-nascidos. Tendo em consideração o aumento das infecções por EGB em adultos não gestantes, assim como a intenção do uso desta vacina nos humanos, o primeiro objectivo desta tese foi avaliar a segurança da vacina de GAPDH e a sua eficácia contra as infecções por EGB em ratinhos adultos susceptíveis ou diabéticos. De forma a testar a segurança das formulações da vacina, foi avaliado um conjunto extenso de parâmetros toxicológicos e clínicos em animais que receberam várias doses da vacina e nos respectivos controlos. Para testar a sua eficácia, ratinhos adultos susceptíveis foram imunizados com a GAPDH recombinante (rGAPDH) ou apenas com o adjuvante da vacina e foram infectados com EGB de estirpes pertencentes ao serotipo Ia ou V, as mais associadas a infecções invasivas em adultos. Induziu-se a diabetes em ratinhos imunizados com a vacina ou apenas com o adjuvante através da administração de estreptozotocina e em seguida infectaram-se com EGB do serotipo V. Os resultados obtidos provam que a vacina de GAPDH de EGB é segura e confere protecção contra infecções causadas por esta bactéria em ratinhos adultos susceptíveis e diabéticos.

Vacinas baseadas na GAPDH têm sido propostas contra várias doenças bacterianas e parasitárias graças à capacidade desta enzima se ligar a componentes da matriz extracelular e/ou de modular a resposta imune do hospedeiro. O segundo objectivo desta tese foi por isso, testar se esta estratégia

de vacinação poderia ser utilizadas contra outro patogéneo neonatal, a *Escherichia coli*. Nas últimas décadas, a importância desta bactéria Gram-negativa como causadora de sépsis e meningite neonatal, principalmente em prematuros e crianças com muito baixo peso à nascença, tem crescido de forma preocupante. Devido ao aumento de estirpes de *E. coli* resistentes a antibióticos estima-se que o número de infecções neonatais por *E. coli* continuarão a crescer nos próximos anos. É, portanto, urgente desenvolver terapias eficazes contra as infecções neonatais por esta bactéria. A avaliação da reactividade cruzada mostrou que os anticorpos contra a GAPDH recombinante do EGB não são capazes de reconhecer a GAPDH da *E. coli*, e vice-versa, excluindo assim a possibilidade de protecção cruzada. Para os estudos de vacinação, ratinhas fêmeas foram imunizadas com a GAPDH de *E. coli* e a descendência infectada oralmente com o patogéneo. Inesperadamente, a vacinação materna com GAPDH de *E. coli* em vez de conferir protecção aos recém-nascidos contra a infecção aumentou a mortalidade dos animais infectados. Estes resultados provam que a GAPDH não pode ser usada como alvo de vacinação contra infecções por *E. coli* e alertam para o cuidado a ter no desenvolvimento de vacinas baseadas nesta proteína.

A razão pela qual os recém-nascidos, especialmente prematuros, são tão susceptíveis à sepsis e meningite provocadas por estipes de *E. coli* (representadas essencialmente pela *E. coli* K1) não é ainda claro. Por este motivo, o terceiro objectivo desta tese foi caracterizar a resposta imune inata à *E. coli* K1, usando um modelo de murganhos recém-nascidos que respeita a patogénese da transmissão vertical que ocorre bos humanos. Adaptámos um modelo de infecção oral com *E. coli* K1 usada em ratos recém-nascidos, para ratinhos recém-nascidos. Deste modo, ratinhos Balb/c com menos de 24 ou 48 h horas de vida foram infectados oralmente com $2x10^6$ células de *E. coli* K1 e sacrificados a diferentes tempos experimentais. À semelhança do que acontece em humanos, observámos uma dependência da idade para a infecção sistémica com *E. coli,* só os ratinhos infectados antes das 24 h de vida eram susceptíveis a esta bactéria. De forma a determinar que mediadores imunes estão a ser produzidos primeiro na resposta imune à *E. coli* K1, começamos por um estudo cinético da colonização no sangue, pulmão, fígado, baço e cérebro, nas primeiras 48 horas da infecção. Logo aos 30 minutos após infecção, *E. coli* K1 foi detectada no sangue, pulmão e fígados dos

ratinhos recém-nascidos. Contudo, a resposta inflamatória a esta bacteriemia, avaliada pela produção de citocinas pro-inflamatórias e quimiocinas, e recrutamento de fagócitos para os órgãos infectados, foi apenas observada 6 horas após a infecção. Este atraso no desenvolvimento da resposta imune não foi consequência da produção de IL-10, uma vez que o bloqueio da sinalização desta citocina não influenciou o resultado da infecção. Por outro lado, o tratamento com anti-IL1R aumentou a susceptibilidade à *E. coli*, indicando um papel protector da citocina pró-inflamatória IL-1β durante a infecção neonatal por *E. coli* K1. Estas evidências indicam que a *E. coli* K1 é capaz de rapidamente colonizar os ratinhos recém nascidos, contudo estes não conseguem desenvolver prontamente uma resposta imune. A compreensão dos mecanismos moleculares e celulares deste atraso poderá permitir a descoberta de novos alvos terapêuticos contra infecções neonatais por *E. coli*.

Em conclusão, nesta tese, determinámos a segurança e eficácia da vacina GAPDH contra infecções por EGB em adultos e infecções em recém-nascidos por *E. coli*, e caracterizámos um modelo de infecção oral com *E. coli* K1 em murganhos, avaliando a cinética da resposta imune neonatal a esta bactéria a tempos muito iniciais da infecção.

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ABBREVIATION LIST

AMP - antimicrobial peptides

AP - activator protein

AslA - arylsulfatase-like A

AT1R - angiotensin II receptor type 1

BBB - blood-brain barrier

BMEC - brain microvascular endothelial cells

C4 bp - C4-binding protein

C9 - complement factor 9

CD - cluster of differentiation

CDC - Centers for Disease Control and Prevention

CIP - complement interfering protein

CNF - cytotoxic necrotizing factor

CNS - central nervous system

CPS - Capsular Polysaccharide

CSF - cerebrospinal fluid

Csp - cell-surface protease

DAMP - danger-associated molecular patterns

DC - dendritic cell

EOD - Early onset disease

EGB – Estreptococcus do Grupo B

ESBL - extended-spectrum beta-lactamase

EsiB - *Escherichia coli* secretory immunoglobulin A-binding protein

ExPEC - extraintestinal pathogenic *Escherichia coli*

Fbs - Fibrinogen-binding protein

FcγRIa - alpha chain of Fcγ receptor I

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

GAS - Group A *Streptococcus*

GBS - Group B *Streptococcus*

GLP - Good Laboratory Practices

HA - hyaluronan

HBMEC - human brain microvascular endothelial cells

HvgA - surface-anchored hypervirulent adhesin

IAP - *intrapartum* antibiotic prophylaxis

IFN - interferon

iNOS - inducible nitric oxide

Iss - increased serum survival

KO - knock out

Lmb - laminin-binding protein

LOD - Late-onset disease

LPS - lipopolysaccharide

LPxTG - (Leu–Pro–X–Thr–Gly)

LRR - leucine-rich-repeat

LTA - lipoteichoic acid

MAC - membrane attack complex

MET - macrophage extracellular trap

NMEC – neonatal meningitis-associated E. coli

NET - neutrophil extracellular trap

NF - nuclear factor

NLRP3 - NOD-like receptors (NLRs) family pyrin domain- containing 3

NOD - nucleotide-binding oligomerization domain

OmpA - outer membrane protein A

PAMP - pathogen-associated molecular patterns

PBP - Penicillin-binding protein

PRR - pattern recognition receptors

ROS - reactive oxygen species

SEPEC - sepsis causing *E. coli*

SIgA - secretory immunoglobulin A

Siglecs - immunoglobulin-like lectins

Sip - surface-immunogenic protein

SodA - superoxide dismutase

SPF - specific-pathogen free

Srr - serine-rich repeat protein

ssRNA - single-stranded RNA

ST - Sequence Type

TLR - Toll-like receptor

TSP-1 - thrombospondin 1

TT - tetanus toxoid

UK - United Kingdom

UPEC - uropathogenic *E. coli*

US - United States

VLBW - very low birth weight

β-H/C - β-haemolysin/cytolysin

THESIS OUTLINE

The present thesis is organized in three main chapters: Introduction, Results and Final Discussion.

The experimental work reported in this thesis focus on three related projects.

In Chapter I, a general introduction, reviewing the main topics addressed in this thesis, is presented. It intends to give the state of the art concerning the relevance of the two human pathogens: Group B *Streptococcus* and *Escherichia coli* infections in neonates and adults, the significance of GAPDH as virulence factor and vaccination target, and the pathophysiology of neonatal infections caused by *Escherichia coli*.

In Chapter II, the Results section, compiles a set of studies and are presented in the form of three manuscripts. Thus, experimental data are accompanied by an abstract, introduction, methods, results and discussion, and references.

The general discussion of the work is presented in Chapter III, and intends to illustrate the major contributions of this research for the immunobiological knowledge of GBS and *E. coli*, and also presents/discuss new research directions.

Introduction and Discussion chapters were written trying to avoid repeating ideas also presented in the Results section. Nevertheless, some information presented in this thesis is reiterated in different sections in order to highlight the importance of the presented data.

SCOPE OF THE THESIS

This work is divided in three major goals each one connected with the previous:

I. To investigate the safety, stability and effectiveness of the neonatal rGAPDH$_{GBS}$ vaccine on an adult susceptible mouse model of GBS infection.

II. To determine whether a GAPDH-based vaccine could be used against *Escherichia coli* neonatal infection

III. To develop a mouse model of *E. coli* K1 infection that address the pathogenesis of human vertical transmission and to characterize the neonatal innate immune response against this pathogen.

CHAPTER I.
INTRODUCTION

I - STREPTOCOCCUS AGALACTIAE OR GROUP B STREPTOCOCCUS

Streptococcus agalactiae also referred to as Group B *Streptococcus* (GBS) is known as the leading cause of life-threatening bacterial infections in newborns [1,2]. However, in the last decades its importance as a pathogen in immunocompromised adults increased to levels that cannot be dismissed [3,4].

I.1 From a veterinary to a human pathogen

GBS, a Gram-positive β-hemolitic diplococcus bacterium, was first reported in 1887 by Norcard and Mollereau as a veterinary pathogen causing bovine mastitis [5]. One of the most relevant consequence of bovine mastitis is the loss of ability to produce milk, a characteristic that rendered the *Streptococcus agalactiae* (*agalactie* is latin for no milk) designation to this in pathogen [6]. In 1933, Rebecca Lancefield, based on the serological cross-reactivity to the cell wall carbohydrate antigens, divided the hemolytic streptococci into groups. In this classification *S. agalactiae* was the only specie belonging to the serogroup B leading to the use of the term “Group B *Streptococcus*” [7].

Only in the late 30’s, was GBS recognized as human pathogen, after Hare and Colebrook noticed that the hemolytic streptococci isolated from vaginal samples resembled those found in mastitis in cattle and when Fry described 3 cases of fatal puerperal sepsis caused by GBS [8,9]. In 1960’s GBS emerged as the leading cause of neonatal infections in the United States (US) and in Europe, due to a replacement of the human GBS population by tetracycline resistant clones [10]. Despite the advances in diagnosis, treatment and prophylaxis, GBS remains the leading agent of neonatal diseases causing morbidity and mortality that affect 0.3 to 3 newborns in every 1000 live births with a mortality rates between 4 and 10% [11-16]. In Portugal, the overall incidence of invasive GBS disease is 0.54 per 1000 live births, with a mortality rate of 6.6% [17]. However, the mortality increases if data is reported to premature infants, with 15.2% fatality rates vs. 6.4% for term infants [16].

I.2 – GBS, a neonatal pathogen

GBS is a common colonizer of the gastrointestinal and genitourinary tracts of healthy adults [18,19]. The presence of GBS in the genital microflora of pregnant women is the major determinant of colonization and infection in neonates [20-22] (Figure1). Maternal colonization increases 24 times the risk of neonatal disease in the first week after birth [21]. It is estimated that one third of pregnant women is chronic, transient or intermittently colonized with GBS in the vagina and/or rectum, making them at 50-70% risk of transmitting this pathogen to their newborn infant in the perinatal period [23-25]. On the other hand, only 5% of infants born from culture-negative woman become colonized during the first 48 h of life. About 2% infants vertically colonized with GBS will develop invasive disease and this risk is proportional to the density of maternal colonization [26,27]. Black race, maternal age of <20 years, low parity, and diabetes are some of the risk factors for positive maternal GBS colonization [28,29].

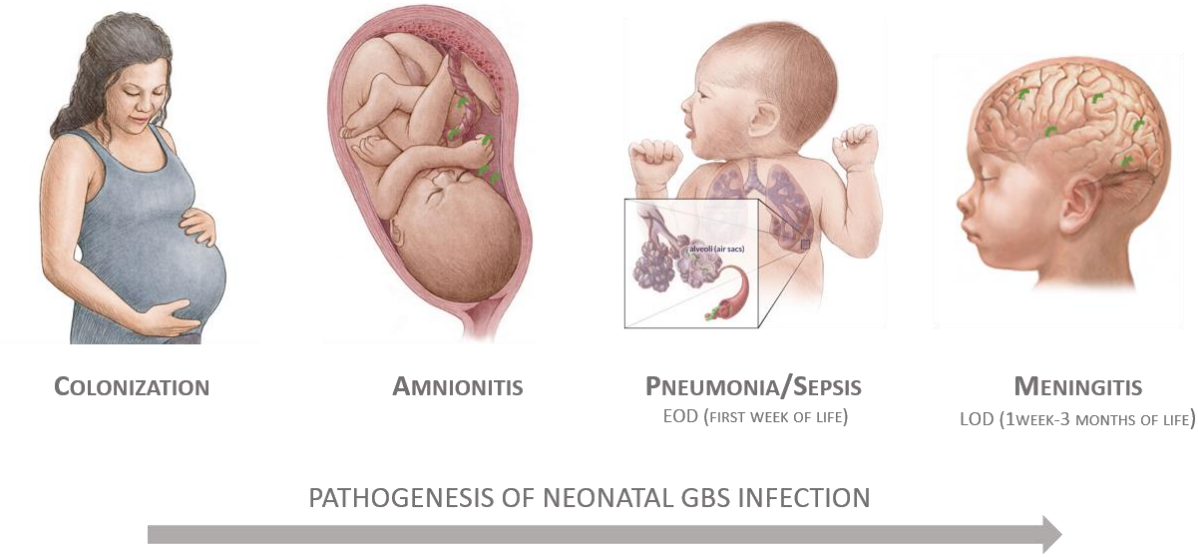

Figure 1. Stages of pathogenesis of neonatal GBS infection. GBS colonizes the gastrointestinal and genitourinary tracts of pregnant women. The fetus can acquire the bacterium *in utero*, leading to amnionitis. When the newborn acquires the bacteria during birth, it causes pneumonia, sepsis or meningitis. The first two are usually early onset manifestations of disease (that occurs in the first week of life), while meningitis is characteristic of late onset disease (occurs after the first week up to three months of age). *Modified from [27]. Illustrations from Amanda Montanez.*

Intrauterine infection of the fetus occurs either by the ascension of GBS to the amniotic cavity or by fetal aspiration of contaminated fluids. This can lead to placental membrane rupture, triggering premature delivery and stillbirths. Alternatively, the newborn can acquire the bacteria during passage through the birth canal, by ingestion or aspiration of infected vaginal fluids, leading to pneumonia and sepsis [2,27,30] (Figure1).

Two distinct syndromes of neonatal invasive GBS disease have been described, according to the timing of manifestation of the symptoms. Early-onset disease (EOD) occurs in the first 7 days after birth, with symptoms being more clinically apparent on the first 24-48 hours in the case of term infants, or in the first 6 hours in the case of preterm newborns [13,31]. Gestational age, maternal *intrapartum* fever, chorioamnionitis, and prolonged rupture of membranes are some of the known risk factors that increase the risk of GBS' EOD [31]. The most typical clinical manifestations of EOD are sepsis (80-85%), pneumonia (10-15%) and meningitis (5 to 10%) [13] (Figure1).

Late-onset disease (LOD) is defined by a GBS infection that starts after the first week up to 90 days of age. LOD cases are characterized by septicaemia (65%) and a high frequency of meningitis (25%). GBS meningitis remains associated with high mortality and leaves 25-50% of surviving infants with long-term health problems, including developmental disabilities, paralysis, seizure disorder, hearing and vision loss [32].

GBS is an encapsulated bacterium that can be subclassified, according to the immunogenic capsular polysaccharides (CPS), into ten serotypes (Ia, Ib, II–IX). Global colonization rates reveal significant regional variations in prevalence and serotype distribution. Currently, serotype Ia, II, III and V are the most frequent GBS isolates from vaginal-rectal cultures of women in Western Europe, North America, South Africa and China [33-36]. Portugal follows a similar profile, with serotypes III (35%), V (33%), Ia (16%) and II (10%) the most commonly found [37]. Serotypes VI and VIII were most frequently detected in Japan and serotype IV was common in United Arab Emirates [38,39].

Regarding the neonatal invasive-disease isolates responsible for EOD, serotype III and Ia were found to be overrepresented [13,40,41]. In the US the most frequent serotypes

associated with EOD were Ia (30%), III (28%), V (18%), and II (13%) [13] while in Portugal, are the serotypes Ia (31%) and III (29%) [42].

In case of LOD the majority, 50-90%, depending on the region, are caused by serotype III infections [13,34,40,42-44]. Almost all cases of meningitis are a consequence of an infection with a highly virulent clone of serotype III that belongs to bovine-derived sequence type (ST) 17 [44]. The impact of ST-17 among invasive neonatal infections is worldwide recognized due to its rapid dissemination and successful adaptation to human neonates [45]. The ability of this clone to cause neonatal meningitis relies on a ST-17-specific surface-anchored protein, the hypervirulent GBS adhesin (HvgA) that enhances GBS adherence and translocation across intestinal epithelium and the blood-brain barrier (BBB) [46].

I.3 The burden of GBS infections in pregnant and non-pregnant adults

GBS colonization in pregnant women in frequently asymptomatic. Nevertheless, in about 0.12 per 1000 live births, it leads to maternal invasive disease, a rate that has been declining since the implementation of IAP [47-50]. Pregnancy increases five times the risk for GBS disease in women and the majority of pregnancy-associated disease occur in the postpartum period [48]. Half of pregnancy-associated invasive GBS disease resulted in upper genital tract, placental and aminiotic infections and resulted in fetal death. The remained cases included manifestations as bacteremia without focus (31%), endometritis without fetal death (8%), chorioamnionitis without fetal death (4%), pneumonia (2%), and puerperal sepsis (2%) [13]. Most obstetric patients with GBS infection, even in the presence of bacteremia, show a rapid response after initiation of antimicrobial therapy. Potential fatal outcomes, although rare, can occur, and include meningitis [51,52], endocarditis [53,54], sepsis [55], epidural abscess [56] and necrotizing fasciitis [57].

In the past 3 decades, invasive GBS infections in non-pregnant adults started to represent a significant and growing health burden worldwide [4]. The age distribution of invasive GBS cases in United Kingdom (UK) and in US shows a clear shift from

neonatal towards non-pregnant adult [4,58]. Data from the UK shows that, between 1991 and 2010, the rate of GBS infection in adults increased from 0.92 to 2.39 per 100,000 individuals [58]. Likewise, the incidence of infections among the adult community in the US more than doubled between 1990 and 2007, from 3.6 to 7.3 cases per 100.000 individuals [4]. The incidence increased in all adult age groups but was most preeminent among older patients (ages from 65 to 79 years old) reaching the value of 22 cases per 100,000 individuals (an increase of 114.7% when compared with adults with <65 years old) [4]. Disease rates also seem to discriminate color and sex of the individuals, with black and males being affected more than their white and female counterparts [59,60]. These infections are associated with substantial morbidity and mortality, particularly in individuals with chronic underlying conditions. Diabetes mellitus is the most frequent comorbid condition associated with GBS infection, typically presented in 20-50% of non-pregnant adults with GBS disease [4,61-63]. Other comorbidities include liver disease (such as cirrhosis), obesity, cardiovascular diseases, cancer, renal disease and neurologic disease [3,4,64,65]. The majority of GBS infected patients presented at least one of these conditions [4,66]. In the case of elderly adults, residence in nursing homes, bedridden state and gastrointestinal disease also appear as factors associated with GBS infections [67-69]. Some studies point out that aging of the population and the rising numbers of patients with chronic disease such as obesity and diabetes, are part of the reason for the observed increase of GBS infections in adults. Another important aspect of these infections is its recurrence (4.3-28%) rate, usually caused by the same strain from the first infection, indicating a bacterial reinvasion from a colonizing site [19,66,70].

The fatality ratio is strikingly higher amongst non-pregnant adults (8%–24%) than the one reported for neonates with GBS invasive disease (4-6%) [4,13] and this proportion increases in the older age groups, showing the importance for the development of a vaccine for these risk group of individuals.

Clinical manifestations of GBS infections in adults can be presented in a large spectrum of pathologies. The most common are bacteremia without focus and skin and soft-tissue infections, mainly in the form of cellulitis [4]. Conditions such as lymphedema, vascular insufficiency, chronic dermatitis, radiation-induced injury and mastectomy are frequently predisposing factors for GBS-caused cellulitis [60,65,71].

Decubitus, foot ulcers are other relevant clinical presentations of skin and soft-tissue GBS infections, with the latter occurring predominately in patients with diabetes. Bone and joint infections, especially osteomyelitis, are present in 10% of GBS adult infections [4] and most often linked to vascular insufficiency and overlying ulcers and spread from adjacent skin and soft tissue infection [72]. GBS pneumonia and urinary tract infections generally occur in in elderly adults [73], the first in older adults with neurological impairments and the latter is normally associated with prostate disease, prior cases of urinary tract infections, urinary catheters and anatomic abnormalities of the urinary tract [60,74-76]. GBS-caused meningitis and endocarditis, although less frequent, due to their poor outcome represent a concern among physicians. Among GBS infected non-pregnant adults only 2-5% presented meningitis, representing up to 4% of all cases of bacterial meningitis in adults [4,50,61,77]. Most cases occur in postpartum women, elderly adults, or adults with significant underlying diseases. Symptoms are generally abrupt in onset, and bacteremia is present in approximately 80% of cases [77,78]. In elderly patients, GBS meningitis is often fatal with an overall case-fatality rate of 27-34%, and 56% among adults with more than 65 years of age [77]. A proportion of survivors (7%) are left permanently deaf [77].

In recent studies, GBS endocarditis accounted for 3-10% of the invasive disease in adults [4,61,79]. In the last decades the focus of GBS endocarditis reports changed from parturient women to non-pregnant adults, with a higher incidence in the elderly [61,80]. Diabetes mellitus, chronic obstructive pulmonary disease, neoplasms, urological disorders and chronic liver disease were frequently associated with this manifestation of GBS invasive disease [79]. These infections often require cardiac surgery due to a rapid destruction of mitral or aortic valves. Complications include metastatic infection, heart failure, major emboli, myocardial abscess, and complete heart block [79-83]. Despite the decrease in GBS endocarditis in the past few years, its rates remain high. In young patients, fatality rates can reach 21% while among the elderly these rates can go as high as 45% [68,79,80].

GBS serotypes distribution in non-pregnant adults is distinct from the one associated with vaginal colonization of pregnant women and invasive infection of neonates. In the US, serotype V was the most prevalent GBS serotype associated with adult disease (29%), followed by serotype Ia (24%), II (12,5%) and III (11,4%) [4]. In France and UK,

the serotype III was most commonly found (25-26%), followed by V and Ia [61]. Nevertheless, when stratified by age, serotype V was predominant in individuals with more than 40 years old in both the US and France [4,61]. A markedly increase in the frequency of this serotype among adults (from 9% in 1995 to 24% in 2010) was observed in the UK [58]. In Portugal, serotype Ia was responsible for the majority of invasive infections in all age groups (34.7%). In the subpopulation with less than 65 years, serotype Ia was isolated three times more frequently than other serotypes (44.2%), such as III (14.7%), V (14.7%), and II (11.6%). In the elderly, although serotype Ia was still the most prevalent (27.7%), serotypes such as V (23.0%) and III (15.4%) were almost as frequent [59].

In eastern countries like Japan and Taiwan, serotype Ib is the main identified cause of invasive GBS infections in non-pregnant adults, immediately followed by serotype V [84,85]. In these countries, serotype IV (that has a negligible incidence in the US and Europe) was responsible for 8 -18% of GBS infections in adults [84,85].

The incidence of serotype V is steadily increasing since the early 90's. A recent report determined that 92% of bloodstream infections caused by serotype V in Houston and Toronto were caused by genetically related ST-1 strains and that these strains were a single loci variant of the first serotype V strains identified in humans. This evidence indicates that the emergence of serotype V GBS-causing invasive disease in non-pregnant adults was not driven by major genetic alteration on this serotype but is, most probably, a result of the phenotypic diversity resulted from small genetic changes [86].

I.4 GBS virulence factors

The uninterrupted and growing impact of GBS as a human pathogen, first in newborns and currently in non-pregnant adults, is a clear reflex of its ability to colonize and invade host tissues, to resist and evade host immune defenses and to adapt/resist to antibiotic treatments. To be successful in all those stages, GBS possesses a diverse array of surface-associated and secreted virulence factors that mediate specific host-cell interactions and interfere with immune clearance mechanisms (Figure 2).

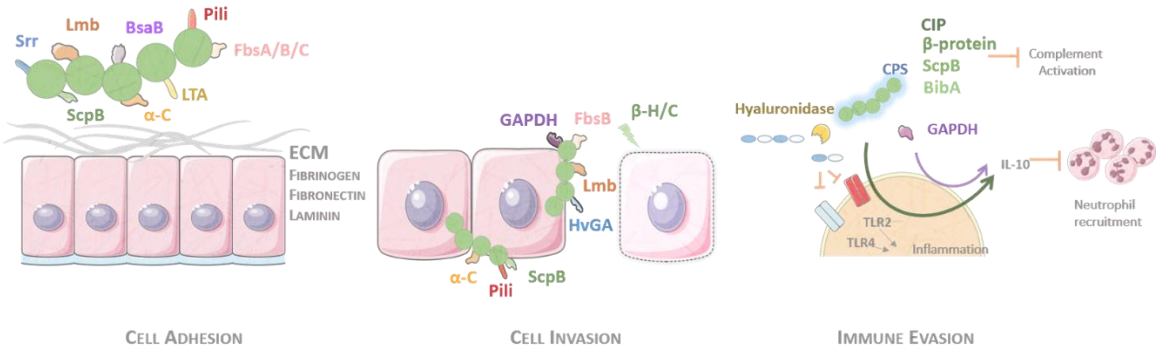

Figure 2. GBS virulence factors associated with the different stages of neonatal infection. Surface-expressed proteins FbsA/B/C, ScpB, Srr, pili, BsaB, LTA, α-C proteins and Lmb mediate GBS binding to host cells and extracellular matrix components, such as fibrinogen, fibronectin and laminin, contributing to cell adhesion. Some of those proteins are also involved in degradation of the extracellular matrix components in which they adhere, promoting cell invasion. Secreted β-haemolysin/cytolysin promotes GBS invasion, by breaking down host barriers to reveal novel receptors on the basement membrane and by inducing cell death. GBS GAPDH activates host plasminogen and degrade the extracellular matrix. CIP, β-protein, ScpB and BibA are associated with inhibition of complement activation after GBS infection. Secreted GBS hyaluronidase degrades pro-inflammatory hyaluronan fragments to disaccharides that block TLR2/4 signaling. GBS GAPDH induces an early IL-10 production that inhibits neutrophil recruitment to infected organs. See text for references.

I.4.1 Adherence and cell invasion

Colonization of the gastrointestinal tract of pregnant and non-pregnant adults as well as vaginal colonization of pregnant women is an early step for the development of a disseminated infection. GBS has the capacity of binding avidly to human epithelial cells of colon and genital tract or vaginal tissue culture, with maximal biofilm formation under the low pH conditions, characteristic of vaginal mucosa [87-89]. GBS cell-wall-associated lipoteichoic acid (LTA) mediates low-affinity interactions with epithelial cells, whereas hydrophobic GBS surface proteins mediate higher-affinity interactions [90]. Many of these interactions involve attachment of the bacterium to extracellular matrix molecules such as fibronectin, fibrinogen and laminin. Some bacterial adhesion

factors were named according to their specific ligand, such as fibrinogen-binding protein A (FbsA), fibrinogen-binding protein B (FbsB), fibrinogen-binding protein C (FbsC) and laminin-binding proteins (Lmb) [91,92]. Bacterial surface adhesin of GBS (BsaB) and streptococcal C5a-peptidase (ScpB) binds to fibronectin [93,94], serine-rich repeat protein (Srr)-1 binds to human keratin 4 and fibrinogen [95,96] and Srr2 binds to both fibrinogen and plasminogen [97]. The Srr1 interaction with fibrinogen was associated with GBS virulence in an animal model of meningitis and contributed to the pathogenesis of infective endocarditis [95,98]. GBS surface protein containing the leucine-rich-repeat (LRR) motifs, binds to human lung and cervical epithelial cells in a dose-dependent manner [99].

GBS alpha C proteins and Srr proteins are adhesion factors characterized by the highly conserved LPxTG (Leu–Pro–X–Thr–Gly) motif at the C- terminus. LPxTG is cleaved between Thr and Gly by the transpeptidase Sortase A, which covalently binds GBS to the cell wall promoting colonization and invasion [87,100,101].

Additional structures involved in adhesion are Pili, a filamentous cell surface appendages. GBS pili are constituted by three subunits: a major shaft subunit, the backbone protein (PilB), which is critical for pilus assembly; a pilus-associated adhesion (PilA) and a component anchoring the protein assembly to the cell wall (PilC). PilA contributes to the adherence of GBS to vaginal and brain endotelium [100,102-104].

The regulation of some of these and other GBS adherent factors is controlled by the two-component gene regulation system CovRS which, in turn, is modulated by acidic epithelial pH, high glucose levels and passage through intestine [105,106]. Deletion of CovRS, results in increased bacterial adherence but decreased invasion of vaginal epithelial cells [107].

Following cellular adherence and colonization, GBS needs to promote bacterial entry and survival within host cells in order to promote a systemic infection. Some of the factors associated with cell adherence are also associated to cell invasion, which is the case for FbsB, Lmb, ScpB and alpha C proteins [101,108-110]. It was described, for instance, that Lmb is important for GBS translocation across the intestinal epithelium and the blood–brain barrier (BBB) [108]. GBS ST17 possesses a surface-

anchored hypervirulent adhesin (HvgA) as a specific virulence factor. Both HvgA and pili are involved in the colonization and invasion of the intestine and confer meningeal tropism in neonatal mice [46,111].

The invasion of epithelial cells provides an intracellular niche for GBS survival but can also promote tissue damage and inflammation, contributing to the disease's pathology.

GBS β-haemolysin/cytolysin (β-H/C), a pore-forming toxin, is responsible for the cellular damage of both choroamniotic and lung tissue, compromising their barrier function [112-114]. Several studies demonstrated the crucial role of GBS β-H/C in disruption of the maternal-fetal barrier and subsequent vertical transmission of GBS, causing preterm births and abortions [113,114]. Moreover, β-H/C expression was proved to be essential for GBS penetration of pulmonary barriers and development of systemic infections [112,115].

Another important GBS virulence factor is the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This protein is present on the cell surface of the bacteria and can bind to lysine residues of the host's plasminogen [116,117]. The association of plasminogen to GBS surface activates the bound pro-enzyme to plasmin, thereby gaining the ability to degrade the host's matrix proteins such as fibronectin. This surface modification facilitates the GBS traversal of the extracellular matrix barriers and tissue penetration leading to an increase blood-brain-barrier bacterial invasion [118].

I.4.2 Host immune evasion

As soon as GBS reaches the bloodstream and establishes an infection, the host mounts an immune response to eliminate the pathogen. The high ability of the GBS to survive in the host is a reflex of the aptitude of its virulence factors for resistance and evasion of the bactericidal host responses.

A first step of any immune response is the recognition of danger- or pathogen-associated molecular patterns (DAMPs and PAMPs) by the immune cells. In response to infectious or sterile tissue injury, hyaluronan (HA), a glycosaminoglycan polymer of the extracellular matrix of nearly all tissues, is rapidly degraded by reactive oxygen

species or host hyaluronidases. The end products of these reactions are tetramic HA or larger fragments that have immunostimulatory activity, in part for their recognition by Toll-like receptor (TLR) 2 and/or TLR4 leading to the activation of pro-inflammatory responses. GBS secretes a hyaluronidase that degrades the host or pro-inflammatory HA into disaccharides. These disaccharides block TLR2/4 signaling by both pro-inflammatory HA and TLR2/4 agonist inhibiting the protective inflammatory response. By this mechanism GBS hyaluronidase promotes bacterial dissemination [119].

CPS is another virulence factor of the GBS that avoid the host immune recognition. The distinct types of CPS are created by arrangements of four monosaccharides into unique repeating units. However, every single CPS contains a terminal sialic acid bound to galactose in a α2 → 3 linkage. This conserved component is identical to a sugar epitope widely displayed on the surface of human cells. This not only avoids immune recognition, but also allows direct binding to immunoglobulin-like lectins (Siglecs) on leukocytes, inhibiting complement C3 activation and dampening the oxidative burst and bactericidal activities of phagocytic cells [120-123].

GBS is not only able to avoid immune recognition, but can also modulate the immune response triggered after its recognition. It is widely recognized that newborns have an undeveloped immune system that immediately after birth has to deal with the colossal challenge of efficient elimination of potentially dangerous microorganisms and the tolerance of commensals on skin and mucosal surfaces. To control the inflammatory responses, the neonatal immune system is polarized to an anti-inflammatory status [124-126]. GBS take advantage of this regulatory environment to evade the immune response. Bacterium recognition through TLR2 and secretion of GBS GAPDH to extracellular space induces an early and exacerbated production of the anti-inflammatory cytokine IL-10, in newborns [127-129]. This IL-10 impaired neutrophil recruitment into infected organs thus preventing bacterial clearance. Blocking either TLR2, IL-10 signaling or GAPDH, restores the recruitment of neutrophils to infected organs and confers neonatal resistance to lethal infections with GBS hypervirulent strains [127,128].

In addition to CPS, several others GBS virulence factors contribute to the inhibition of activation and deposition of some key molecules of the complement cascade (from

both classical and lectin pathways). ScpB mediates the proteolytic C5a inactivation [130], BibA binds to human C4-binding protein, a regulator of classical complement pathway [131], β-protein binds to factor H, enabling the unbound active region to block C3b deposition on the bacterial cell surface [132], and complement interfering protein (CIP) binds to C4b, preventing the formation of the C4bC2a convertase [133]. Complement activation is also inhibited by factors that induce the coating of the bacterial surface with human proteins, as in the case of GBS beta-antigen of the c protein complex, that leads to a unspecific IgA-binding on bacterial surface by GBS [134] and by cell-surface protease (Csp) A, that produces adherent fibrin-like cleavage products [135].

Some of GBS virulence factors allow its evasion from phagolysosomal processing. GBS is a catalase negative bacterium, yet it is still able to neutralize reactive oxygen species via BibA, β-H/C, superoxide dismutase (SodA), and another unknown factors [136-138].

GBS exploits the cationic nature of most antimicrobial peptides (AMP), and the need of a negatively charged microbial cell surface for its electrostatic attraction, by incorporating positively charged D-alanine residues into its cell-wall LTA, reducing the negative surface charge and, consequently, its affinity for the cationic peptides [139]. This resistance to AMP is further amplified by surface-anchored penicillin-binding protein (PBP) 1a, and pilus backbone protein PilB [140,141].

An alternative bacterial defense mechanism to avoid phagocytic clearance is the induction of apoptosis, or programmed cell death. Several GBS proteins have this ability, depending on the environment. For instance, β-H/C induces cell death in epithelial, meningeal cells and astrocytes in vitro [142], phosphoramidon-sensitive metalloprotease induces apoptosis of human endothelial cells *in vitro* [143] and GAPDH induces apoptosis in murine macrophages [144].

Neutrophils and macrophages, beside their capacity to phagocyte and directly kill pathogens, are able to form extracellular traps (NET in neutrophils and MET in macrophages). NET and MET consist in a high local DNA matrix with elastase, enzymes, proteases, histones and antimicrobial peptides that are able to bind and kill pathogens extracellularly, independently of phagocytic uptake [145,146]. The structure

of NETs and METs, held together by a DNA backbone, is critical to their antimicrobial function. GBS secretes a nuclease A (NucA) that is able to degrade the DNA matrix compromising NET stability. *In vivo* infection studies confirmed that NucA decreases bacterial clearance from lung tissue and increases mortality in infected mice [147].

Bacterial modulation of the immune system doesn't simply rely on shutting down protective immune responses but can also occurs by increase the production of pro-inflammatory mediators towards tissue causing damage. For instance, GBS PilA promotes the activation of host chemokine expression and neutrophil recruitment to brain endothelium, increasing the blood-brain-barrier permeability and inducing bacterial central nervous system penetration [102].

I.5 GBS recognition and immune response

During GBS infection, a wide array of immune responses is induced, including the production of cytokines, cell recruitment to infected organs and production of antimicrobial factors. The recognition of bacterial pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) comprises the first stage of the innate immune response. Amongst the several functionally distinct classes of PRR, TLRs have been extensively studied in the context of GBS infection.

The mammalian TLR comprises a family of cell surface (TLR1, TLR2, TLR6, TLR4 and TLR5) or endossomal transmembrane (TLR3, TLR7, TLR8, TLR9, TLR10, TLR11, TLR12 and TLR13, the last three are not present in humans) germline-encoded membrane glycoproteins that lead to the production of pro- and anti-inflammatory cytokines when stimulated. A central role of TLRs in GBS infection was confirmed by the requirement of the TLR adaptor MyD88 for optimal host defense and pro-inflammatory cytokine production [148-151]. GBS lipoproteins (but not LTA) stimulate the production of pro-inflammatory cytokines by a mechanism involving TLR2/TLR6. These receptors, however, appear to be essential only for the recognition of released factors from GBS, whereas whole GBS organisms potently induce cytokines in a TLR2-, TLR6-independent fashion *in vitro* [152]. A recent study implicates the TLR (and excluded nucleotide-binding oligomerization domain (NOD)-dependent) signaling in

the activation of dendritic cells (DCs) by GBS. The same article showed that abrogation of TLR2 in GBS-infected DCs only partially affected the production of IL-6 and CXCL1, indicating that other TLR should have an importance on cytokine production on DC in response to GBS [153].

The role of TLR2 in GBS infection deeply depends on the model and the bacterial dose used. Interaction of GBS with TLR2 was beneficial to the host in a low dose GBS neonatal infection model [148] but had, however, a detrimental role in neonatal models of GBS lethal infections [127,148]. In the case of adult mice models, TLR2 deficiency was associated with enhanced severity of infection in a sub-lethal (or low-mortality) GBS infection [148,154,155]. However, in a model with an inoculum that leads to the death of 90% WT mice, the lack of TLR2 signaling conferred resistance to GBS infection [148].

TLR2 engagement and/or MyD88 activation during GBS infection is associated with the production of both pro-inflammatory and anti-inflammatory cytokines. The protective role of each one of the cytokines that are produced during GBS infection is highly dependent on the magnitude and timing of their production. GBS is a potent inducer of TNF-α [156] which is assumed to be one of the main molecular mediators in the pathophysiology of sepsis, in mice and humans [157]. A dramatic increase in TNF-α and other pro-inflammatory cytokines has been observed in adult GBS-susceptible mice when compared with resistant strains [148,155]. However, the treatment with anti-TNF serum prior to the GBS infection didn't alter the GBS-induced lethality of adult mice [158] and, in a mice model of GBS-induced arthritis, the chemical block of TNF-α production showed a negligible involvement of this cytokine in the pathogenesis of the disease [159]. Also, in neonatal infections, the role of this cytokine is controversial. A study using a neonatal model of GBS subcutaneous infection showed that, depending on the inoculum, the blockage of TNF-α could result in increased resistance (high dose) or susceptibility (low dose) [148]. By contrast, treatment of neonatal mice and rats with TNF-α specific antibodies, prior to the lethal infection with GBS, did not affect mortality, simply delaying it 24 h in one of the studies [127,160]. The expression of TNF-α during GBS infection implicates the activation of nuclear factor (NF)-κB and activator protein (AP)-1 and is greatly amplified by the activation of the alternative complement pathway [156,161].

IL-1β is another pro-inflammatory cytokine highly associated with the septic-shock and that is markedly elevated during GBS disease. Production of IL-1β is comprised by two phases, the induction of pro-IL-1β synthesis and the caspase-1 cleavage of the inactive precursor to the active “mature” cytokine. GBS infection is able to induce both phases [151,162]. Higher maternal IL-1β plasma concentrations were associated with early term births and with an increased risk for GBS infection [163]. IL-1R signaling pathway has distinct effects to the pathogenesis of GBS infection, depending on GBS inoculum. Infections with sub-lethal GBS doses proved the importance of IL-1β production and signaling in protecting the host against GBS infection by inducing the production of the chemokines KC and MIP-1α and the recruitment of polymorphonuclear leukocytes to sites of GBS infection [149,151,164]. In a model of GBS arthritis, however, IL-1 was strongly associated with the pathogenesis of the disease [159,165] and, in a neonatal mouse model with a lethal GBS infection, abrogation of IL-1R signaling was able to partially reduce mortality [127].

IL-10 is an important anti-inflammatory cytokine that limits the production of pro-inflammatory cytokines, chemokines and adhesion molecules. High concentration of plasma and cord blood IL-10 in preterm neonates during sepsis was associated with mortality. Several studies stated that an early and exacerbated production of this cytokine during neonatal GBS infection facilitated pathogen immune evasion by inhibiting protective immune responses [127,128,166]. In a model of arthritis induced by GBS, IL-10 helped to control the inflammation that is associated with the worst prognosis of the disease [167]. In another study, however, the neutralization of IL-10 with specific antibodies had no effect on lethality of neonatal GBS infection and the administration of recombinant IL-10 at 4 or 24 h before bacterial challenge resulted in improved survival [168]. The reasons for these discrepancies in the role of this anti-inflammatory cytokine are still uncertain.

Others TLRs have also been associated with GBS recognition. For instance, GBS activation of TLR7 in lysosomes by single-stranded RNA (ssRNA) potently induced type I interferon production in conventional DCs [169]. This and other studies suggest the involvement of the chaperone protein UNC93B1 (that is required for the function of intracellular but not cell surface-associated TLRs) in GBS ssRNA recognition [169,170]. This type I interferon (IFN) production in murine macrophages and dendritic

cells is critical to the host's defense against GBS [171,172]. This cytokine primes immune cells to increase pro-inflammatory responses and, upon GBS infection in the absence of type I IFN signaling, a robust impairment of the production of TNF-α and IFN-γ was observed [171].

IFN-γ is an important cytokine that enhances the recruitment of phagocytes and increases the effector functions of these cells. IFN-γ production during GBS infection seems to have a protective role since administration of recombinant IFN-γ to GBS infected pups increased survival and decreased GBS colonization [173]. Therapeutic use of IFN-γ in a mouse model of GBS-induced arthritis led to the development of milder manifestations of the disease but prophylactic use induced the opposite results [174]. *In vitro* studies, with human umbilical vein endothelial cells and cord blood monocyte-derived macrophages, concluded that the activation of the cells by IFN-γ decreased the viability of intracellular GBS [175,176]. IL-12 is a heterodimeric cytokine and a potent IFN-γ inducer that directs the generation of T-helper type 1 responses. The protective effects of this cytokine are similar to the ones already described for IFN-γ. For instance, administration of recombinant IL-12 had a protective effect in GBS infected pups, not only therapeutically, but also prophylactically [177]. A deficiency of this cytokine, like a deficiency in IFN-γ, contributed to an increased susceptibility of newborns to GBS infection [178,179] and had beneficial effects on GBS-induced arthritis [167]. Additionally, it was demonstrated the important role of IL-18 in control of GBS infection and inducing host survival, by inducing IFN-γ production [179,180]. The protective role of Th1-dependent responses in GBS adult infections was further confirmed by evaluation of the Th2-associated cytokine IL-4. The deficiency of this cytokine decreased GBS-induced lethality due to an up-regulation of pro-inflammatory cytokines, leading to a higher bacterial clearance in IL-4 KO animals than in controls [181]. However, the increased production of pro-inflammatory cytokines in IL-4 KO mice intensified the severity of arthritis caused by GBS [181]. Treatment with recombinant IL-6 also amplified the severity of this disease in mice infected with GBS [159].

In addition to TLRs, intracellular receptors and CD11b/CD18 β2-intregrin (complement receptor 3, CR3), have been described as GBS signaling receptors [182]. GBS β-hemolysin induce the secretion of IL-1β on mouse dendritic cells through activation of

the intracellular NOD-like receptors (NLRs) family pyrin domain- containing 3 (NLRP3; also known as cryopyrin or NALP3) inflammasome [151]. This NLRP3 inflammasome role was proven to be crucial for *in vivo* anti-GBS defense [151]. Alternatively, the association of GBS with CR3 mediates not only the phagocytosis of the bacteria, but contributes to the cytokine production, such as TNF-α and lL-6, *in vitro* [156,183-185]. Despite these findings, a study with peritoneal macrophages from CD11b KO mice showed a decrease in opsonic uptake of the GBS but a normal pro-inflammatory response [150].

Aside from systemic immune responses, GBS also induces a mucosal immune response. A correlation was reported between rectal or cervical GBS colonization and levels of serotype-specific IgA and IgG in cervical secretions [19,186]. The induction of specific antibodies on mucosal sites may prevent genital colonization and decrease the vertical transmission of the bacteria.

Overall, it is clear that the nature of the immune response produced upon GBS-infection will define the fate of the host and the level of pathology induced.

I.6 Treatment

In 1996 the Centers for Disease Control and Prevention (CDC), in collaboration with relevant professional Societies, published guidelines that several countries adopted as a policy for the use of *intrapartum* antibiotic prophylaxis (IAP) to prevent perinatal GBS diseases [187,188]. In 2002 and 2010 the guidelines were revised and republished but the foundations remained unchanged [31,189]. Following these recommendations, pregnant women should receive IAP if: the vaginal-rectal screening for GBS colonization at 35-37 weeks give positive, the women had delivered a previous infant with GBS disease, the GBS status is unknown at deliver, during the labor women developed a fever >38°C, or if the women has a rupture of membranes for 18 hours or longer [31].

The recommended antibiotics for IAP are penicillin and ampicillin and, in case of allergy to β-lactams, the antibiotic of choice should be cefazolin, erythromycin or clindamycin

[31]. These are also the first-line agents against the treatment of GBS infections in adults.

In the US, as a result of an active prevention, there was more than 80% decline in the incidence of GBS EOD, from 1.7 per 1000 live births in the early 1990s to less than 0.3 per 1000 live births in 2013 [31,190-192]. Nevertheless, IAP did not have an impact on the incidence of GBS-caused LOD, stillbirths or premature births [193,194].

Moreover, the use of antibiotic, as prophylaxis and treatment, raises several important questions due to its limitations and side effects. One of the most relevant and well known consequences of antibiotic use is the emergence of antibiotic-resistance strains [195].

Clinically isolated GBS have been considered to be uniformly susceptible to β-lactams, however very rare isolates with reduced susceptibility to penicillin have been identified in Japan and the US [85,196,197]. Of higher concern, however, are the increasing resistances to macrolides and clindamycin among GBS invasive isolates [198]. Streptococcal resistance to macrolides is commonly mediated by two major mechanisms: (i) alteration of the antibiotic binding site, that can confer inducible or constitutive resistance to macrolides, lincosamides and streptogramin B (MLS_B phenotype) and (ii) increase of macrolide-specific efflux mechanism (M phenotype), encoded by the *mefA* gene [199,200].

In Portugal, erythromycin resistance among colonizing strains of GBS, were similar to those described in France and Canada (18%) [37,199,201] with a higher percentage relatively from the one reported in Czech Republic (3,8%) [202] but lower than the one reported in the US (38–41.9%) [203,204].

Regarding antibiotic resistance among invasive GBS strains, serotype V has been pointed out as having a central role. The resistance is mainly observed against erythromycin and clindamycin. A report from Korea, for instance, showed that, among 37 studied erythromycin-resistant strains, 29.7% of them belonged to serotype V, with a rate of erythromycin-resistance for this serotype of 68.8% [205]. In the US 44-63% of erythromycin-resistant GBS belong to serotype V, with a rate of erythromycin and/or clindamycin resistance for this serotype of 40-48% [204,206]. Moreover, a study from

southwest Germany focused on neonatal and maternal GBS isolates, showed that 37% of the erythromycin-resistant GBS isolates belonged to serotype V, representing the majority of the erythromycin-resistant strains [207].

Antibiotic use during pregnancy has been shown to have not only an effect on the emergence of GBS-resistance strains, but also to be associated with the shift to *Escherichia coli* neonatal infections [208-210]. Other studies however, rebut that association [211,212].

The shaping of GBS epidemiology has been influenced by antibiotic use since its emergence as a human pathogen. It was recently proposed that the widespread use of tetracycline helped drive the adaptation of GBS to neonatal hosts [10].

In the last year, our knowledge on the importance of gut microbiota in several aspects of the human development as increased significantly. It is now known that the colonizing microorganisms play a key role in driving postnatal maturation of the gut and development of the immune system [213,214]. Antibiotic exposure near the time of birth reduces the diversity and composition of intestinal microbiota of both the mother and child and vaginal microbiota of the mother, delaying the appearance of beneficial bacteria in the child [215,216]. These alterations may interfere with the development and maturation of the child's immune system and are linked to an increased susceptibility to the development of LOD, allergic diseases, asthma, rheumatoid arthritis, atopic dermatitis and autoimmune diseases [217,218]. A study using neonatal mice model identified a role of the microbiota in regulating postnatal granulocytosis and increasing resistance to sepsis. In this study, antibiotic-exposed neonatal mice were more susceptible to *E. coli* and *Klebsiella pneumoniae* infection than control mice [219]. Furthermore, a recent study where maternal gut microbiota is altered by administration of non-absorbable antibiotics during pregnancy showed an influence of maternal gut microbiota on behavior of the offspring [220].

Overall, the long-term consequences of actual antibiotic prophylaxis and treatment, as well as its limitations, impose a need for an effective alternative against GBS infections.

I.7 Vaccination

Vaccination represents the most attractive strategy for GBS disease prevention. Several efforts are being made to find an efficient vaccine that would stimulate the production of antibodies that would not only confer protection against GBS infections in adults, but should also be transferable by placenta and milk, conferring protection against the neonatal forms of the disease.

A real investment has been made in GBS CPS-based vaccines. The interest in the use of these antigens began with the detection of a correlation between low levels of maternal antibodies against CPS with neonatal susceptibility to GBS infection. The development of neonatal GBS disease was associated with low or undetected maternal levels of CPS-specific antibodies and, on the other hand, among mothers of neonates that didn't developed GBS diseases, the majority had antibodies with CPS-binding capacity [221-224]. Moreover, a poor neutrophil-mediated functional activity against GBS serotype V in healthy elderly adults was associated with low concentrations of type V CPS–specific antibodies [225].

The conjugation of CPS antigens to a carrier protein enhanced the immunogenicity and induced a long-lasting immune response against the polysaccharide. Monovalent conjugate vaccines against serotypes Ia, Ib, II, III and V have been prepared coupled to tetanus toxoid (TT) and were already tested in phase I and II of clinical trials. Each vaccine was able to induce functionally active serotype-specific IgG and, CPS III–TT vaccine in pregnant women, induced titers of protective IgG antibody against type III CPS that persisted through at least 2 months of life [226-229]. Currently, a trivalent vaccine against CPS of serotypes Ia, Ib and III, is already in phase II of clinical trials [230]. The results of the bivalent CPS-vaccine trial in healthy adults showed that the vaccine was well tolerated and lead to the production of higher IgG titers in those subjects with pre-existing anti-capsular antibody to GBS [231]. Similar results were obtained in vaccinated healthy elderly adults. A single intramuscular dose of serotype V GBS CPS–TT vaccine induced specific-antibodies that were able to promote opsonophagocytic killing of serotype V GBS in vitro [232]. Although capsular conjugate vaccines are in an advanced stage of development, the constant variation of serotype distribution, the existence of non-typable GBS isolates, as well as serotype

replacement/switching, hint to potential problems in the global coverage of CPS-based vaccines [35,233,234].

A GBS effective vaccine should be based on immunogenic antigens that should not only be surface-exposed but should also be conserved among all invasive strains.

Some GBS surface proteins appeared as promising antigen to GBS vaccination as the α and β components of the C protein complex [235,236], alpha-like protein 3 [237], Rib protein [238], FbsA fragments [239], and C5a peptidase ScpB [240,241]. Although highly immunogenic and effective against GBS infections, all these protein lack the universality aspect of a suitable GBS vaccine.

Surface-immunogenic protein (Sip) [242] and a combination of 3 antigens of pili proteins were pointed as highly conserved antigens able to induce cross-protective immunity against all invasive GBS serotypes [243]. Nevertheless, due their structural role, it is likely that under selective pressure vaccination with these antigens will lead to selection of GBS clones expressing new antigen variants, as already happened with the *Neisseria gonorrhoeae* pili vaccine [244].

Our group developed a vaccine based on the GBS immunomodulatory protein GAPDH. This glycolytic enzyme was found at surface and in the supernatants of every analyzed invasive strain belonging to the different GBS serotypes [116,128]. Maternal vaccination with recombinant GAPDH as well as passive immunization of neonates with anti-GAPDH IgG antibodies confer protection to the offspring in a mouse model of lethal GBS infections. The mechanism responsible for the observed protection is the blockage of GAPDH modulatory effect on the neonatal immune system, since the anti-GAPDH F(ab') 2 fragments were as effective in improving survival and bacterial clearance as whole antibodies [128].

Although GAPDH is ubiquitously expressed in all type of cells, including mammalian cells, antibodies produced against either native or denatured GBS GAPDH are not able to recognize human or mouse GAPDH [128].

The efficacy of GAPDH vaccination, associated with the fact that this protein is highly conserved and essential for bacterial survival (thus being less susceptible to selective pressure), make it an ideal target antigen for the development of a universal vaccine.

Vaccination would substantially reduce the burden of infant GBS disease in low-income settings, where prenatal screening and *intrapartum* antibiotics are generally not feasible [245], and in theory, could help the control of GBS diseases in elderly and/or immunocompromised adults.

The first aim of this thesis was to evaluate if the neonatal GAPDH vaccine developed by our group could be used against GBS infection in adults.

II MICROBIAL GAPDH AS A VIRULENCE FACTOR

GAPDH is an essential cytoplasmic enzyme involved in the glycolytic pathway and catalyzes the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate. Despite the lack of secretion signal or transmembrane domain this enzyme has been found at the surface or in the supernatant of several bacteria, fungi, protozoans and even humans. In GBS and *Streptococcus pneumoniae*, GAPDH extracellular presence is, at least in part, associated with bacterial cell lysis [144,246]. In enteropathogenic *E. coli,* cytoplasmatic GAPDH is secreted through a type III secretion system [247]. In this unexpected location, GAPDH exhibits various functions facilitating colonization and invasion of host tissues, as well as immune evasion.

In group A *Streptococcus* (GAS), when surface export of GAPDH was prevented (by insertion of a hydrophobic tail at the C-terminal end) virulence was seriously reduced [248].

II.1 GAPDH, a protein associated with adhesion and invasion

Some bacterial GAPDH has the capacity to bind to extracellular matrix components increasing pathogen adhesion, and in some cases invasion, of host tissues. That is the case of GAS GAPDH which attaches to fibronectin, actin and the ATPase domain of myosin, suggesting a role in streptococcal colonization, particularly on injured tissues. The extracellular-GAPDH ability to bind cytoskeletal and extracellular matrix proteins has also been described in GBS, enteropathogenic and enterohemorrhagic *E. coli* and *Candida albicans* [116,249-251].

Furthermore, GAPDH of GAS, GBS, *Staphylococcus spp*, *S. pneumoniae* and *Mycoplasma pneumoniae* binds to plasminogen, which is then converted to the proteolytically active plasmin form. The bacterial coverage with plasmin plays an important role in the pathological process of infection, allowing it to escape from blood clots and tissue invasion [117,252-254].

II.2 GAPDH in immune evasion

As previously stated, GBS exploits GAPDH's ability to induce an early IL-10 production in neonates, delaying the protective neutrophil influx to infected organs and, therefore, evading the host innate immune response [128].

GAPDH of other microorganism are also able to modulate and escape the immune system. For instance, GAS' GAPDH can capture the complement C5a protein (an essential step for its cleavage by streptococcal C5a peptidase), which inhibits the recruitment of neutrophils and H_2O_2 production [255]. *S. pneumoniae* and parasitic nematode *Haemonchus contortus* GAPDH interacts with C1q and C3 complement protein, respectively, inhibiting their activity [246,256].

II.3 GAPDH-based vaccines

Beside the previously mentioned GBS GAPDH vaccine developed by our group, others GAPDH-based vaccines were already proposed, not only for human but also for veterinary diseases. A DNA vaccine encoded for GAPDH, for instance, was tested against *Haemophilus parasuis,* the causative agent of swine bacterial polyserositis, polyarthritis, and meningitis [257]. GAPDH protein of *Streptococcus zooepidemicus*, an important agent of for septicemia, meningitis and mastitis in horses, pigs, sheep, cows, and several other mammalian species, was also proved to be immunogenic [258].

Surprisingly, GAPDH vaccination has been a widely studied strategy against infection in the fishing industry. This vaccination against *Edwardsiella tarda*, *Edwardsiella ictaluri*, *Vibrio anguillarum*, *Streptococcus iniae*, *Aeromonas hydrophila*, *Vibrio harveyi* and *Lactococcus garvieae*, induced a protective immune response in fish [259-264].

Glycolytic enzymes associated with the cell surface of *S. pneumoniae*, of which GAPDH, were proved to be antigenic in humans and elicited protective immune responses in mice [265].

A new approach to vaccine design consisted in using antigen presented cells loaded with peptide antigens as vectors. A study with DCs loaded with the *Listeria monocytogenes* $GAPDH_{1-22}$ peptide conferred higher protection and security against listeriosis than the most explored *L. monocytogenes* peptide [266].

Overall, GAPDH seems to be a highly conserved protein between several pathogens, both in terms of sequence and importance as virulence factor. Nonetheless, the protective effect of GAPDH-based vaccination was not yet characterized in several microorganism where the association of this protein with virulence have been already described. *E. coli* is one of these cases. Thus, the second aim of this thesis was to understand if a GAPDH could be used as a vaccination target against *E. coli* infections, in a neonatal model of infection.

III *ESCHERICHIA COLI*

E. coli is a non-sporulating Gram-negative rod-shapped bacteria with approximately 0.5 µm in width and 2 µm in length. This bacteria is a typical colonizer of the intestine of warm-blooded animals and, in the human digestive tract, is the most common aerobic organism [267]. It is normally located in the large intestine, residing in the mucus layer that covers the epithelial cells, especially in the caecum and the colon. Usually, the host colonization by *E. coli* is beneficial for both species. On the one hand, the bacteria benefits from the host with a steady supply of nutrients, a stable environment and protection against some stresses, as well as a means of transportation and dissemination, while on the other hand, the induction of a resistant colonization by the normal *E. coli* microbiota prevents the colonization of the host digestive tract by pathogens [268]. Besides the innocuous commensal *E. coli,* this specie also comprises strains and variants with pathogenic potential. Some of these strains have the ability to cause extraintestinal infections, being therefore designated ExPEC (from Extraintestinal Pathogenic *E. coli*). The pathotypes implicated in extraintestinal infections are usually divided in three different sub groups: uropathogenic *E. coli* (UPEC), neonatal meningitis-associated *E. coli* (NMEC) and sepsis-causing *E. coli* (SEPEC) [269].

III.1 *E. coli* as a neonatal pathogen

NMEC and SEPEC pathotypes are the most common cause of gram-negative neonatal sepsis and meningitis, with a case fatality rate of 15-40% [211,270,271]. Moreover, they also cause severe neurological defects in up to 50% of the survivors [211,272]. Over the past years, the incidence of *E. coli* neonatal infections seems to be increasing, representing about 24-29% of all EOS cases, 44% of all early-onset meningitis [211]. This bacterium became progressively recognized as the leading sepsis pathogen among preterm, being responsible for 81% of all EOS occurring in these infants. When very low birth weight (VLBW) infants are considered separately, *E. coli* surpasses GBS as the most frequent cause of EOS, accounting for 33.4% of all episodes [273]. The

rise of pathogenic *E. coli* in neonatal infections can be in part explained by the increase in preterm births and in the survival of VLBW [28,212].

In the 1997-2006 period in the US, *E. coli* EOS in VLBW infants occurred in 10.22 cases per 1000 VLBW admissions whereas LOS was observed in 21.66 cases per 1000 VLBW admissions and 8.23 cases per 1000 admissions of term infants [212].

As already described for GBS neonatal infections, *E. coli* vaginal colonization of pregnant women seems to be an important step for vertical transmission. Early and heavy *E. coli* vaginal colonization during pregnancy is associated with higher risk of VLBW and prematurity [274]. The presence of this bacterium in vaginal microbiota was observed in 13–15% of pregnant women [275,276].

E. coli strains fall into 5 major taxonomic lineages, A, B1, B2 and D, and are commonly classified by their three major surface antigens: the O antigen (that is part of the lipopolysaccharide, LPS), the H antigen (flagellum) and the K antigen (capsular polysaccharide) [277]. Neonatal sepsis and meningitis are associated with a limited number of phylogenetic groups, in sharp contrast with the wide genetic diversity of human commensal *E. coli* isolates. Most of *E. coli* strains that cause these diseases belong to the highly clonal B2 group [272,278] and 81-83% of NMEC and 60% of SEPEC strains express the K1 capsule antigen [278-281]. These strains come from the intestinal flora and are positively select for their ability to adapt to the vaginal environment and to the amniotic fluid during pregnancy [278].

The ability of *E. coli* K1 strains to pass over to the bloodstream after gastrointestinal colonization has been vastly documented in neonatal rat models [219,282-286]. Oral or intragastric administration of *E. coli* K1 resulted in stable and persistent gastrointestinal colonization of both adults and pups but systemic infection was only detected on the latter group. The ability to induce a systemic infection was closely dependent with the age of the neonates. Two-day old pups were considerably more susceptible to bacterial invasion than nine-day-old ones [285,286]. At early stages of infection, a small number of *E. coli* K1 were found in the mesenteric lymphatic system indicating that this route could be important for the invasion of the blood circulation [283,285].

Two-day old pups colonized in the gastrointestinal tract by *E. coli* K1 are unable to produce defensin peptides in the lumen of the small intestine. This dysregulation, associated with a poor maturation of the mucus barrier in newborn pups was insufficient to prevent translocation of *E. coli* K1 from gut lumen to blood circulation. An integral mucus barrier is already formed at day nine after birth, preventing systemic invasion [286]. In a study with bioluminescent *E. coli* K1 infection, both keratinized and non-keratinized surfaces of esophagi were colonized to a considerably greater extent in susceptible two-day old pups than in corresponding tissues from infection-resistant olds pups [285]. The bacteria appeared to damage and penetrate the non-keratinized esophageal epithelium of infection susceptible animals. This result raises the possibility that the esophagus represents an additional place of translocation of *E. coli* K1 to the blood [285].

Currently, there are no guidelines for prophylaxis of *E. coli* neonatal diseases. During EOS, the antibiotics are selected empirically, based on the likelihood of etiologic pathogens, and typically consist of ampicillin and gentamicin. Usually, bacterial identification, in blood, cerebrospinal fluid (CSF) and/or urine, is made only after the initial treatment. In several cases, if *E. coli* infection is confirmed, cephalosporins are added to the initial treatment [28,287].

E. coli resistance to antibiotics has been increasingly reported, particularly by the production of extended-spectrum beta-lactamase (ESBL). These enzymes confer resistance to nearly all β-lactam antimicrobial drugs, including the third-generation cephalosporins. Resistance to aminoglycosides was also observed in ESBL producers [211,288]. In Poland, 91,7% of all *E. coli* neonatal isolates were resistant to ampicilin, 54,2% to amoxicillin, 41,7% to trimethoprim-sulfamethoxazole and 33.3% to aztreonam [272].

III.2 *E. coli* virulence factors

From the initial site of colonization, *E. coli* transcytoses the gastrointestinal tract into the bloodstream, and from there, colonizes several organs or traverses the BBB into the central nervous system (CNS). Since a successful CNS invasion requires a high

bacteremia, NMEC must survive in the bloodstream. Bacterial invasion, survival and replication within the host cells are traits mediated by bacterial virulence factors.

Isolates associated with ExPEC and specifically to NMEC and SEPEC, express specialized virulence genes when compared to the isolates of other groups. That is the case of the K1 capsule, fimbriae, alpha-hemolysin, rough lipopolysaccharide, brain endothelium invasion proteins (Ibe), outer membrane protein A (OmpA), cytotoxic necrotizing factor (CNF) 1, Traj, TraT, Nlpl, arylsulfatase-like A (aslA) and increased serum survival protein (Iss) (Figure 3).

Since CSF and human serum are iron-deficient environments, iron acquisition genes and proteins have also been implicated as important factors for *E. coli* virulence. NMEC and SEPEC possess iron uptake systems that are not found in non-virulent fecal *E. coli* strains from both human and animal sources [272,289].

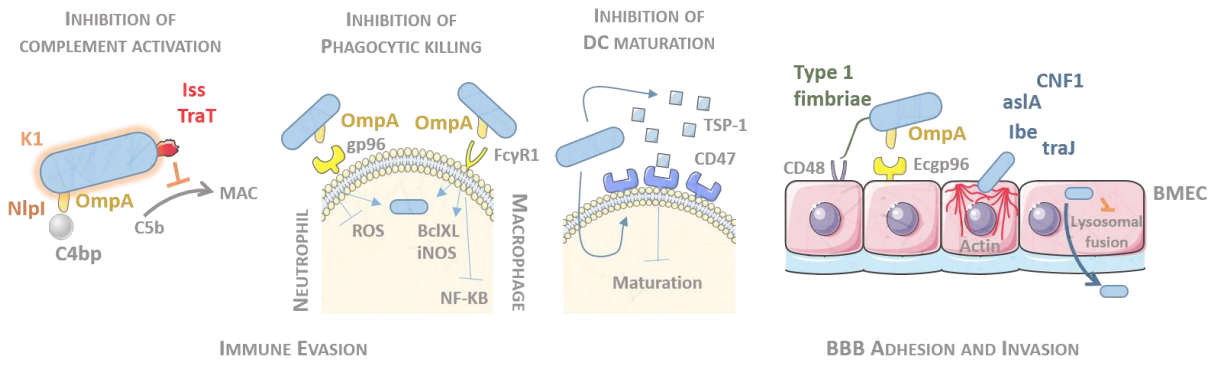

Figure 3. *E. coli* K1 virulence factors associated with the different stages of neonatal infection. Some of the *E. coli* K1 virulence factors associated with immune evasion stages (inhibition of complement activation, phagocyte intracellular killing and dendritic cell (DC) maturation) and blood-brain barrier (BBB) adhesion and invasion. K1 capsule impairs complement opsonization. *E. coli* K1 Iss and TraT inhibit the formation of complement membrane attack complex. OmpA, with the help of Nlpl binds to C4 bp, a classical complement pathway regulator, to block the complement cascade reaction. The association of *E. coli* K1 OmpA with its receptors in phagocytes induces bacterial internalization but inhibits the microbicidal mechanism (ROS formation, apoptosis and pro-inflammatory cytokine production dependent of NF-KB), while increases iNOS production and consequent up-regulation of OmpA receptor, allowing the intracellular survival of the bacterium. *E. coli* K1 infection induces the expression of CD47 and its ligand TSP-1, inhibiting DC maturation. The association of *E.*

coli K1 fimbriae and OmpA to their receptors, as well interaction of CNF1, aslA, Ibe and traJ proteins with brain endothelium cells, allow bacterial adherence and induce cytoskeletal rearrangements essential for *E. coli* K1 BBB invasion. See text for references.

III.2.1 Adherence and cell invasion

Blood-brain barrier (BBB) adherence and invasion is probably the most studied feature of NMEC. It is widely accepted that *E. coli* successful crossing of BBB requires a threshold level of bacteremia [290], invasion of brain microvascular endothelial cells (BMEC), and specific host cell signal-transduction pathways [291]. *E. coli* invasion of BMEC monolayers was visualized by electron microscopy and intracellular *E. coli* organisms were found within intracellular vacuoles [292].

Several *E. coli* proteins have been associated with bacteria-BMEC interactions with important roles in central nervous system invasion. *E. coli* attachment to the BBB is essentially mediated by OmpA and Type 1 fimbriae (Figure 3).

Type 1 fimbriae are filamentous surface organelles produced by *E. coli* that are regulated by a phase variation, in which each individual bacterium can alternate between fimbriated and nonfimbriated states. In *E. coli* K1, type 1 fimbriae have been shown to be important for oropharyngeal colonization in a neonatal rat model, although not relevant for intestinal colonization or bloodstream invasion [293]. The fimbriae are composed of a major FimA protein and a small tip structure containing FimF, FimG, and FimH [294]. Type 1 fimbriae contribute to the interaction between the meningitis-causing *E. coli* K1 and human BMEC (HBMEC) [295] in part by association of the lectin-like adhesin FimH with glycosylphosphatidylinositol-anchored receptor CD (cluster of differentiation) 48 on HBMEC's surface [296]. This association triggers host cell signaling cascades that are involved in *E. coli* K1 invasion of HBMEC [296] .

OmpA is one of the major outer membrane proteins in *E. coli* and its expression significantly enhances the *E. coli* invasion of BMEC [297,298]. OmpA binds to the GlcNAc1-4GlcNAc epitopes of Ecgp96, a homologue of endoplasmic reticulum specific gp96, which is present specifically on BMEC but not on systemic endothelial cells [299]. Blocking of *E. coli* K1 association with Ecgp96 with a derivative of telmisartan, an angiotensin II receptor type 1 (AT1R) blocker, prevented the onset of neonatal *E. coli*

meningitis [300]. Although the contributions of type 1 fimbriae and OmpA to *E. coli* K1 binding to and invasion of HBMEC are independent of each other or at least partially additive, the deletion of OmpA genes in *E. coli* K1 is associated with a decrease in the expression of type 1 fimbriae [301].

Deletion of E*. coli* K1 proteins, such as Ibe proteins [302], traJ [303], aslA [304], and CNF1 [305] decreased the ability of the bacteria to invade BMEC. This invasion is a prerequisite for penetration into the central nervous system and requires actin cytoskeletal rearrangements (Figure 3). *E. coli* internalization into BMEC was inhibited by more than 90% when microfilament-disrupting agents were added to infected cultures [292]. The Rho family GTPases, including RhoA, Rac1, and Cdc42, have been identified as molecular switches that regulate actin cytoskeleton organization. FimH and CNF1 are likely to contribute to HBMEC invasion via RhoA activation, whereas Ibe proteins and OmpA contribute to HBMEC invasion via Rac1 activation and cytosolic phospholipase A2. The addition of human transforming growth factor-beta (TGF-β) in *E. coli* K1 infected HBMEC increased invasion in a RhoA-dependent way [306].

III.2.2 Host immune evasion

Newborns are especially susceptible to infection by ExPEC, before or soon after birth, due to particular characteristics of their immune system which is adapted for early postnatal life [307]. Both innate and adaptive immunity are distinct at birth relative to adulthood, as the development of the immune system entails a number of age dependent maturation, during the first years of life.

Upon birth, newborns are exposed for the first time to a wide range of foreign antigens and, since they don’t possess immunologic memory, they are thought to be heavily dependent on their innate immune system for protection against infection [308].

Complement system

One of the first serological defenses of the innate immune system is the complement system. The concentration of all circulating complement proteins and complement

mediated opsonic capabilities are decreased in the neonatal plasma, when compared to the levels in adults [308-310]. Complement levels increase after birth and take months to reach adult concentrations [309]. These reduced levels are associated with deficient opsonization and impaired bacterial killing. In particular, the serum concentration of complement factor C9, essential for the formation of the membrane attack complex (MAC), is profoundly decreased in the newborns. Supplement of C9 protein to neonatal human serum enhanced the capacity to kill *E. coli* K1, although less efficiently than pooled adult sera [311].The K1 antigen is a polysialic acid immunochemically indistinct from the capsular antigen of serogroup B *Neisseria meningitidis*. It confers invasiveness to the bacteria by impairing complement opsonization and consequent phagocytosis [312]. *E. coli* K1 OmpA binds to C4-binding protein (C4 bp), a classical complement pathway regulator, to block the complement cascade reaction, and thereby avoid bacteriolysis and recognition by immune cells [313]. *E. coli* lipoprotein NlpI seems to contribute for this effect on the complement pathway [314]. Moreover, *E. coli* TraT and Iss protein have been associated with an interference with MAC formation [315,316] (Figure 3).

Innate immune cells

The increased susceptibility of the neonate to bacterial infections has also been associated with a quantitative and qualitative age-dependent deficit in the neonatal innate cell population [317,318]. Neutrophils, macrophages and dendritic cells develop and mature during fetal life, but at different times, and the function of all components of innate cell immunity is weak in newborns compared to later in life [307,319]. At the end of the third trimester there are already mature neutrophils, which increase in number, shortly before birth, due to stimulation with granulocyte-colony-stimulating factor [307]. Their number then returns to a stable level within days however, in response to infection, newborns showed a limited capacity to increase neutrophil numbers, probably due to a diminished bone marrow storage pool [320]. Additionally, neonatal neutrophils show a reduced bactericidal function and NET formation [321-323], poor responses to inflammatory stimuli, reduced adhesion to endothelial cells and diminished chemotaxis when compared to neutrophils from adult counterparts [324,325].

The survival of *E. coli* in neutrophils appears to be the first step in the pathogenicity of these bacteria and has already been described in peripheral blood-derived human neutrophils [326]. *E. coli* K1 infection of neutrophils increases the cell surface expression of gp96, enabling the association with *E. coli* OmpA. The interaction of OmpA with its receptor on neutrophils allows the internalization of the bacteria and inhibits the production of reactive oxygen species (ROS), even in the presence of external stimuli such as LPS. This suppression of the oxidative burst allows bacterial survival inside neutrophils (Figure 3). Proof of concept was established by neutrophil depletion that prevented the onset of meningitis in three-days old newborn mice intranasally infected [327].

Moreover, ExPEC express a secretory immunoglobulin A-binding protein (EsiB) that, by interaction with secretory immunoglobulin A (SIgA), interfered with SIgA-induced neutrophil chemotaxis and respiratory burst [328]. Conversely, neutropenia was associated with impaired host defense and increased susceptibility to *E. coli* K1 infection in a model of microbiota disruption in five-day old intraperitoneally infected mice [219] and a decrease on neutrophil recruitment was associated with higher colonization levels of TRIF KO neonatal [329].

Monocytes, macrophages and dendritic cells of preterm and newborn infants have both quantitative and qualitative differences when compared with adults. Monocytes have reduced MHC II expression, which potentially contributes to impairment of antigen presentation [330,331]. Despite human newborns having a basal TLR expression in blood monocytes similar to adults (with exception of preterm that have diminished TLR4 expression), the functional consequences of the activation of the signaling pathways is quite different [308].

As neutrophils, macrophages have been characterized as permissive niches for *E. coli* K1 survival. Bacterial entrance, survival, and intracellular replication were observed in both murine and human macrophage cell lines, as well as in monocytes and macrophages of newborn rats [332]. Again, OmpA is the central protein for this effect. The interaction of this protein with the alpha chain of Fcγ receptor I (FcγRI, CD64) is required for *E. coli* K1 entry into macrophages, for which IgG opsonization is not necessary [333,334] (Figure 3). The bacteria increases FcγRI expression on

macrophages by inducing pterin production, which, not only helps the entry of *E. coli* K1 in macrophages but also suppresses reactive oxygen species [335]. *E. coli* internalization and survival within professional phagocytes during early dissemination seems to also be dependent on TraJ expression [336]. Bacterial entry and multiplication within monocytes and macrophages permits the high grade bacteremia crucial to the pathogenesis of meningitis in neonates [336]. Nevertheless, in TRIF KO neonatal mice, the susceptibility to *E. coli* infection was associated with a decreased in the recruitment of peritoneal neutrophils and macrophages [329] and in a model of intracerebral injection of *E. coli* K1, the depletion of neutrophils and monocytes increased the susceptibility to the infection [337]. Overall, the detrimental/ protective effect of phagocytes in *E. coli* infection *in vivo* seems to be dependent on the animal model and type of inoculation that is used.

To survive within phagocytes, *E. coli* K1 uses several strategies. One of the approaches is the induction of anti-apoptotic protein BclXL expression and consequent blockade of mitochondrial cytochrome c release and inhibition of macrophages apoptosis [338]. Another, and unexpected, strategy for survival inside macrophages appears to be the induction of inducible nitric oxide (iNOS). Peritoneal macrophages and polymorphonuclear leukocytes isolated from iNOS KO mice demonstrated enhanced uptake and killing of *E. coli* K1 compared with macrophages and polymorphonuclear leukocytes from wild-type mice [339]. This counterintuitive effect of iNOS in *E. coli* infection is in part linked to the increase of OmpA receptor expression by NO production [340].

E. coli K1 infection of monocytes suppresses the production of cytokines and chemokines (TNF-α, MIP-1α, IL-1β and IL-8). This suppression is dependent of OmpA surface expression. OmpA prevents the phosphorylation and degradation of inhibitor KB, thereby blocking the translocation of nuclear factor (NF)-KB to the nucleus and consequent cytokine and chemokine production [341].

E. coli K1 invasion, intracellular survival and replication were also observed in myeloid DCs. Once more, OmpA deletion impairs this *E. coli* capability. Exposure of DCs to live *E. coli* K1, significantly up-regulates the expression of CD47, an integrin-associated protein, and its natural ligand thrombospondin 1 (TSP-1). CD47 is one of the molecules

that have been implicated in the tolerogenicity of immune cells and, in this case, prevented DCs from progressing in their maturation process [342] (Figure 3). The inhibitory effect on DC maturation was confirmed by failure to upregulate costimulatory molecules, CD40, HLA-DR, and CD86 and by an increased production of IL-10 as well as TGF-β and decreased production of IL-6, TNF-α, IL-1β, and IL-12p70 [343]. Blocking of OmpA with specific antibodies abrogated the suppressive effects of *E. coli* on DC maturation and the inhibition of CD47 expression conferred protection to infected pups in a model of *E. coli* K1-induced meningitis [342,343].

Moreover, immunosuppressive $CD71^+$ erythroid precursor cells have been shown to compromise neonatal host defenses against *E. coli* infection. These cells are enriched in neonatal mice and human cord blood and have distinctive immunosuppressive properties, essentially due to the expression of the enzyme arginase-2. The depletion of $CD71^+$ cells in neonatal mice, or the decline in number of these cells as postnatal development progresses, restored resistance to *E. coli* infection [344].

Toll-like Receptors and cytokines

The proper development of an immune response by innate immune cells is not only dependent on the inherent properties of the cells, but also on the identification of the pathogen and on the subsequent cell signals.

As previously mentioned, TLR basal expression and cellular distribution in monocytes from term newborns occurs at adult-like levels. Moreover, similar activation of neonatal and adult mononuclear samples is achieved in response to several tested TLR ligands [345]. Nevertheless, decreased expression and activation of intracellular signaling intermediates was detected in cord-blood cells [346-348].

Another important neonatal PRR is the β2-integrin CR3. Beside its role in binding complement, it also binds LPS and other microbial surface components [349]. The expression of this receptor is decreased on neonatal PMN and monocyte cells, with reduced levels in preterm neonates [350,351].

Although not observed on all studies, in response to many stimuli, including most TLR agonists, human neonatal cord blood cells exhibit a market polarization, with reduced

capacity to produce TH1-polarizing cytokines such as TNF-α, INF-γ and IL-12p70 and increased production of TH2/TH17 and anti-inflammatory cytokines such as IL-6, IL-10, IL-17, IL-1β and IL-23 [308,345]. Interestingly the capacity to produce IL-10 is even greater in preterm infants, whereas production of IL-6 and IL-23 are dominant in term infants [345,352].

TNF-α and IL-1β production during infection is associated with premature labor and favors abortion. This is, possibly, a relevant reason for the bias of maternal and fetal immune responses towards Th2-polarizing cytokines [353]. Plasma adenosine, an endogenous plasma metabolite that rises with hypoxia and stress, is elevated in newborn blood plasma and is, at least in part, responsible for the shift towards a high IL-6/TNF-α production ration by newborn cells [354].

TLR signaling proceeds through one of three different pathways: MyD88 alone, TRIF alone or the unique case of TLR4 in which both MyD88 and TRIF pathways are activated. TRIF KO mice neonates were more susceptible to intraperitoneal *E. coli* infection than WT or MyD88 KO pups [329]. Consistently, newborn TLR2 KO mice were proved to be more resistant to *E. coli* K1 meningitis, while TLR4 KO ones succumb to infection sooner than WT. In this study, resistance of TLR2 KO mice was associated with a decrease expression of Ecgp96 at the surface of BMEC [299]. The presence of the K1 capsule appears to be an important factor for the modulation of innate immune recognition of *E. coli.* The lack of CD14 (co-receptor with TLR4 and MD-2 of LPS) expression had no effect in survival of adult mice to *E. coli* K1, whereas CD14 KO mice presented higher resistance to K1-negative *E. coli* infection than WT mice [355].

Surprisingly, Toll-like receptor pre-stimulation appears to confer protective effects to immune cells, both in vitro and in vivo, against *E. coli* K1 infection. Stimulation of murine microglial cells with both TLR1/TLR2, TLR3, TLR4 and TLR9 agonists increases the phagocytic activity of the cells, promoting the killing of the bacteria as well as the production of TNF-α and neutrophil chemoattractant CXCL1 [356,357]. Moreover, intraperitoneal prophylaxis with CpG oligodeoxynucleotides (TLR9 agonist) protected neutropenic adult susceptible mice against intracerebral *E. coli* K1 infection [358].

The presence of cytokines, such as TNF-α, IL-1β, and IL-6 in CSF and blood is a hallmark of the pathogenesis of neonatal meningitis and sepsis. *E. coli* LPS is a well-known inducer of pro-inflammatory cytokines and several studies show a pro-inflammatory cytokine production after LPS or *E. coli* challenge. In neonates, however, *E. coli* K1 infection of monocytes blocked the production of cytokines and chemokines, even after stimulation with exogenous LPS [341], indicating that suppression of pro-inflammatory response in the replication stage is advantageous to *E. coli* for the establishment of meningitis. Nevertheless, the production of the anti-inflammatory cytokine IL-10, that is triggered during neonatal intranasal infection with *E. coli* K1 infection [359], is required for host survival, pathogen control and prevention of hyper-inflammatory immune responses [359]. IL-10 administration prior infection or during high grade bacteremia improved the outcome of *E. coli* K1 sepsis and meningitis [359,360]. This IL-10 administration induced the up-regulating CR3 expression on neutrophils and macrophages of infected mice, whereas infected and untreated mice displayed increased expression of FcγRI and TLR2 [359], receptors associated with bacterial survival inside host cells [299,335]. A protective effect of IL-10 administration on newborns was also observed on a model of maternal *E. coli* intrauterine inoculation and neonatal white matter injury [361]. Moreover, anti-TNF antibody pre-treatment was not capable of preventing the occurrence of *E. coli* K1 meningitis in newborn mice [359]. However, in a model of *E. coli* neonatal sepsis, TNF-α blocking resulted in a significant improvement in survival rate compared with not treated mice. [360]

Overall, the neonatal immune response to *E. coli* still needs to be further elucidated. So, the third aim of this thesis was to decipher some of the key aspects of *E. coli* K1 interaction with the neonatal immune system, by using a mice model with a natural route of infection.

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CHAPTER II.
RESULTS

MANUSCRIPT I

A safe and stable neonatal vaccine targeting GAPDH confers protection against Group B *Streptococcus* infections in adult susceptible mice

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Short Title: GBS GAPDH is a safe and stable vaccine

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ABSTRACT

Group B *Streptococcus* (GBS), a commensal organism, can turn into a life-threatening pathogen in neonates and elderly, or in adults with severe underlying diseases such as diabetes. We developed a vaccine targeting the GBS glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme detected at the bacterial surface, which was proven to be effective in a neonatal mouse model of infection. Since this bacterium has emerged as an important pathogen in non-pregnant adults, here we investigated whether this vaccine also confers protection in an adult susceptible and in a diabetic mouse model of infection. For immunoprotection studies, sham or immunized adult mice were infected with GBS serotype Ia and V strains, the two most prevalent serotypes isolated in adults. Sham and vaccinated mice were also rendered diabetic and infected with a serotype V GBS strain. For toxicological (pre-clinical) studies, adult mice were vaccinated three times, with three concentrations of recombinant GAPDH adjuvanted with Allydrogel, and the toxicity parameters were evaluated twenty-four hours after the last immunization. For the stability tests, the vaccine formulations were maintained at 4°C for 6 and 12 months prior immunization. The results showed that all tested doses of the vaccine, including the stability study formulations, were immunogenic and that the vaccine was innocuous. The organs (brain, blood, heart, and liver) of vaccinated susceptible or diabetic adult mice were significantly less colonized compared to those of control mice. Altogether, these results demonstrate that the GAPDH-based vaccine is safe and stable and protects susceptible and diabetic adult mice against GBS infections. It is therefore a promising candidate as a global vaccine to prevent GBS-induced neonatal and adult diseases.

INTRODUCTION

Streptococcus agalactiae, or Group B *Streptococcus* (GBS), is the leading cause of life-threatening bacterial infections in newborns [1]. In the past three decades, this bacterium have emerged as a major cause of invasive infections in non-pregnant adults, mainly in individuals with more than 65 years old or with underlying medical conditions [2-5]. Diabetes mellitus appears as the most common predisposition for GBS bacteremia in this group [2,5]. The case fatality rates are higher in adults than in neonates [2,6]. Eighty percent of human GBS isolates are resistant to tetracycline and it has been recently proposed that the widespread use of this antibiotic from 1948 was responsible for the selection of few tetracycline-resistant clones particularly adapted to the human host, thereby causing the emergence of GBS diseases in neonates in the 60s [7]. GBS express a capsular polysaccharide (CPS) and ten serotypes have been described to date (Ia, Ib, and II–IX). While GBS serotype III strains are strongly associated with neonatal meningitis, serotype V isolates emerged in the United States as the most frequent serotype causing invasive disease in nonpregnant adults, followed by serotypes Ia and III [5]. These three capsular serotypes are also associated with the vast majority of invasive infections in several European countries [8-10]. The only available treatment against GBS infections is based on the use of antibiotics. The implementation of the *intrapartum* antibiotic prophylaxis (IAP) in colonized pregnant women contributed to the decrease of the early-onset manifestations of GBS diseases (infection occurring in the first week of life). However, the IAP treatment has contributed to the emergence of antibiotic-resistant clones [6] with no effect on the late-onset neonatal disease (infections occurring between the first week and the first month of life). The rate of adult GBS disease has not declined until now and the use of antibiotics will likely cause increased resistance as observed with neonatal GBS isolates. Therefore, the development of a vaccine, as an alternative approach to the current use of antibiotics, will benefit neonates, pregnant and nonpregnant adults [11].

GBS vaccines have been initially developed by coupling capsular polysaccharide (CPS) antigens to immunogenic protein carriers but the existence of distinct epitope-specific capsular serotypes has hampered the development of a global GBS vaccine [12]. To avoid the selection of mutants that escape immune recognition, the ideal

human GBS vaccine should be directed against structurally conserved antigens that are essential for GBS virulence and/or growth, but none of the hitherto described candidate antigens fulfills these requisites.

We showed in a mouse model of neonatal GBS infection that GBS glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a valuable vaccine candidate. Maternal vaccination with the recombinant form of this protein was highly effective in protecting the offspring against a lethal infection with GBS [13]. Importantly, the antibodies raised against the bacterial GAPDH did not react with the human GAPDH [13]. However, before being used in clinical practice, a vaccine must pass several rigorous pre-clinical tests to evaluate its safety, effectiveness, or possible side effects [14]. Therefore, in this study, we conducted a comprehensive series of experiments to evaluate the stability, systemic toxicity, and local reactogenicity of the rGAPDH vaccine. These experiments were designed to identify any potential systemic and organ-specific toxicity, and to evaluate the stability of the vaccine formulation. Moreover, given the increased cases of adult infections with this pathogen, we assessed the effectiveness of rGAPDH vaccine against the infection caused by GBS in adult susceptible mice using two GBS strains, A909 and 2603V/R, belonging to serotypes Ia and V, respectively. Since diabetes is present in 20-40% of non-pregnant adults infected with GBS [2,4,5], we also tested the efficacy of rGAPDH vaccine in mice rendered diabetic. The obtained results showed that rGAPDH vaccine is highly immunogenic and stable for at least 12 months at 4°C, and no systemic or organ specific toxicity were observed. The protective assays proved that the vaccine constituted by GAPDH is effective against the infections caused by GBS in susceptible and diabetic adult mice. These results identify GBS GAPDH as a valuable global human vaccine to prevent neonatal and adult GBS diseases.

MATERIALS AND METHODS

Animals

Balb/cAnNCrl mice were purchased from Charles River (Italy). All the animals were kept in the animal facilities of the Institute Abel Salazar during the time of the experiments. Mice were 6-8 weeks of age at the beginning of experiences. They were housed in Techniplast ventilated polycarbonate cages under positive pressure with hardwood bedding and provided with Mucedola Diet and fresh tap water, *ad libitum*, throughout the study. All animals were housed in environmentally controlled cages with 40 air changes per hour.

Ethics statement

This study was carried out in strict accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and Directive 2010/63/EU and Portuguese rules (DL 113/2013). The animal experimental protocol was approved by the competent national authority Direção Geral de Alimentação e Veterinária (DGAV) (Protocol Permit Number: 0420/000/000/2008). All animal experiments were planned in order to minimize mice suffering.

Bacteria

GBS A909 (NEM2526) and 2603V/R (NEM2433) belong to capsular serotype Ia and V, respectively. They were grown in Todd-Hewitt broth or agar (Difco Laboratories) at 37°C.

Purification of recombinant GAPDH

E. coli BL21 (DE3) strain (Novagen) and the pET28a plasmid (Novagen) were used for production of the recombinant GAPDH (rGAPDH) protein from GBS NEM316 as described previously [15].

Formulations

rGAPDH vaccine for protection and safety studies were formulated prior to immunization with 10 µg (V10 group), 20 µg (V20 group), or 40 µg (V40 group) of protein, a dose corresponding to 0.5, 1 and 2 mg/kg of rGAPDH, respectively, in a 1:40 PBS-Alhydrogel suspension. The sham-immunized control animals received 200 µL of PBS (Vehicle control without Alhydrogel) or a 1:40 PBS-Alhydrogel suspension (Vehicle control with Alhydrogel). The vaccine used in stability studies was formulated immediately after rGAPDH purification with 20 µg of rGAPDH in a final volume of 200 µL of a 1:40 PBS-Alhydrogel suspension (Aluminium hydroxide Gel; Brenntag) and was maintained at 4°C for 0, 6, and 12 months (S0, S6 and S12 groups, respectively).

Vaccine safety studies

A total of 30 female Balb/c mice, in groups of 6, were injected s.c. three times, with a three-week intervening period, with 200 µL of V10, V20, or V40. The sham-immunized controls received 200 µL of PBS or 1:40 PBS-Alhydrogel suspension.

Cage side observations were performed daily and included evaluation of mortality, morbidity, general health, and signs of toxicity. Clinical observations were performed twice a week. Body weight was determined twice a week. Reactogenicity of immunization site was scored for edema, ulcer, and erythema using a scale from zero for no symptoms to four for severe symptoms. Reactogenicity scoring was performed on all the mice in each study group after each dosing. One day after administration of the last dose (Day 43), all animals were fasted overnight and euthanized for interim necropsy. Prior to necropsy, terminal blood was collected from all mice under Rompum/Imalgene 1000 anesthesia. Plasma was used for clinical chemistry analyses which included alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), albumin, amylase, lactate dehydrogenase (LDH), complement component 3 (C3), creatine kinase (CK), creatine kinase MB (CK-MB), total protein, creatinine, total bilirubin, glucose and Clara cell secretory protein 16 (CC16). Moreover, quantitative tests of 24-hour urine were also carried out with metabolic cages, for the evaluation of the levels of creatinine, N-acetyl-beta-D-glucosaminidase (NAG), and urea. All reagents were obtained from PZ Cormay

S.A with exception of NAG reagents that were obtained from Diazyme Europe GmbH.

Plasma biochemical parameters were measured in duplicate on an AutoAnalyser (PRESTIGE 24i, PZ Cormay S.A). Urinary urea, creatinine and total proteins were measured in duplicate according to previously described methods [16,17]. Plasmatic Clara cell 16 was quantified with an Enzyme Linked Immunosorbent Assay kit (USBiological) used according to the manufacturer's instructions.

Animals were subjected to a full gross necropsy. External features suggesting any abnormality, especially evidence of lymph node enlargement was register. After opening the chest and abdominal cavities, an *in situ* examination was done. Heart, lungs, liver, spleen, kidneys, gastrointestinal tract, pancreas, thymus and injection site were studied. The individual organs (liver, spleen, kidney, lung and heart) were removed and re-examined for gross morphology changes. After examination, they were weighed, adequately sliced and fixed in 4% (v/v) neutral buffered paraformaldehyde by diffusion, for 24h, and subsequently dehydrated with graded ethanol and included in paraffin blocks. Xylene was used in the transition between dehydration and impregnation. Sections of 5 µm were cut from paraffin blocks on a microtome (Leica Microsystems, Model RM2125) and mounted on silane coated slides. After dewaxing with xylene and rehydrated with graded alcohol, slides were stained with hematoxylin/eosin and examined under a light microscope (Zeiss Axio ImagerA1) by a certified veterinary pathologist. For every visual field, the histopathological evidences of tissue damage were analyzed as previously described [18] and a total histopathological score was calculated for each organ, allowing the comparison among all groups.

Vaccine stability studies

A total of 20 female Balb/c mice, in groups of 4, were injected subcutaneously (s.c.), three times, with a three-week intervening period, with 200 µL of S0, S6 and S12 preparations containing 20 µg of rGAPDH in a 1:40 PBS-Alhydrogel suspension. The sham-immunized control animals received 200 µL of PBS or a 1:40 PBS-Alhydrogel suspension. Cage side and clinical observations as well as evaluation of

the reactogenicity of immunization site were performed as described above for safety vaccination studies.

Immunoprotection studies

Balb/c mice were injected subcutaneously (s.c.), three times, with a 3-week intervening period with 20 µg dose of rGAPDH in a 1:40 PBS-Alhydrogel suspension. The sham-immunized control animals received 200 µL of 1:40 PBS-Alhydrogel suspension. Mice were infected i.p. with 0.3 ml of PBS containing $3x10^6$ CFU of GBS A909, or $3x10^7$ CFU of GBS 2603V/R, and sacrificed at indicated timepoints. Survival curves were determined over a 20-day experimental period. Prior to necropsy, terminal blood was collected from all mice under Rompum/Imalgene 1000 anesthesia. Blood was collected and analyzed for GBS counts and the serum was used for cytokine analysis. The analyzed organs were aseptically removed, homogenized in PBS and serial dilutions of homogenized organs were plated on Todd-Hewitt agar to enumerate bacterial CFU.

Diabetes mouse model

Balb/c mice were injected subcutaneously (s.c.), three times, with a 3-week intervening period with 20 µg dose of rGAPDH in a 1:40 PBS-Alhydrogel suspension. The sham-immunized control animals received 200 µL of 1:40 PBS-Alhydrogel suspension. One week after the second immunization, diabetes was induced by i.p. administration of a single dose of streptozotocin (Sigma) at 200 mg/kg freshly dissolved in 0.05M citrate buffer, pH 4.5 [19]. Plasma glucose levels were measured by OneTouch Verio blood glucose meter system (LifeScan, Johnson and Johnson Company). Serum glucose levels of control animals ranged from 75-195 mg/dl. Mice showing non-fasting serum glucose levels above 600 mg/dl at the time of the third immunization STZ-injection were considered diabetic and used for the study. Mice were infected i.p. with 0.3 ml of PBS containing $3x10^7$ CFU of GBS 2603V/R one week after the last immunization and sacrificed 18h post-infection. Prior to necropsy, terminal blood was collected from all mice under Rompum/Imalgene 1000 anesthesia. Peritoneal lavage was performed with 5 mL of ice-cold PBS. The analyzed organs were aseptically removed, homogenized in PBS

and serial dilutions of homogenized organs were plated on Todd-Hewitt agar to enumerate bacterial CFU.

Quantification cytokines and CRP

TNF-α, IL-6, IL-1β and C-reactive protein (CRP) were quantified with an Enzyme Linked Immunosorbent Assay kit (eBioscience) used according to the manufacturer's instruction.

Antibody titration

Total IgG and rGAPDH-specific IgG antibodies titers were assessed in the plasma of immunized mice by Enzyme Linked Immunosorbent Assay. Briefly**,** serial dilutions of the serum of immunized mice were added to the wells of a microtiter plate (NUNC) coated with rGAPDH (5 µg/mL) for two hours at room temperature.

A conjugated goat anti-mouse IgG-HRP (H+ L, 1:1000, SouthernBiotech) antibody was then added and the plate was incubated for two additional hours at room temperature. The *o*-phenylenediamine substrate solution (Pierce) was added and, after addition of the stop solution, the color reaction was measured immediately by the absorption at 450 nm using a spectrophotometer (Thermo Multiskan Ex).

Statistical analysis

All statistical analyses were performed in GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, California). For safety studies, one-way ANOVA with post-hoc Dunnett's Multiple Comparison Test with 95% of confidence was used to analyze the differences between all groups and Alhydrogel group. For stability studies, a one-way ANOVA with post-hoc Tukey’s t-test with 95% of confidence was used to compare the different tested groups. For colonization and cytokine analysis, unpaired Student’s t-test was used to determine the differences between GAPDH-immunized and sham-immunized groups. For survival curve analysis, Mantel-Cox test was performed. Considering the abnormal distribution of the histopathological score data, differences among groups were tested using the nonparametric Kruskal-Wallis test followed by Dunn's test. A P value < 0.05 was considered statistically significant.

RESULTS

Safety studies evaluation

Mortality and clinical observations

All mice survived to the assigned end point and appeared outwardly healthy after exposure to the vaccine, with no visible physical disability or behavior alterations.

Reactogenicity

Small nodules (< 3 mm) were only observable after necropsy at the site of each injection in mice of the groups V10, V20, V40 and Alhydrogel alone. The nodules were too small to be noticed by touch during reactogenicity observations. This was considered as a normal reaction resulting from the vehicle components. Signs of edema or erythema were not observable in any animal during the time of experiment.

Body weight and body weight increase

No significant effects on body weight and body weight increase were observed during the study among all test groups. Overall, most animals gained weight throughout the study.

Anatomical pathology and histopathology

There were no significant changes in organ weights (Table 1) and no gross morphological changes. Microscopic analysis was performed on several sections from different locations in every organ with a magnification of 40x in order to guarantee a global and precise organ overview. This examination took into account the severity of tissue organization, the degree of cellular degeneration, the amount of interstitial inflammatory cells, and the existence and extension of tissue necrotic areas. In all groups, none of these histopathological traits was detected in the vital organs studied and the total histopathology score calculated for each organ did not show significant differences among groups (data not shown).

Immunogenicity

The efficacy of a vaccine is closely associated with the strength of the induced immune response [14]. To confirm the immunogenicity of vaccine, titers of IgG specific for rGAPDH were determined in the serum of the animals twenty-four hours after the last immunization.

All tested vaccine doses (V10, V20, and V40) induced the production of IgG antibodies against rGAPDH (Fig 1). The titer values show a tendency to increase with the dose of rGAPDH-Alhydrogel injected, but the observed differences are not statistically significant. As expected, rGAPDH-specific IgG antibodies were not detected in the serum of the controls (Vehicle and Alhydrogel).

Plasma and urine chemistry

The serological and urine biochemical parameters were evaluated in all animals and found to be in normal range after repeated exposure to the vaccine compounds (Table 2). The parameters evaluated were: hepatotoxicity by quantifying the levels of alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH) and albumin [20]; cardiotoxicity by assessing the levels of creatine kinases (CK) and specifically its isoform creatine kinase-MB (CK-MB) [21]; and nephrotoxicity by measuring the levels of urea, creatinine and N-acetyl-beta-D-glucosaminidase [22]. Moreover, to evaluate pancreatic and lung toxicity, the amylase and clara-cell 16 were analyzed, respectively [23,24]. The systemic toxicity was also considered by quantifying the serum levels of glucose and total proteins.

Inflammatory parameters

Acute-phase proteins were used as markers for acute inflammation induced after rGAPDH immunization. These acute-phase proteins are produced by cells or tissues in response to inflammatory stimulus. The degree of inflammation can be evaluated by measuring the serum level of these proteins [25].

The levels of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 in the serum of the animals were below detection level in all groups and the levels of C-reactive protein were not significantly different between groups (Table 2).

Stability studies evaluation

The shelf-life is an important feature for any vaccine or drug to be used in humans, especially when intended for use in low-income countries where it is often difficult to provide appropriate storage conditions [26]. The vaccine formulations were maintained at 4°C for 6 (S6) and 12 (S12) months before immunization. An immunization protocol similar to that of the safety studies was used and the potency of the formulations was evaluated by comparing the resulting specific antibody titers with those obtained with a fresh prepared formulation (S0).

Antibody titers

The titers of IgG antibodies against rGAPDH induced by vaccines stored at 4°C for 6 and 12 months were similar to those induced by a freshly prepared vaccine (Fig 2). Moreover, as expected, rGAPDH-specific IgG antibodies were not detected in the serum of the controls (Vehicle and Alhydrogel) (Fig 2). The levels of total IgG were similar between all groups (vaccinated and controls, data not shown). The storage of the vaccine did not alter the potency or the specificity of the response.

Safety assessment of S0, S6, and S12 formulations

Treatment with S6 and S12 formulations had no life-threatening effect. All mice survived to the assigned end point and appeared healthy outwardly after exposure to the vaccine, with no visible physical disability or behavior alterations, and with no difference in body weight associated with the immunization. The treatment with the vaccines and adjuvant alone produced small nodules only observable after necropsy at the site of injection. The levels of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 in the serum of the animals were below detection threshold in all groups. The levels of C-reactive protein were not significantly different between groups (Data not shown).

rGAPDH vaccination protects susceptible and diabetic adult mice from GBS infections

To assess the effectiveness of rGAPDH vaccine against GBS infections in adult mice, immunized adult Balb/c mice were infected with A909 (serotype Ia) or 2603V/R (serotype V). As shown in Fig 3 and 4, rGAPDH immunization confers protection to adult mice against GBS infections caused by both serotypes. The survival of rGAPDH-vaccinated mice, either infected with serotype Ia or V, were significantly increased compared with the respective sham-immunized group (Fig 3 and Fig 4). Indeed, none of the immunized mice succumbed to the infection (100% survival) whereas ~40% and ~60% of sham-immunized mice infected with serotype Ia and V, respectively, died within the first 48h (Fig 3A and 4A). To investigate whether the longer survival of rGAPDH-vaccinated mice was associated with an early control of GBS growth, we next determined organ colonization at 6h and 18h post-infection. When infected with GBS serotypes Ia or V, lower numbers of viable bacteria were found in blood, heart, liver, and brain of the rGAPDH-immunized mice, as compared with the sham-immunized controls (Fig 3B and 4B). Lower bacterial levels were also found in the lung of rGAPDH-immunized animals infected with serotype Ia (Fig 3B). Since non-pregnant adult humans with diabetes are highly susceptible to GBS infections, we also evaluated the efficacy o rGAPDH vaccine in mice rendered diabetic by streptozotocin induction and infected with GBS 2603V/R, i.e. a strain belonging to the emerging serotype causing invasive disease in non-pregnant adults. As shown in Table 3, rGAPDH vaccination rendered the diabetic mice more resistant to GBS infection, compared with sham-vaccinated diabetic mice. Indeed, 18h post-infection, lower levels of GBS CFUs were observed in blood, heart, liver, peritoneum, kidney, spleen and brain of the rGAPDH-immunized compared with the sham-immunized controls (Table 3).

To characterize the pro-cytokines associated with protection in rGAPDH-immunized mice, IL-6, TNF-α, and IL-1β were analyzed 3h post-infection in blood, liver and spleen of both groups. As shown in Fig 3C and 4C, an increased production of the pro-inflammatory cytokines, mainly IL-6 and IL-1β, were observed in vaccinated mice. Indeed, compared to the sham-immunized controls, the rGAPDH-immunized mice infected with either GBS serotype Ia or V, presented higher levels of IL-6 in

sera, spleen, and liver, and of IL-1β in liver. Higher levels of TNF-α were observed in the spleen of immunized mice infected with GBS serotype V (Fig 4C).

DISCUSSION

In the past few decades GBS infections have been responsible for a significantly high morbidity and mortality rates amongst nonpregnant adults, particularly in elderly or individuals with chronic underlying conditions, such as diabetes [2-5]. Currently, the invasive disease in adults is increasing and represents about two-thirds of all cases of GBS infections, with a case fatality ratio of about 10-20% [4]. The antibiotic use as prophylactic measure in pregnancy and as treatment in adult against GBS infections raised several obvious concerns regarding the emergence and dissemination of antibiotic-resistant clones [6,7,27]. Moreover, while the use of IAP for prevention of GBS disease since 1996 was highly effective at preventing early-onset diseases, it did not changed the rate of late onset diseases, as well as the rates of stillbirths and prematurity caused by GBS infections [28]. Thus, vaccination is considered as a promising and complementary alternative since it could be easily and broadly applicable and could protect not only adults, but also newborns without the inherent limitations and problems associated with antibiotic use [29].

Since low levels of maternal antibodies to capsular polysaccharide antigens correlate with neonatal susceptibility, efforts were concentrated in the production of a vaccine based on the GBS capsular polysaccharide [30,31]. However, this strategy is not consistent with the fact that the distribution of the ten GBS serotypes can vary in space and time [32,33]. Moreover, capsular polysaccharide switching has been recently reported in GBS [34]. The possibility of a shift in serotype prevalence supports the need for a universal GBS vaccine based on a different antigen [35]. Accordingly, Grandi and colleagues developed a vaccine utilising a component of each of the three pilus identified so far in GBS [36]. However, a change in the pili variant was described to occur in the case of *Neisseria gonorrhoeae*, rendering a promising vaccine ineffective [37]. Based on this observation, one might predict that, following vaccination with pilus antigen, the immune selective pressure will select GBS pilus variants to escape immunity.

We have identified GAPDH as a valuable universal GBS vaccine candidate [13,15]. In a mouse model of GBS infection, maternal vaccination with rGAPDH or passive

immunization with anti-rGAPDH IgG antibodies conferred neonatal protection [13]. Since GAPDH is ubiquitously expressed in all type of cells, including mammalian cells, we demonstrated that the antibodies produced against the GBS GAPDH do not react with its human counterpart [13]. Eukaryotic and prokaryotic GADPH sequences display only two 10-aminoacid long identical segments and we consistently showed that antibodies produced against either native or denatured GBS GAPDH do not recognize human GAPDH [13].

In the present work, the protective effect of this vaccine was extended to adult susceptible mice. We showed that adult mice vaccinated with rGAPDH are protected against GBS infections caused by the two serotypes, Ia and V, mostly associated with the human invasive disease [5]. The survival of rGAPDH-vaccinated adult mice is significantly increased compared with sham-vaccinated animals. Mice vaccinated with rGAPDH presented decreased number of bacterial counts in blood, brain, and heart, compared with sham-immunized animals. This observation is particularly important since endocarditis and meningitis are the two clinical presentations of GBS invasive infections with the worst prognosis in terms of morbidity and mortality [38,39]. The rising incidence rate of GBS infections in non-pregnant adults has been associated with the aging of the population and with the increasing prevalence of individuals with underlying comorbidities, like diabetes mellitus [4,5]. Thus, to mimic the impact of diabetes in the susceptibility to GBS infections, we determined the efficacy of the rGAPDH vaccine in a mouse model of streptozotocin-induced diabetes. Our results show that vaccination with rGAPDH protects adult diabetic mice against GBS serotype V infection.

GAPDH is an essential cytoplasmic enzyme involved in the glycolytic pathway which, despite the lack of standard signal sequences, has been found at the surface of unrelated GBS isolates [13]. This abundant enzyme is most likely released upon cell lysis to then bind to the surface of living bacteria [40]. GAPDH possesses a critical metabolic function, being essential for bacterial growth in blood, and plays an important role in GBS virulence. Alike GAPDH, other metabolic and cytosolic proteins have been also detected at the surface of numerous microorganisms where they exert a distinct function, being therefore called “moonlighting” proteins [41]. In pathogens, extracellular “moonlighting” proteins are often involved in colonization

and invasion of host tissues and we previously reported that cell surface bound GAPDH confers to GBS the ability to bind plasminogen and fibrinogen [42,43] and displays immunomodulatory properties that contributes evasion from the host immune system [13,15]. The potential of these “moonlighting” proteins as a vaccine target have also be experimentally assayed [44-47] in other streptococci. For example, the α-enolase of *Streptococcus sobrinus* and *Streptococcus suis* [46,47], the fructose-bisphosphate aldolase *of Streptococcus pneumoniae’* [45] and the arginine deiminase and the trigger factor of *Streptococcus pyogenes* [44] were characterized as protective antigens.

To move a candidate vaccine from the laboratory to the clinic, preclinical tests including safety studies in animals are mandatory [14,48]. The evaluation of toxicological parameters of repeated administration of rGAPDH-Alhydrogel formulations showed no effect on mortality, clinical appearance, behavior, or body weight change. In the first months of life, newborns protection against infectious diseases is highly dependent on passive immunity mediated by mother’s specific IgG antibodies that are transferred trough the placenta [49]. Therefore, a maternal vaccine aimed at protecting the newborn should elicit high levels of IgG antibody [48]. Consistently, our results showed that all tested doses of GAPDH were immunogenic and induced high and similar levels of specific rGAPDH IgG antibodies. Gross and histopathological examinations revealed no obvious abnormalities in organs and tissues of vaccinated mice. Moreover, the biochemical characterization of serum samples confirmed the innocuity of the vaccine deduced from the histological data. The serum levels of acute phase proteins or organ specific markers were not altered by the immunization, which indicates that rGAPDH vaccination does not induce acute inflammation or organ toxicity. Therefore, our study demonstrates the safety and immunogenicity of the rGAPDH vaccine. The WHO guidelines for nonclinical and clinical evaluation of vaccines stress a need for stability data to support clinical trial approval [50] and we showed here that the rGAPDH vaccine conserves its potency and safety, without significant alteration, at 4°C for at least 12 months.

This study predicts a potential application of rGAPDH vaccine in humans to prevent GBS-induced adult and neonatal diseases.

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Figure Legends

Fig 1. rGAPDH vaccine is immunogenic at all tested doses. rGAPDH-specific IgG titers of 6 female Balb/c mice per group, immunized three times with a three week interval, 24h after the third immunization with 10 (V10), 20 (V20) or 40 (V40) µg of rGAPDH with 1:40 Alhydrogel, alhygrogel alone, or PBS. The ELISA plates were coated with rGAPDH and revealed with goat anti-mouse IgG - HRP. Titers are represented as minimal serum dilution necessary for the lost absorbance signal.

Fig 2. rGAPDH vaccine preserves its potency after prolonged storage at 4ºC. rGAPDH-specific IgG titers of 4 female Balb/c mice per group, immunized three times with a three week interval, 24 h after the third immunization with PBS, Alhydrogel and 20 µg of rGAPDH in a 1:40 PBS/Alhydrogel suspension prepared fresh (S0) or conserved at 4°C for 6 (S6) and 12 (S12) months. The ELISA plates were coated with rGAPDH and revealed with goat anti-mouse IgG - HRP. Titers are represented as minimal serum dilution necessary for the lost absorbance signal. One-way ANOVA with post-hoc Tukey's t-test. ns – not significant.

Fig 3. rGAPDH vaccination improves survival and induces protection against serotype Ia GBS infection in an adult mouse model. A) Kaplan–Meier survival curves. The lethality was monitored for 20 days. The numbers in parentheses represent the number of animals that survived out of the total number of infected animals. B) Blood, liver, lung, heart and brain colonization 6 and 18 hours post-infection (6 hours - Sham and GAPDH-immunized n=7; 18 hours - Sham n=7, rGAPDH-immunized n=6) and C) sera, liver and spleen cytokine production 3 hours post-infection (n=6 for both groups). Balb/c mice were immunized three times, with a three week interval, with 20 µg of rGAPDH in 1:40 Alhydrogel or treated with Alhydrogel alone and infected i.p. with $3x10^6$ CFU of A909 (serotype Ia). Unpaired Student's t-test. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$

Fig 4. rGAPDH vaccination improves survival and induces protection against serotype V GBS infection in an adult mouse model. A) Kaplan–Meier survival curves. The lethality was monitored for 20 days. The numbers in parentheses

represent the number of animals that survived out of the total number of infected animals. B) Blood. liver. lung. heart and brain colonization 6 and 18 hours post-infection and (6 hours - Sham n=8. GAPDH-immunized n=7; 18 hours - Sham n=9. rGAPDH-immunized n=8) C) sera. liver and spleen cytokine production 3 hours post-infection (n=6 for both groups). Balb/c mice were immunized three times, with a three week interval, with 20 µg of rGAPDH in 1:40 Alhydrogel or treated with Alhydrogel alone and infected i.p. with $3x10^7$ CFU of 2603V/R (serotype V). Unpaired Student's t-test. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$

Table 1. Organ weight in mice with different vaccine doses

Organ	Vehicle control (w/out Alhydrogel)	Vehicle control (w/ Alhydrogel)	rGAPDH (with Alhydrogel)		
			V10	V20	V40
Heart	0.006 ± 0.001	0.006 ± 0.001	0.006 ± 0.001	0.006 ± 0.001	0.006 ± 0.001
Lungs	0.52 ± 0.04	0.50 ± 0.04	0.59 ± 0.11	0.58 ± 0.09	0.51 ± 0.05
Kidneys	2.02 ± 0.33	2.14 ± 0.29	2.11 ± 0.34	2.40 ± 0.39	2.19 ± 0.20
Liver	8.91 ± 1.41	9.11 ± 1.34	8.99 ± 1.31	7.84 ± 1.42	9.74 ± 0.92
Spleen	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01

Values are expressed as mean ± S.D; Number of animals per goup = 6; V10 – 10 µg s.c.; V20 – 20 µg s.c.: V40 – 40 µg s.c. One-way ANOVA with post-hoc Dunnett's Multiple Comparison Test, $p > 0.05$.

Table 2. Biochemistry of plasma and urine collected 24h after the last vaccine injection

	Vehicle control (w/out Alhydrogel)	Vehicle control (w/ Aldhydrogel)	rGAPDH (with Alhydrogel)		
			V10	V20	V40
Plasma Parameters					
Albumin (g/L)	32.61 ± 2.21	29.19 ± 4.65	29.86 ± 1.19	31.89 ± 1.81	30.91 ± 2.22
Total proteins (g/L)	51.84 ± 3.41	48.04 ± 1.09	47.22 ± 1.96	48.98 ± 3.08	47.34 ± 2.24
Glucose (mg/dL)	85.7 ± 30.7	106.6 ± 29.9	90.5 ± 25.8	86.2 ± 10.8	87.0 ± 25.8
CK-MB (U/L)	227.9 ± 30.3	230.9 ± 38.8	236.0 ± 97.6	224.1 ± 67.9	196.2 ± 53.8
CK (U/L)	1111 ± 265	1192 ± 397	1754 ± 721	1460 ± 476	1175 ± 546
ALAT (U/L)	31.7 ± 2.9	35.1 ± 11.5	34.0 ± 2.9	29.4 ± 4.7	31.0 ± 3.5
ASAT (U/L)	99.0 ± 19.2	92.5 ± 20.7	111.1 ± 37.2	112.1 ± 33.4	90.2 ± 26.7
LDH (mg/dL)	743.6 ± 360.7	633.6 ± 234.7	625.6 ± 107.7	677.4 ± 65.6	623.9 ± 203.5
Amylase (U/L)	966.2 ± 262.2	870.5 ± 197.6	1000 ± 251.9	867.2 ± 167.9	806.8 ± 101.9
C3 (mg/mL)	11 ± 3	11 ± 3	14 ± 4	9 ± 3	14 ± 5
Urea (mg/dL)	46.3 ± 11.3	47.9 ± 10.3	53.2 ± 22.6	45.5 ± 5.8	45.3 ± 9.7
Creatinine (mg/dL)	0.35 ± 0.08	0.35 ± 0.05	0.28 ± 0.12	0.35 ± 0.05	0.30 ± 0.10
CC16 (ng/mL)	125.1 ± 43.1	154.9 ± 29.3	161.3 ± 37.9	121.2 ± 55.5	116.4 ± 25.6
Urine Parameters					
Urea (mg/24h)	4.47 ± 3.40	5.18 ± 2.68	5.48 ± 3.68	4.46 ± 2.35	4.91 ± 2.41
Creatinine (mg/dL)	57 ± 24	65 ± 14	70 ± 18	67 ± 22	66 ± 25
NAG (U/L)	12.04 ± 2.63	11.71 ± 3.95	15.80 ± 1.95	14.57 ± 3.6	11.56 ± 3.5
Inflammatory Parameters					
IL-1β (pg/mL)	BDL	BDL	BDL	BDL	BDL
IL-6 (pg/mL)	BDL	BDL	BDL	BDL	BDL
TNF-α (pg/mL)	BDL	BDL	BDL	BDL	BDL
CRP (ng/mL)	2.30 ± 0,76	2.50 ± 0,81	2.30 ± 0,30	2.40 ± 0.31	2.23 ± 0,62

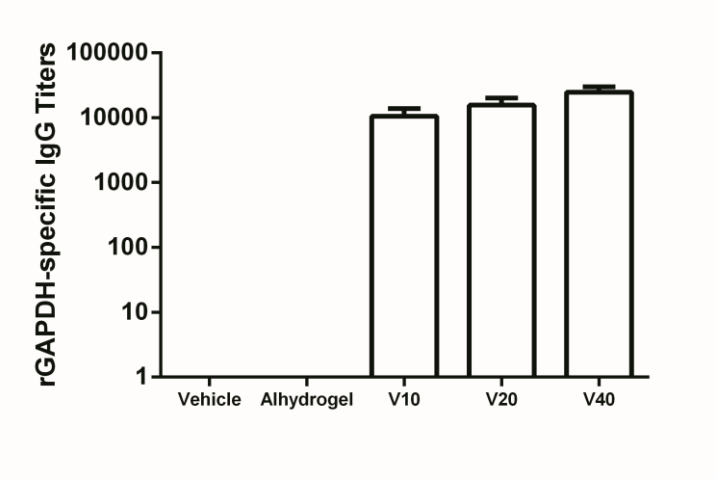
Values are expressed as mean ± S.D; Number of animals per group=6; BDL – Below detection Level; CK - Creatine kinase; CK-MB - Creatine kinase MB; ALAT - Alanine aminotransferase; ASAT - Aspartate aminotransferase; LDH - Lactate dehydrogenase; C3 – Complement component 3; CC16 - Clara cell secretory protein 16; NAG - N-acetyl-beta-D-glucosaminidase; IL – Interleukine; TNF-α – Tumor necrosis factor α; CRP – C-reactive protein. One-way ANOVA with post-hoc Dunnett's Multiple Comparison Test, $p > 0.05$.

Table 3. Organ colonization of rGAPDH and Sham-immunized diabetic mice infected with GBS serotype V strain 2603V/R

Organs	Diabetic mice		P-value[a]
	Sham-immunized (log CFU/organ)	rGAPDH-immunized (log CFU/organ)	
Blood	4.35 ± 0.732	0.902 ± 0.462	0.0010***
Liver	5.22 ± 0.550	3.06 ± 0.216	0.0017**
Spleen	4.76 ± 0.505	3.16 ± 0.460	0.0329*
Lung	4.60 ± 0.665	2.46 ± 0.501	0.0198*
Brain	2.61 ± 0.625	0.189 ± 0.189	0.0014**
Heart	4.43 ± 0.908	1.52 ± 0.511	0.0114*
Kidney	4.18 ± 0.754	1.92 ± 0.300	0.0107*
Peritoneum	5.69 ± 0.887	2.93 ± 0.621	0.0204*

Mice were killed 18h post-infection and the organs collected for bacterial counts. Values are expressed as mean ± S.E.M.; Number of animals Sham-immunized = 8. rGAPDH-immunized = 9; [a]Student's t Test. *p<0.05; **p<0.01. ***p<0.001

Figure 1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100000
10000
1000
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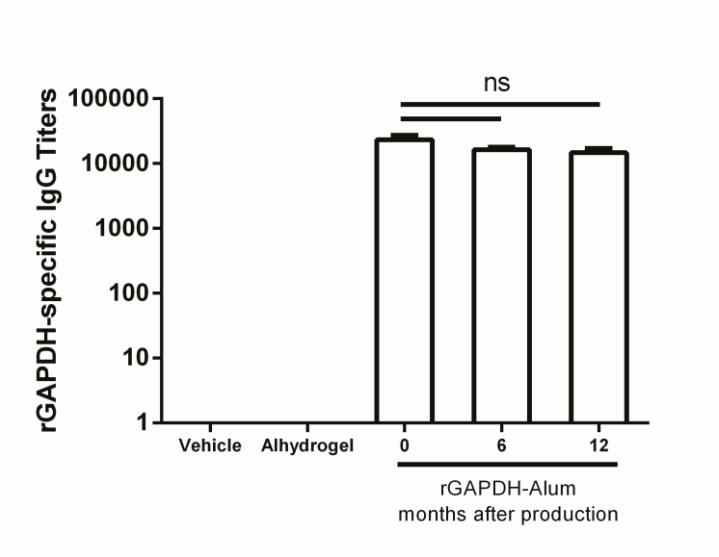
10
1
Vehicle
Alhydrogel
V10
V20
V40

Figure 2


ns
rGAPDH-specific IgG Titers
100000
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Vehicle
Alhydrogel
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12
rGAPDH-Alum
months after production

Figure 3

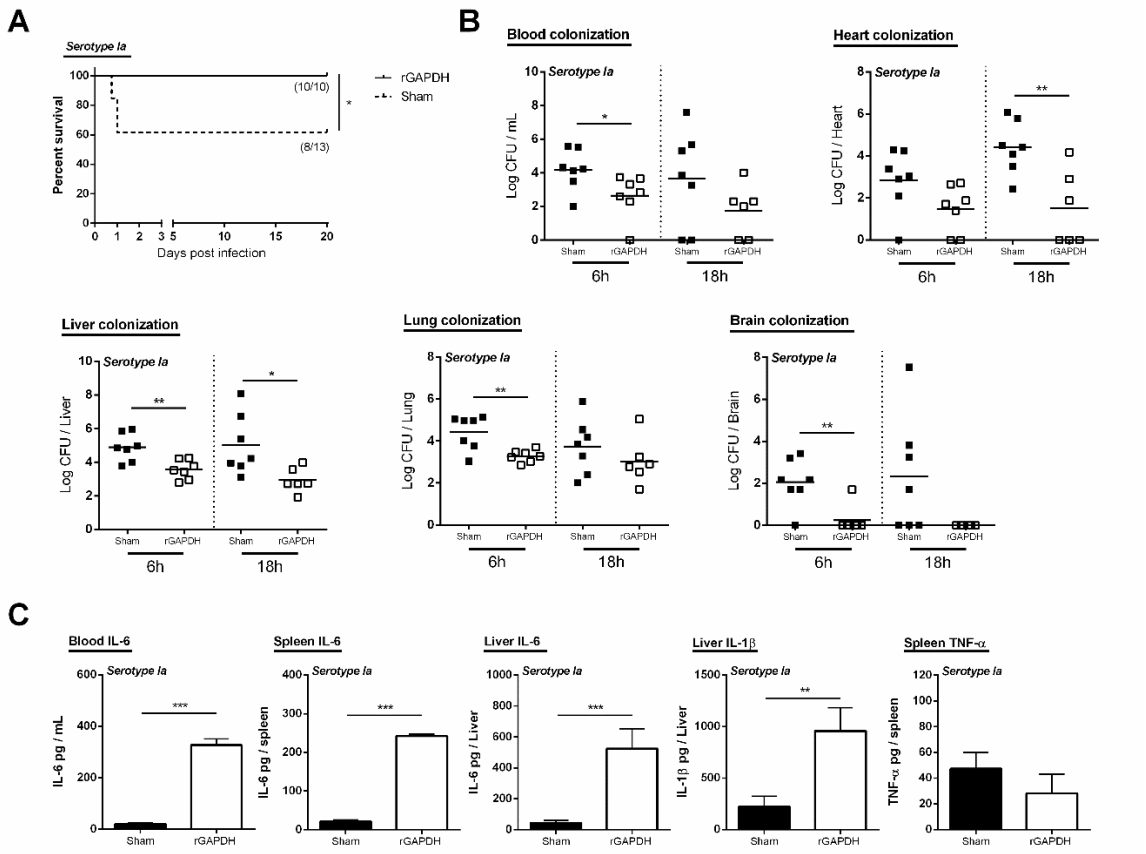
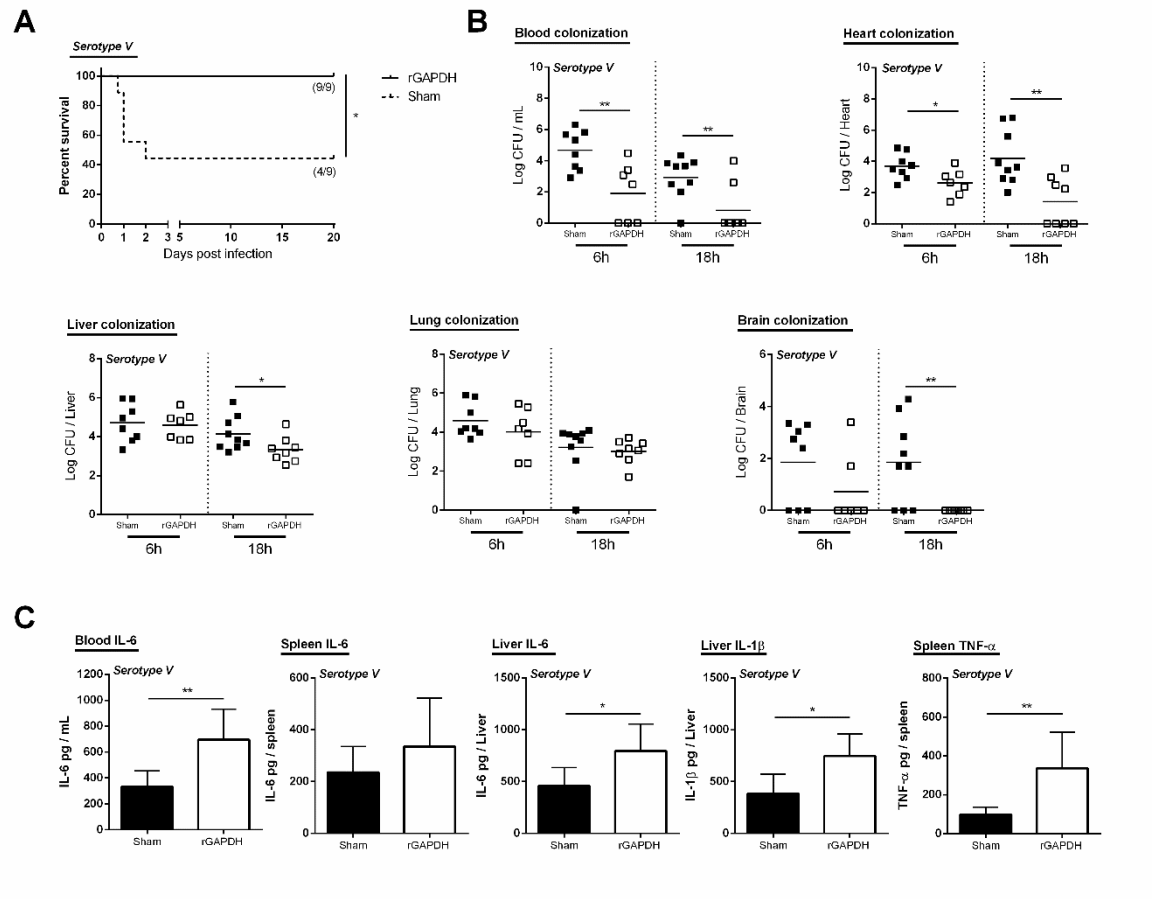

A
Serotype Ia
Percent survival
Days post infection
rGAPDH
Sham
(10/10)
(8/13)
B
Blood colonization
Heart colonization
Liver colonization
Lung colonization
Brain colonization
Log CFU / mL
Log CFU / Heart
Log CFU / Liver
Log CFU / Lung
Log CFU / Brain
Sham
rGAPDH
6h
18h
C
Blood IL-6
Spleen IL-6
Liver IL-6
Liver IL-1β
Spleen TNF-α
IL-6 pg / mL
IL-6 pg / spleen
IL-6 pg / Liver
IL-1β pg / Liver
TNF-α pg / spleen

Figure 4


A
Serotype V
Percent survival
Days post infection
rGAPDH
Sham
(9/9)
(4/9)
B
Blood colonization
Heart colonization
Liver colonization
Lung colonization
Brain colonization
Log CFU / mL
Log CFU / Heart
Log CFU / Liver
Log CFU / Lung
Log CFU / Brain
Sham
rGAPDH
6h
18h
C
Blood IL-6
Spleen IL-6
Liver IL-6
Liver IL-1β
Spleen TNF-α
IL-6 pg / mL
IL-6 pg / spleen
IL-6 pg / Liver
IL-1β pg / Liver
TNF-α pg / spleen

MANUSCRIPT II

An unexpected increase of the susceptibility to *Escherichia coli* infection in offspring of mother vaccinated with GAPDH from this pathogen.

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Short Report *In preparation*

ABSTRACT

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme that has been associated with virulence of several microorganisms. Vaccines containing GAPDH have been tested and proved effective against infections with several pathogens. One of those vaccines, against the neonatal infectious agent Group B *Streptococcus* (GBS), was developed by our group. Since GAPDH was reported as an extracellular virulence factor for *Escherichia coli,* another important neonatal pathogen, here we investigated whether the recombinant GAPDH of GBS (rGAPDH$_{GBS}$) or the recombinant GAPDH of *E. coli* (rGAPDH$_{E.coli}$) could be used as a vaccine against the neonatal infections caused by this pathogen. The cross-reactivity of the antibodies produced against rGAPDH$_{GBS}$ and of rGAPDH$_{E.coli}$ was tested but, despite the 53% homology between the two GAPDHs, the antibodies produced against one of the GAPDH were unable to recognize the other. Having in mind the properties already described for rGAPDH$_{GBS}$, the ability of rGAPDH$_{E.coli}$ to stimulate splenic B cell populations from neonatal and adult mice and induce IL-10 production in these cell cultures was evaluated at different timepoints. However, the rGAPDH$_{E.coli}$ did not induce splenic B lymphocytes activation in neonatal or adult mice cell cultures and did not promote IL-10 production. For vaccination studies, female Balb/c mice were immunized with rGAPDH$_{E.coli}$ and the offspring was orally infected with *E. coli* K1. Unexpectedly, maternal rGAPDH$_{E.coli}$ immunization instead of conferring resistance to its offspring against *E. coli* infection increased its susceptibility to this pathogen. In conclusion, GAPDH of *E. coli* cannot be used as a target antigen for vaccination against *E. coli* infections and this study highlights the need for precaution in the generalization of the use of GAPDH-based vaccines.

INTRODUCTION

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) is a key glycolytic enzyme that is responsible for the reversible conversion of glyceraldehyde-3-phosphate to 1-3 di-phosphoglycerate. However, in the last years, several other functions have been identified and associated with this highly conserved protein, both in eukaryotic and prokaryotic cells. In mammals, for instance, GAPDH has been associated with gene regulation and maintenance of DNA integrity, and appears to be involved in several cellular processes as apoptosis and autophagy (reviewed in [1]). In prokaryotic cells GAPDH has been vastly associated with the virulence of the microbe. Indeed, beside its intracellular localization, important for its glycolytic function, GAPDH has been found associated with the surface or excreted by several pathogens (reviewed in [2]). Its ability to bind to matrix proteins like plasminogen and fibrinogen is an important aspect for adherence and cell invasion of Gram-positive and [3-7], as well as Gram-negative bacteria [8]. Bacterial GAPDH has also been associated with iron uptake (by association with haemoglobin, haem and transferrin) [9,10] and immune evasion (by induction of the anti-inflammatory cytokine IL-10) [11]. Because of these properties, several groups have already invested efforts in developing GAPDH-based vaccines that proved to be efficient against human and veterinary infectious diseases (reviewed in [12]). One of those GAPDH-based vaccines was developed by our group against the bacterium Group B *Streptococcus* (GBS) responsible for serious illness and mortality in newborns and immune-compromised individuals [13]. We proved that GBS' recombinant GAPDH vaccine is effective in a neonatal and adult mouse model of infection [11,14]. Having in mind that GAPDH is a highly conserved protein we hypothesized that GAPDH could be a suitable target for a vaccine to control neonatal infections caused by other pathogens. *E. coli* is the second most common cause of neonatal infections and, in preterm and especially very-low birth weight infants *E. coli* is considered the main bacterial cause of neonatal death [15]. Moreover, GAPDH was already described as an extracellular virulence factor of enteropathogenic and enterohemorragic *E. coli*, binding to human plasminogen and fibrinogen and interacting with intestinal epithelial cells [16,17]. Therefore, in the present study, we tested if maternal vaccination with $rGAPDH_{E.coli}$ confers protection of their offspring against *E. coli* infections. Surprisingly, GAPDH-based maternal

vaccination instead of induced protection to infected pups, increased its mortality. This harmful effect of *E. coli* GAPDH-vaccine should be taken into account in future studies with GAPDH-based vaccines.

MATERIAL AND METHODS

Animals

Balb/cAnNCrl mice, with a specific-pathogen free (SPF) status, were purchased from Charles River (Italy). All the animals were kept in the animal facilities of the Institute Abel Salazar during the time of the experiments. Mice were housed in Techniplast ventilated polycarbonate cages under negative pressure with hardwood bedding and provided with Mucedola Diet and fresh tap water, *ad libitum*, throughout the study. The temperature was maintained at 21–23˚C and the relative humidity at 50% ± 20% with a 12 h light/dark cycle. All animals were housed in environmentally controlled cages with 40 air changes per hour.

Ethics statement

This study was carried out in strict accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and Directive 2010/63/EU and Portuguese rules (DL 113/2013). The animal experimental protocol was approved by the competent national authority Direção Geral de Alimentação e Veterinária (DGAV) (Protocol Permit Number: 0420/000/000/2008). All animal experiments were planned in order to minimize mice suffering.

Western Blot

0.4 µg of purified rGAPDH$_{E.coli}$ and rGAPDH$_{GBS,}$ contained on 10% polyacrylamide gel were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences) according to the manufacturer's instructions. The membranes were probed with the mouse anti-GAPDH$_{E.coli}$ or mouse anti-GAPDH$_{GBS}$ antibody (1 µg/mL) in Tris-buffered saline with Tween 20 for 2 h at room temperature, followed by incubation with an alkaline phosphatase-conjugated goat anti-mouse IgG antibody (SouthernBiotec, 1:5000 in TBST) for 1 h at room temperature. Proteins were revealed by incubation with NBT/BCIP (Roche) in AP buffer (0.01 M Tris, 0.01 M NaCl, 0.5 mM $MgCl_2$ (pH 9.5)).

In vitro splenic cell cultures

Spleen cells from 8 weeks old (adult) and 2 days old (newborn) Balb/c mice were obtained by gently disruption in RPMI 1640 (Sigma-Aldrich) supplemented with penicillin (100 IU/ml, Sigma-Aldrich), streptomycin (50 µg/ml, Sigma-Aldrich), 2-ME (0.05 M, Sigma-Aldrich), and 10% FBS (Biowest). Cells were distributed in 96-well plate (Nunc, 1 x 10^6 cells/well) in 200 µL and cultured at 37°C in a humidified atmosphere containing 5% CO_2. All cells were treated with 6 µg/mL of Polimixin B (Sigma-Aldrich) to inhibit possible endotoxin contamination. Splenic cells were incubated with medium alone or stimulated with 100 ng/mL of Pam3CSK4 (InvivoGen), 25 µg/ml of $rGAPDH_{E.coli}$ or 25 µg/ml of $rGAPDH_{GBS}$. At specific timepoints, 150 µL of cell culture supernatant were collected and stored at -80°C and cells were used for flow cytometry analysis.

Cytokine analysis

IL-6, TNF-α, IL-1β and IL-10 were quantified by ELISA (eBioscience, with exception of IL-10, R&D Systems), according to the manufacturer's instructions. Detection levels of 8 pg/ml, 16 pg/ml, 16 pg/ml and 62.6 pg/ml, respectively.

Flow cytometry analysis

Splenic cell activation and death was evaluated by flow cytometry analysis. Briefly, 6, 12 and 18 h after stimulation, cells were harvest from cell culture plates and stained. To analyze the proportion of dead/live cells in a culture, 1 µL of propidium iodide (PI) was added to 99 µL of PBS. To analyze B cell activation, cells were first incubated with anti-mouse CD16/32 (clone 2.4G2) to block Fc receptors. FITC anti-mouse CD19 Ab (clone MB19-1; Biolegend) was used as B cell marker, PE anti-mouse CD5 (clone 53-7.3; Biolegend) was used to distingue B cell subpopulations (B1a - $CD19^+CD5^+$ or B1b/B2 - $CD19^+CD5^-$) and PE/Cy5 streptavidin plus biotin conjugated anti-CD69 (clone H1.2F3, Biolegend) were used as activation marker. Fluorescence was analyzed using an Epics XL cytometer (Beckman Coulter), and data were analyzed with FlowJo software (TreeStar).

Purification of recombinant GAPDH

E. coli BL21 (DE3) strain (Novagen) and the pET28a plasmid (Novagen) were used for production of the recombinant GAPDH protein from GBS and *E. coli*. Standard recombinant techniques were used as described in [18]. The GBS gapC gene (gbs1811; http://genolist.pasteur.fr/SagaList/) and *E. coli* gapA gene (GenBank: ADE90611.1) were PCR amplified and cloned into pET28a to produce a rGAPDH containing a carboxylic histidyl tag. *E. coli* BL21 cells were transformed with the resulting recombinant plasmid (pET28aΩgapC and pET28aΩgapA). Following a 3 h IPTG-induced expression of the fusion protein, the cells were harvested by centrifugation and suspended in phosphate buffer containing 10 mM imidazole (Sigma). The sample was incubated on ice for 30 min in the presence of 100 µg/ml lysozyme and 10% Triton X-100. After sonication, the insoluble material was removed by centrifugation and the supernatant was filtered through a 0.45-µm pore size filter (Millipore) and applied to a His-trap column (GE Healthcare). The rGAPDH was eluted with 300 mM imidazole under native conditions and the eluant concentrated by vacuum dialysis and equilibrated in PBS buffer before endotoxin removal on Endotoxin Removal Spin Columns (Pierce).

Maternal Immunizations

$rGAPDH_{E.coli}$ was used for maternal immunization assays and both $rGAPDH_{E.coli}$ and $rGAPDH_{GBS}$ were used in immunization protocols for antibody production . For both immunization protocols mice were injected sub-cutaneously (s.c.) three times, with a 3-week intervening period, with 200 µL of a preparation containing 20 µg of rGAPDH in a 1:40 PBS/alum suspension (Aluminium hydroxide Gel). The sham-immunized control animals received 200 µL of a 1:40 PBS/alum suspension. For maternal immunization assays, female immunized mice were paired with male immediately after the third injection. Elevated titers against $rGAPDH_{E.coli}$ were confirmed after the second immunization and after birth. For antibody purification, terminal blood was collected ten days after the third immunization and IgG antibodies purified using a Protein G HP affinity column (GE Healthcare Life Sciences).

Neonatal Passive immunization

Antibody treatments were performed in newborn mice 12 h prior to *E. coli* challenge, with 30 µg (i.p. in 60 µL) of mouse anti-rGAPDH$_{E.coli}$. Control animals received the same amount of mouse control IgGs. Pups from each litter were randomly assigned to control or to experimental groups, marked, and kept with their mother.

Neonatal *E. coli* infection

Neonatal (< 24 h old) Balb/c mice were infected orally with $2x10^6$ CFU (in 10µL of PBS) of extraintestinal pathogenic *E. coli* (ExPEC) IHE3034 of serotype O18:K1:H7, ST95, a neonatal meningitis-associated strain isolated in Finland in 1976 [19]. The bacteria was growth to exponential phase in liquid Todd-Hewitt (TH, Difco Laboratories) media for 3 h at 37 °C. After several washes, absorbance of the bacteria in PBS was adjusted to 0.450 at 600 nm (Jenway 6300 Spectrophotometer), corresponding to 2 x 10^8 CFU/mL. Newborns were kept with their mothers during the experiment. All pups were euthanized if they were in moribund state (lethargic, darker red colour and with no observable milk spot) due to ethical reasons. Survival curves were determined over a 15-d experimental period.

Statistical analysis

All statistical analyses were performed in GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, California). For ELISA and flow cytometry data, two-way ANOVA with Multiple Comparison Test was used to analyse the differences between all groups. In the case of ratios, the values were normalized using the formula: Arcsin(√(Value/100)) x 180/π, previous to statistical analysis. In case of survival analysis the graphs were obtained using the Kaplan-Meier method and differences were calculated using the log-Rank test. A P value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Antibodies produced against GAPDH of GBS do not recognize the GAPDH of *E. coli*

In *E. coli,* GAPDH is encoded by two genes, gapA and gapC. Many laboratory *E. coli* strains have accumulated mutations in gapC gene, generating stop codons which result in a truncated, non-functional protein [20,21]. In enterrohemorragic and enteropathogenic *E. coli* strains, GAPDH was detected at the surface of the bacteria and expression of the gapA but not the gapC gene accounted for the production of GAPDH [16,17]. To examine the amino acid homology between the different GAPDH isoforms the protein sequences of gapA (*E. coli* IHE3034; GenBank: ADE90611.1) and gapC (*E. coli* IHE3034; GenBank: ADE90514.1), gapC (GBS NEM316; GenBank: CAD47470.1), *Mus musculus* (GenBank: AAH83149.1) and *Homo sapiens* (GenBank: CAA25833.1) GAPDH were compared using the multiple sequence alignment programs ClustalOmega and Aline (Figure 1A). *E. coli* gapA GAPDH displays 47.64 % amino acid identity with *E. coli* gapC GAPDH. The *E. coli* gapA GAPDH used in this study displays a 53.38 % similarity with GBS GAPDH and 67.79 and 64.88 % similarity with mouse and human GAPDH, respectively (Figure 1A). In order to evaluate if the $rGAPDH_{GBS}$ could also be used as a target for vaccination against *E. coli* infection the cross-reactivity of the antibodies produced against the $rGAPDH_{GBS}$-vaccine and the gapA-encoded $rGAPDH_{E.coli}$ were tested. However, as shown in Figure 1B, neither the antibodies produced against $rGAPDH_{GBS}$ recognized the $rGAPDH_{E.coli}$ nor the antibodies produced against $rGAPDH_{E.coli}$ recognized the $rGAPDH_{GBS}$ (Figure 1B). Therefore, the $rGAPDH_{GBS}$ could not be used as a vaccine against *E. coli* infections.

E. coli GAPDH is unable to trigger B cell activation or induce cytokine production *in vitro*

A stimulatory effect on splenic B cells and an early induction of IL-10 have already been described for the GAPDH of GBS [22]. Therefore, we tested if *E. coli* rGAPDH

also possess this ability. For that purpose, adult and neonatal mouse splenic cells were cultured with rGAPDH$_{GBS}$ or with rGAPDH$_{E.coli}$. To exclude the possibility of contamination with endotoxins, the absence of endotoxins was confirmed with the highly sensitive e-toxate test and polimixin B was added to cell cultures. Moreover, since we have previously observed that TLR2 activation in GBS infection was associated with an early IL-10 production and consequent susceptibility to GBS infection [23], we also stimulate the cultures with Pam3CSK4, a TLR1/2 agonist, as positive control. B cell activation was assessed by expression of early activation surface marker CD69 on B1a ($CD19^+CD5^+$) and B2/B1b ($CD19^+CD5^-$) splenic cell population 18 h after the stimulatory treatment. As shown in Figure 2 and 3, rGAPDH$_{GBS}$ induces an up-regulation of CD69 on B2 population of both adult and neonatal splenic cells and is able to induce activation of newborns' B1a cells. In contrast, rGAPDH$_{E.coli}$ did not induce an up-regulation of CD69 in any of the studied populations. This result showed that contrary to GAPDH$_{GBS}$, the GAPDH$_{E.coli}$ did not induce B lymphocyte activation.

Regarding cytokine production, rGAPDH$_{GBS}$ was able to induce IL-10 and TNF-α production in the supernatants of neonatal splenic cell cultures, as soon as 6 h post-stimulation (Figure 4A). However, rGAPDH$_{E.coli}$ was unable to induce IL-10 or other pro-inflammatory cytokine production, in neonatal or adult cells cultures, at any time point evaluated (Figure 4). These results showed that the rGAPDH$_{E.coli}$ does not induce IL-10 production or B cell stimulation in contrast to the rGAPDH$_{GBS}$.

In order to understand the irresponsiveness of both adult and newborn cultures to rGAPDH$_{E.coli}$ we analysed cell viability post-stimulation by PI incorporation. As observable in Figure 5, rGAPDH$_{E.coli}$ induced cell dead in adult but not in neonatal splenic cell cultures after 12 h of stimulation (57% vs 73% of live cells in control). Neonatal spleen has a different frequency of the different cell populations than the adult counterparts. In neonatal mice the spleen is a site of hematopoiesis, a characteristic that is lost with post-natal development [24]. Therefore hematopoiesis progenitor cells only exist in the neonatal spleen. Moreover, B cell, T cell and dendritic cell populations are distinct in number and properties in neonatal and adult spleen [25]. These differences in cell populations could explain the observed

resistance to cell dead induced by rGAPDH$_{E.coli}$ in neonatal splenic cultures, when compared to the adult ones.

In contrast to rGAPDH$_{E.coli}$, rGAPDH$_{GBS}$ did not induced cell dead in any of splenic cultures. Actually, at 18 h post-stimulation, the percentage of live cells in newborn splenic culture was higher in cells stimulated with rGAPDH$_{GBS}$, than that in the RPMI control (Figure 5A). Having in mind that capacity of rGAPDH$_{GBS}$ to induce cell death has already been described on a murine macrophage cell line [18], the result of Figure 5 indicates that this property of rGAPDH$_{GBS}$ may be dependent on the cell type.

Notwithstanding the similarities between the amino acid sequences of the two GAPDH, their ability to induce B cell activation, IL-10 production or cell death is clearly distinct.

Maternal vaccination with rGAPDH$_{E.coli}$ increases neonatal susceptibility to *E. coli* oral infection

GAPDH have been proposed as suitable target for a vaccination against numerous pathogens [12]. In order to understand if rGAPDH$_{E.coli}$ vaccination induces protection against neonatal *E. coli* infection, female Balb/c mice were immunized with rGAPDH in Alum adjuvant. Control mice were sham-immunized with the adjuvant alone. After the last immunization, females were mated and progeny was orally infected with *E. coli* K1, an *E. coli* strain associated with neonatal sepsis and meningitis [26]. Unpredicted, all but one mouse born from rGAPDH-immunized mothers succumbed to infection (13% survival) whereas 8 out of 15 infected pups survived to the *E. coli* challenge in the control group (53% survival) (Figure 6A). To confirm that the lack of protection was an effect of the antibody response to rGAPDH$_{E.coli}$ and not by induced maternal alterations by immunization, pups were treated 12 h prior to infection with specific anti-rGAPDH$_{E.coli}$ or mouse IgG control antibodies. Again, antibody treatment was unable to confer protection to infected pups and, although not statistically significant due to the number of used mice, the pups treated with

anti-rGAPDH$_{E.coli}$ antibodies started to die sooner than control treated ones (Figure 6B).

CONCLUDING REMARKS

The GAPDH-based vaccines have been gaining some attention, due to their success against human and veterinary diseases in animal models [12]. GAPDH have already been identified as an extracellular virulence factor of enteropathogenic and enterohemorragic *E. coli* [17] but whether this enzyme could be used as a target for vaccination had never been investigated. The obtained results show that maternal immunization with $GAPDH_{E.coli}$ increases the susceptibility of the neonates to *E. coli* infection by increasing their mortality. This is, to our knowledge, the first time that an increase in mortality was associated with a GAPDH vaccine. This harmful effect of $GAPDH_{E.coli}$ vaccination highlight the need for precaution in the generalization of the use of GAPDH-based vaccines.

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Figure Legends

Figure 1. Multiple sequence alignment and cross-reactivity analysis of the antibodies specific for rGAPDH$_{GBS}$ or specific for rGAPDH$_{E.coli}$. (A) Multiple sequence alignment of the GAPDH proteins. (B) Western blot analysis of rGAPDH$_{GBS}$ and rGAPDH$_{E.coli}$ revealed with mouse anti-GAPDH$_{E.coli}$ IgG antibodies (right membrane) and mouse anti-GAPDH$_{GBS}$ IgG antibodies (left membrane).

Figure 2. Expression of the early activation marker CD69 on neonatal B lymphocytes stimulated with different rGAPDH detected by flow cytometric analysis. (A) Expression of CD69 on B1a ($CD19^+CD5^+$) and B2 ($CD19^+CD5^-$) of splenic cells of neonates Balb/c mice stimulated with: medium alone (RPMI), 100 ng/mL of Pam3CSK4, 25 µg/ml of rGAPDH$_{GBS}$ or 25 µg/ml of rGAPDH$_{E.coli}$ at the indicated time points. At top the representative dotplots of 18 h post-stimulus. At bottom the bars indicate the mean value of fold expression [(%CD69 positive cells - %CD69 positive cells in RPMI) / %CD69 positive cells in RPMI] ± SEM of two wells per stimulus and correspond to one representative experiment of two independent experiments. Two-way ANOVA with multiple comparison *p<0.05; **p<0.01, ***p<0.001.

Figure 3. Expression of the early activation marker CD69 on adult B lymphocytes stimulated with different rGAPDH detected by flow cytometric analysis. (A) Expression of CD69 on B1a ($CD19^+CD5^+$) and B2 ($CD19^+CD5^-$) of splenic cells of adult Balb/c mice stimulated with: medium alone (RPMI), 100 ng/mL of Pam3CSK4, 25 µg/ml of rGAPDH$_{GBS}$ or 25 µg/ml of rGAPDH$_{E.coli}$ at the indicated time points. At top the representative dotplots of 18 h post-stimulus. At bottom the bars indicate the mean value of fold expression [(%CD69 positive cells - %CD69 positive cells in RPMI) / %CD69 positive cells in RPMI] ± SEM of two wells per stimulus and correspond to one representative experiment of two independent experiments. Two-way ANOVA with multiple comparison *p<0.05; **p<0.01, ***p<0.001.

Figure 4. Cytokine production of splenic cultures stimulated with rGAPDH$_{GBS}$ and rGAPDH$_{E.coli}$. Production of IL-10, IL-6 and TNF-α by splenic cells of neonates

(A) or adult (B) Balb/c mice stimulated with: medium alone (RPMI), 100 ng/mL of Pam3CSK4, 25 µg/ml of rGAPDH$_{GBS}$ or 25 µg/ml rGAPDH$_{E.coli}$ at the indicated times points. The data indicate the mean value ± SEM of two wells per stimulus and correspond to one representative experiment of two independent experiments. Two-way ANOVA with multiple comparison *p<0.05; **p<0.01, ***p<0.001.

Figure 5. Splenic cell viability after stimulation with with rGAPDH$_{GBS}$ and rGAPDH$_{E.coli}$. Cell viability of splenic cells of neonates (A) or adult (B) Balb/c mice stimulated with: medium alone (RPMI), 100 ng/mL of Pam3CSK4, 25 µg/ml of rGAPDH$_{GBS}$ or 25 µg/ml rGAPDH$_{E.coli}$ at the indicated times points. Percentage of viable cells was determined by exclusion of percentage of PI incorporation by flow cytometry. The bars indicate the mean value ± SEM of two wells per stimulus. Two-way ANOVA with multiple comparison *p<0.05; **p<0.01, ***p<0.001 was performed after normalization of the values using the formula: Arcsin(√(Value/100)) x 180/π.

Figure 6. *E. coli* GAPDH-based vaccine increase the susceptibility of neonatal mice to oral infection with *E. coli* K1. (A) Survival of pups from rGAPDH-vaccinated and Sham-immunized mice. Female Balb/c mice were immunized three times, with a three week interval, with 20 µg of rGAPDH$_{E.coli}$ plus Alum or Alum alone. At the day of the third immunization the females were mated and the progeny orally infected with 2x10^6 CFU of *E. coli* IHE3034. (B) Survival of Balb/c pups treated i.p. with 30 µg of mouse anti-rGAPDH$_{E.coli}$ or control IgG 12 h prior to oral infection with 2x10^6 CFU of *E. coli* IHE3034 (<24 h old pups). Newborns were kept with their mothers during the experiment and were evaluated twice a day. All pups were sacrificed if they were in moribund state due to ethical reasons. Survival curves were determined over a 15-d experimental period. Log-rank (Mantel-Cox) test. ns – not significant (p > 0.05) **p<0.01

Figure 1

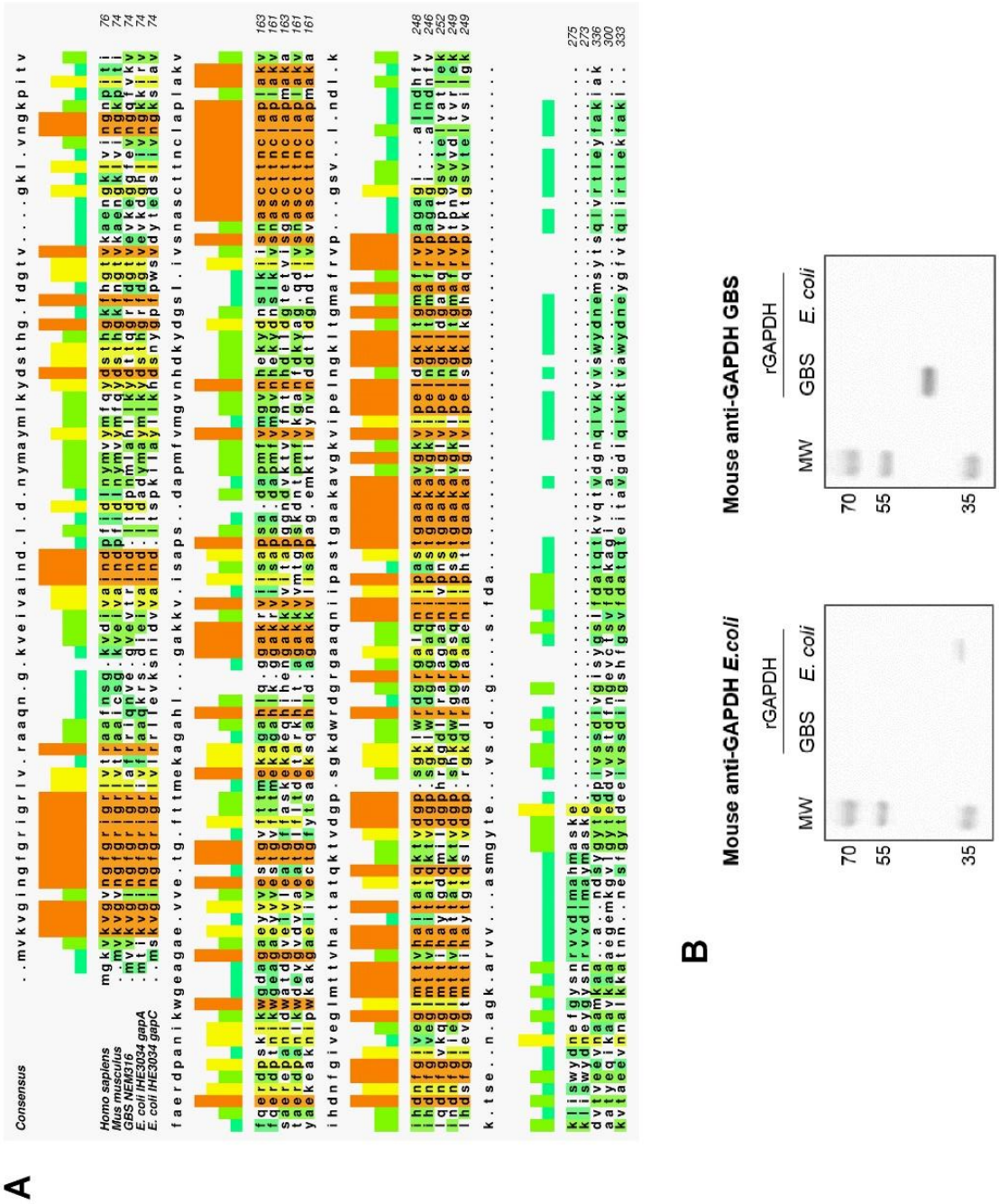

A
Consensus
Homo sapiens
Mus musculus
GBS NEM316
E. coli IHE3034 gapA
E. coli IHE3034 gapC
B
Mouse anti-GAPDH E.coli
Mouse anti-GAPDH GBS
rGAPDH
MW
GBS
E. coli
70
55
35

Figure 2

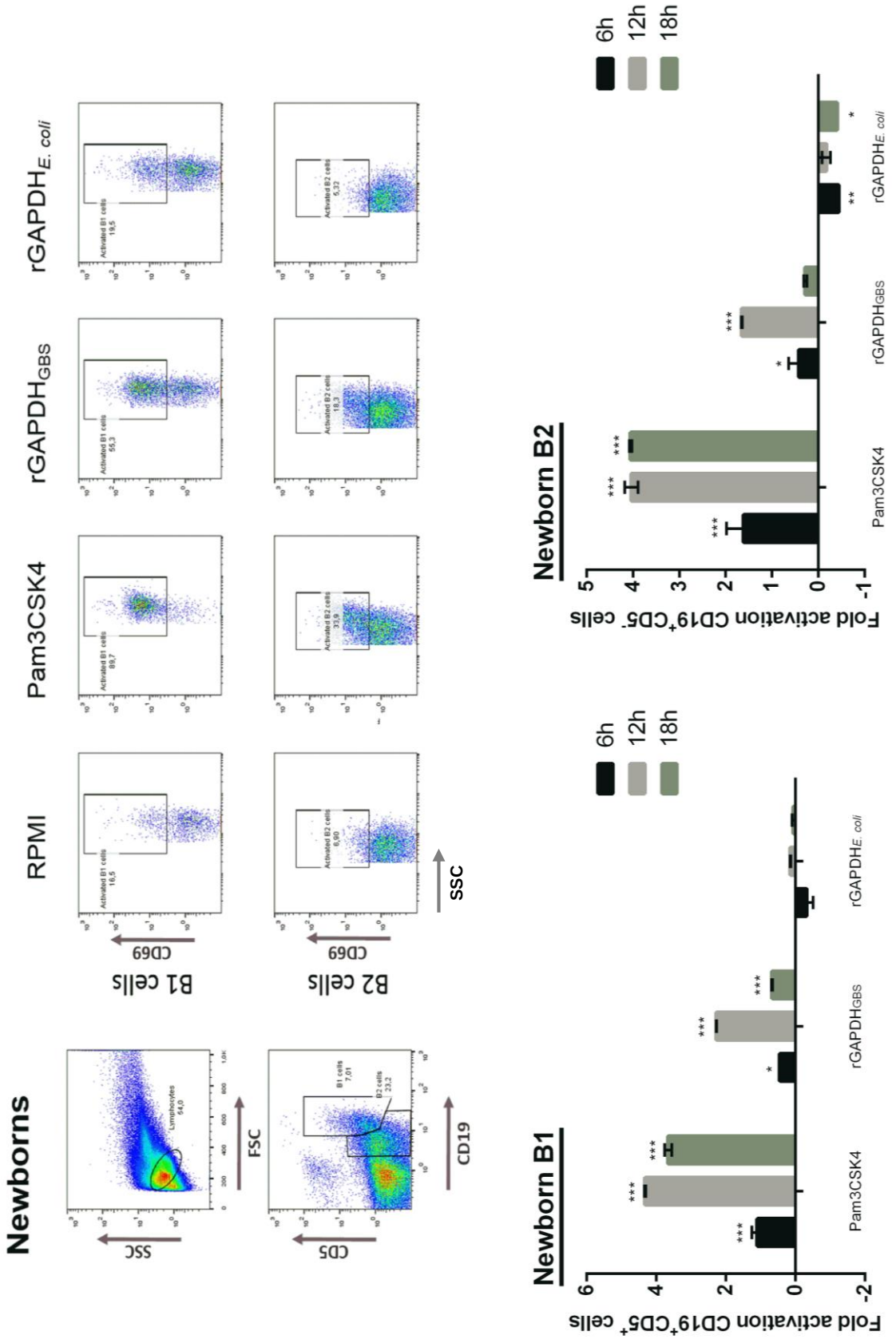

Newborns
SSC
FSC
Lymphocytes
94,0
CD5
CD19
B1 cells
7,01
B2 cells
23,2
B1 cells
CD69
B2 cells
CD69
SSC
RPMI
Pam3CSK4
rGAPDHGBS
rGAPDHE. coli
Activated B1 cells
16,5
Activated B1 cells
89,7
Activated B1 cells
56,3
Activated B1 cells
16,5
Activated B2 cells
6,60
Activated B2 cells
33,9
Activated B2 cells
43,3
Activated B2 cells
5,32
Newborn B1
Fold activation CD19+CD5+ cells
6h
12h
18h
Pam3CSK4
rGAPDHGBS
rGAPDHE. coli
Newborn B2
Fold activation CD19+CD5- cells
6h
12h
18h
Pam3CSK4
rGAPDHGBS
rGAPDHE. coli

Figure 3

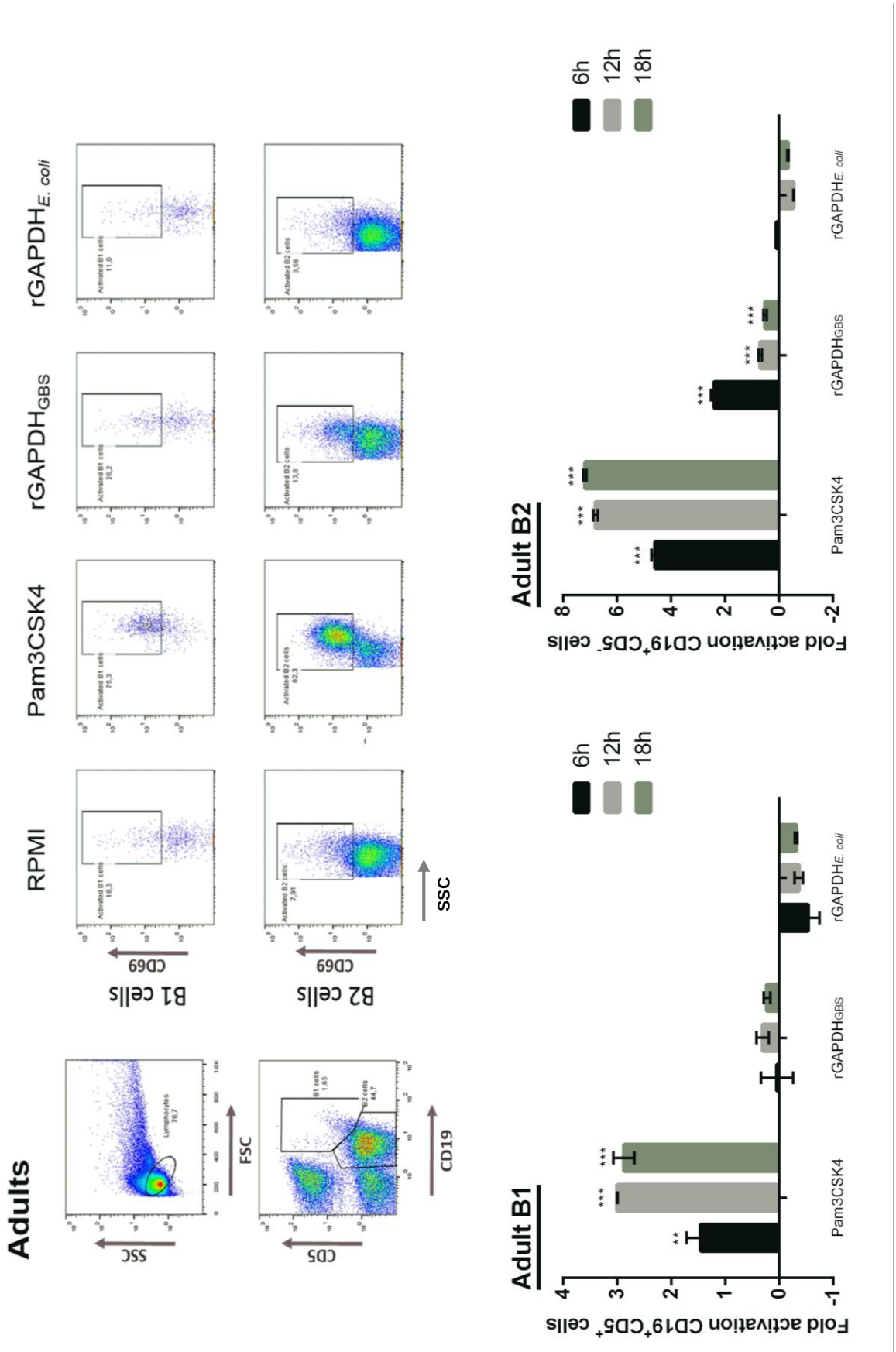

Adults
RPMI
Pam3CSK4
rGAPDHGBS
rGAPDHE. coli
SSC
FSC
CD5
CD19
CD69
B1 cells
B2 cells
ssc
Adult B1
Adult B2
6h
12h
18h
Fold activation CD19+CD5+ cells
Fold activation CD19+CD5- cells
Pam3CSK4
rGAPDHGBS
rGAPDHE. coli

Figure 4

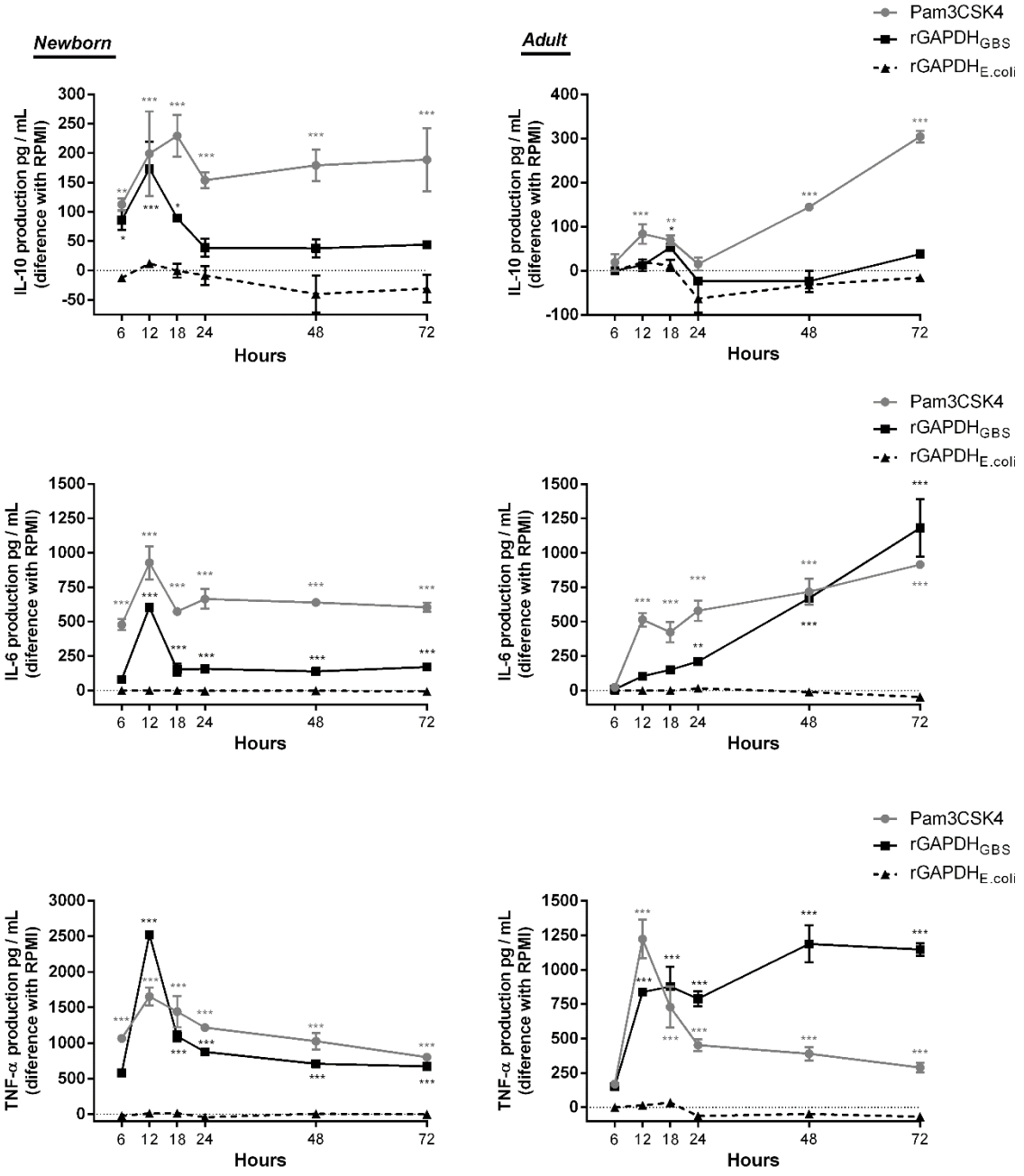

Newborn
Adult
Pam3CSK4
rGAPDH$_{GBS}$
rGAPDH$_{E.coli}$
IL-10 production pg / mL (diference with RPMI)
IL-6 production pg / mL (diference with RPMI)
TNF-α production pg / mL (diference with RPMI)
Hours
6 12 18 24 48 72

Figure 5

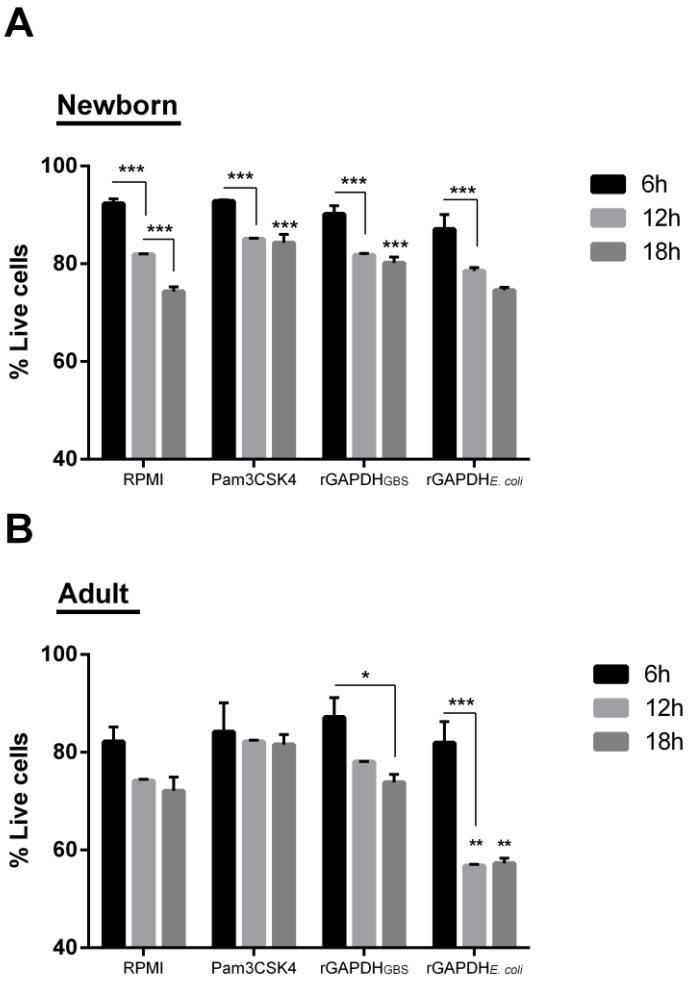
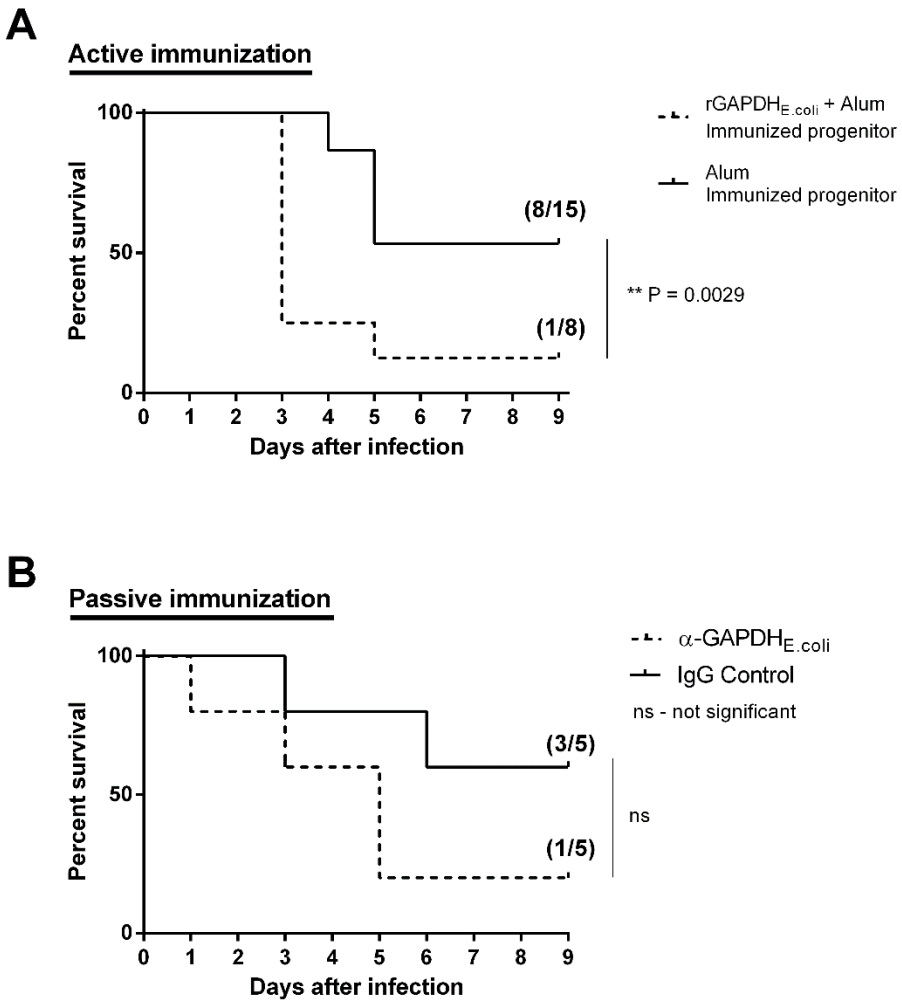

A
Newborn
% Live cells
100
80
60
40
***
***
***
***
***
***
***
***
RPMI
Pam3CSK4
rGAPDHGBS
rGAPDHE. coli
6h
12h
18h
B
Adult
% Live cells
100
80
60
40
*
***
**
**
RPMI
Pam3CSK4
rGAPDHGBS
rGAPDHE. coli
6h
12h
18h

Figure 6


A
Active immunization
Percent survival
100
50
0
0 1 2 3 4 5 6 7 8 9
Days after infection
(8/15)
(1/8)
rGAPDH$_{E.coli}$ + Alum
Immunized progenitor
Alum
Immunized progenitor
** P = 0.0029
B
Passive immunization
Percent survival
100
50
0
0 1 2 3 4 5 6 7 8 9
Days after infection
(3/5)
(1/5)
α-GAPDH$_{E.coli}$
IgG Control
ns - not significant
ns

MANUSCRIPT III

Using a neonatal mice model of *Escherichia coli* oral infection to decipher the innate immune response against this pathogen

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ABSTRACT

Escherichia coli is a common cause of neonatal sepsis and meningitis worldwide, with special incidence among preterm and very low birth weight infants. The high mortality and morbidity associated with these neonatal infections, as well as the increase of *E. coli* antibiotic-resistance, strengthens the need for a prophylaxis treatment. For the development of new therapies it is essential to understand how the bacteria interacts and evades the host immune system and establishes an infection. To characterize the immune response to *E. coli* infection, we adapted a model of oral infection in neonatal mice. For that purpose, Balb/c mice, with less than 24 h old, were orally infected with $2x10^6$ CFU of *E. coli* K1 (a strain associated with neonatal sepsis and meningitis) and were sacrificed at specific time points. The bacterial colonization was evaluated in blood, spleen, liver, lungs, and brain. In addition, the levels of cytokines and chemokines, and the immune cell recruitment were analysed in liver and lungs. The results showed that like in humans, an age-dependency for the systemic *E. coli* infection was observed. Moreover, bacteria were detected both systemically (a characteristic of sepsis) and in the brain tissue (a characteristic of meningitis). Interestingly, despite the non-invasive form of infection, bacteria were detected in lungs, blood and liver as soon as 30 minutes post-infection. However, the inflammatory response to this bacteraemia, evaluated by the production of pro-inflammatory cytokines and chemokines and phagocyte recruitment to infected organs, was only observed 6 h post-infection. This delay in the development of the innate immune response was not due to the induction of the anti-inflammatory IL-10, since the treatment with antibody blocking of IL-10 signalling did not influence the outcome of infection. On the other hand, anti-IL-1R treatment increased the susceptibility to *E. coli*, providing evidence that the pro-inflammatory cytokine IL-1β, has a protective role during *E. coli* K1 neonatal infection.

Taken together, these findings indicate that *E. coli* K1 is able to rapidly colonize the neonatal mice but the infected host does not promptly develop an immune response. Understanding the reason for the delay in immune response activation should enable the characterization of novel therapeutic targets.

INTRODUCTION

From the estimated three million neonatal deaths that occur around in the world each year, more than one-third are caused by infections [1]. Neonatal sepsis and pneumonia, account for approximately half million of those fatalities [2]. The chances of survival are reduced for newborns with a serious infection, in particular for those with very low birth-weight [3,4]. For those who are treated and survive, the consequences may be severe long-term neurodevelopmental impairment and disability [5]. *Escherichia coli* is the most common cause of Gram-negative bacterial neonatal infections, being responsible for a high number of early-onset sepsis and late-onset meningitis [6-8]. When very low birth-weight infants are considered alone, *E. coli* becomes the most frequent cause of early-onset sepsis, accounting for 33.4% of episodes [8]. Despite the use of antibiotics, the morbidity and mortality rates associated with *E. coli* neonatal infections have not decreased over the last few decades, with reports of approximately 30% fatality rates in infected infants [7,9,10]. In fact, due to an emergence of *E. coli* ampicillin-resistant clones (with several reports where this clones represent more than 70% of the isolates), it is estimated that *E. coli* neonatal infections will continue to increase [11-13].

Newborns acquire this bacterium perinatally, from maternal contaminated vaginal fluid [14]. *E. coli* is one of the most common organisms in the genital tract, being detected in 13% of pregnant women [15]. Not all *E. coli* strains have the ability to cause infections in neonates, but those that do, usually belong to B2 group and express the K1 capsular antigen [16-19]. In fact, infants infected with K1 strains have increased morbidity and mortality rates compared to infants infected with other *E. coli* strains [3]. After newborn's colonization, *E. coli* transcytose from gastrointestinal tract into the bloodstream inducing bacteremia, and from there, can traverse the blood brain barrier (BBB) into the central nervous system (CNS), causing meningitis [20,21]. Each one of these stages of the infection require a specific set of molecular determinants, several of which were already identified [19]. Meanwhile, only a few reports shed some light on the first stages of *E. coli* neonatal infection. Neutrophils and macrophages have been implicated on both protective [22-24] and detrimental responses to this bacterium [25-27], and the role of specific inflammatory mediators is still unclear. A better understanding of the neonatal immune response in the first stages of *E. coli* infection

could provide new insights for the susceptibility of neonates to this infection and offer the basis for a new generation of therapeutics and prophylactics. In some animal models, infection is initiated by parenteral or intranasal administration of bacteria, bypassing or ignoring some of the essential steps of the natural process of colonization and dissemination, thus creating an incomplete or artificial infection scenario [22,28,29]. Oral infection in rats was already employed by several works [25,30-34]. Although the neonatal rat has unequivocal advantages when compared with neonatal mice, it limits the use of transgenic animals and the comparison with the majority of infection and immunological studies, that use mice models. In 1988, Pluschke et al, tested oral neonatal *E. coli*-infections in several mice strains with 3-5 days old, but pups only became bacteremic in endotoxin-irresponsive mice and with a success of 21 and 52% (in B57BL/10ScCr and C3H/HeJ^{b} strains, respectively) [35]. The remaining results of the paper were obtained using intraperitoneal or intravenous routes of *E. coli* infections and, until now, the mice model for oral *E. coli* infection has been set aside. In this study, Balb/c pups with less than 24 h old were oral infected with *E. coli* K1. In this model all infected animals presented bacteremia and 43% died. As also described in other studies, and as occurs in humans [13,31,32,36,37], we observed an age-dependency for the systemic *E. coli* infection and the ability of the K1 strain to induce bacteremia and meningitis. We detected bacteria in several organs at very early-time points, 30 minutes post-infection, that are disregarded in the majority of *in vivo* models. We observed that, despite this rapid systemic *E. coli* colonization, the induction of a pro-inflammatory innate immune response only occurred after several hours after infection. Moreover, and as opposite to what was previously observed with GBS neonatal infections, the blocking of IL-10 during infection did not improve neonatal survival to *E. coli* infection, indicating that IL-10 is not responsible for the delay on the development of the innate immune response.

By understanding how bacteria induce or take advantage of the tolerant/suppressive status of the newborn, novel and targeted therapies against *E. coli* neonatal infection could be developed.

MATERIALS AND METHODS

Animals

Balb/cAnNCrl mice, with a specific-pathogen free (SPF) status, were purchased from Charles River (Italy). All the animals were kept in the animal facilities of the Institute Abel Salazar during the time of the experiments. Mice were housed in Techniplast ventilated polycarbonate cages under negative pressure with hardwood bedding and provided with Mucedola Diet and fresh tap water, *ad libitum*, throughout the study. The temperature was maintained at 21–23˚C and the relative humidity at 50% ± 20% with a 12-hours light/dark cycle. All animals were housed in environmentally controlled cages with 40 air changes per hour.

Ethics statement

This study was carried out in strict accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and Directive 2010/63/EU and Portuguese rules (DL 113/2013). The animal experimental protocol was approved by the competent national authority Direção Geral de Alimentação e Veterinária (DGAV) (Protocol Permit Number: 0420/000/000/2008). All animal experiments were planned in order to minimize mice suffering.

Bacteria

The bacterium used in all experiments was extraintestinal pathogenic *E. coli* (ExPEC) IHE3034 of serotype O18:K1:H7, ST95, a neonatal meningitis-associated strain isolated in Finland in 1976. The bacterium was growth to exponential phase in liquid Todd-Hewitt (TH, Difco Laboratories) media for 3 hours at 37°C. After several washes, absorbance of the bacteria in PBS was adjusted to 0.450 at 600 nm (Jenway 6300 Spectrophotometer), corresponding to 2 x 10^8 CFU/mL.

Neonatal mouse model of *E. coli* infection

Neonatal (< 24 h old) Balb/c mice were infected orally with 2x10^6 CFU of *E. coli* IHE3034 in 10µL of PBS. Littermate controls received the same amount of PBS.

Newborns were kept with their mothers during the experiment. All pups were euthanized if they were in moribund state (lethargic, darker red colour and with no observable milk spot) due to ethical reasons. Survival curves were determined over a 9-d experimental period. Blood was collected by facial vein puncture after anesthesia with isoflurane (IsoFlo® Esteve). Liver, spleen, lungs, and brain of infected pups were aseptically removed at indicated time points and homogenized (VWR™ Pellet Mixer) in PBS. Liver and lungs were weight and divided prior to homogenization, a part of the organs was used for flow cytometry analysis. To quantify the bacterial load, serial dilutions in sterile saline were plated on Todd–Hewitt agar and incubated overnight at 37˚C.

Antibody treatments

Antibody treatments were performed in newborn mice 12 h prior to *E. coli* challenge, with 60 µg (i.p. in 60 µL) rat anti-mouse IL-1R (Reg21 Hybridoma), rat anti-mouse IL-10R mAbs (1B1.3a, Schering-Plough Corporation). Control animals received the same amount of control IgGs. Pups from each litter were randomly assigned to control or to experimental groups, marked, and kept with their mother.

Determination of cytokine production

Organ homogenates were incubated on ice for 10 min with 1:1 lyses buffer (200 mM Tris; 300 mM NaCl; 2% Triton X-100; pH=7.4), centrifuged at 14 000 rpm (UEC Micro 14/B centrifuge) and the supernatants stored at -80^{o}C. IL-6, TNF-α, IL-1β, IL-10, MIP-2 and KC were quantified by ELISA (eBioscience, with exception of IL-10, R&D Systems), according to the manufacturer's instructions. Detection levels of 8 pg/ml, 16 pg/ml, 16 pg/ml, 62.6 pg/ml, 15.6 pg/ml and 31.2 pg/ml, respectively.

Flow cytometry analysis

Neutrophil recruitment in the lungs of infected pups was evaluated by flow cytometry analysis. Briefly, 30 min, 1 h, 3 h, 6 h, 12 h, 18 h, 24 h and 48 h after *E. coli* infection, the liver and lungs were removed, gently homogenized in PBS, and passed through glass wool to remove cellular aggregates. Anti-mouse CD16/32 (clone 2.4G2) was

added to each sample to block Fc receptors. FITC anti-mouse CD45 Ab (clone X; BD Pharmingen) was used for leukocyte detection. Neutrophils were identified by biotin anti-mouse Ly6G (clone 1A8; BD Pharmingen) plus streptavidin-PECy5 (BD Pharmingen) staining and macrophages were identified by PE anti-mouse F4/80 (clone BM8; Biolegend) staining. Cell number was evaluated using micro particle size standard based on polystyrene with 10 µm (Sigma Aldrich). Fluorescence was analyzed using an Epics XL cytometer (Beckman Coulter), and data were analyzed with FlowJo software (TreeStar).

Statistical analysis

All statistical analyses were performed in GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, California). For ELISA and flow cytometry data, One-way ANOVA with Multiple Comparison Test with 95% of confidence was used to analyse the differences between all groups. In the case of ratios, the values were normalized using the formula: $\text{Arcsin}(\sqrt{(\text{Value}/100)}) \times 180/\pi$, previous to statistical analysis. For survival curve analysis, Mantel-Cox test was performed. A P value < 0.05 was considered statistically significant.

RESULTS

Survival of neonatal mice to oral infection with *E. coli* K1 is pup age dependent

The study of the neonatal immune response to systemic *E. coli* infection is important for the development of new therapeutics/prophylactics for *E. coli*-associated neonatal sepsis and meningitis. These studies would clearly benefit from animal models of infection that mimic all features of the disease in the human neonate. Oral bacterial feeding parallels the natural route of *E. coli* infection after gastrointestinal colonization and has been used with neonatal rats [25,30-34]. Nevertheless, this rodent model limits the use of several reagents and transgenic animals. In order to overcome these obstacles, we developed a model of *E. coli* oral infection in neonatal mice. BALB/c mice were orally infected with $2x10^6$ CFU of *E. coli* K1. As seen in Figure 1, an age-dependency for neonatal susceptibility to *E. coli* infection was observed, similarly to what is observe in humans [13]. Mortality (~43%) was only observed in pups that were infected before one-day old (<24 h old). After date age, the pups were resistant to oral infection with *E. coli* K1. Therefore, in this study, the pups were infected before one day of life.

E. coli K1 rapidly develop systemic infection in neonatal pups

It is widely accepted that, after *E. coli* aspiration and ingestion during birth, the bacteria invades the epithelia of the lung or of the gastrointestinal tract and disseminates through the bloodstream [20]. In our model, it was also observed a high bacterial colonization in the lungs of infected pups as soon as 30min post-infection (Figure 2). This could be due to part of the inoculum reached the neonatal respiratory tract during the bacterial administration. Indeed, after oral administration of the bacterium small bubbles were observable in pups' nostrils. As shown in Figure 2, the colonization levels were maintained at high levels in neonatal lungs during the first 18 h of infection. Thirty minutes post–infection, all animals presented *E. coli* in bloodstream. To our knowledge, this is the first time that a neonatal *E. coli* systemic colonization is quantified at these early timepoints after a non-invasive type of infection. *E. coli* K1

bacterium was also observed in the blood circulation 30 min post-infection and persists at the same levels during the first 18 h post-infection. As shown in Figure 2, liver colonization starts as soon as bacteria reaches the bloodstream, with 65% of animals presenting colonization in the first half hour of infection. Colonization in this organ increased overtime and, 18 h post-infection, only 1 out of 14 infected animals presented colonization below detection levels. Although *E. coli* was detected in the brain and spleen of some animals at very early timepoints, only at 3 and 12 h post-infection, the levels of colonization in these organs, respectively, were statistically significant.

A delay in the development of the neonatal innate immune response

During the host response to infection, the balance between pro and anti-inflammatory responses, as well as the timing of production of the different immune mediators, establishes the fate of the infection [38]. Therefore, the production of cytokines and chemokines was evaluated in liver and lungs of infected neonatal mice at several timepoints post-infection. As showed in Figure 3, despite the elevated colonization observed in these organs (Figure 2) very early after infection, an increase of the levels of pro-inflammatory cytokines was only observable 6 hours post-infection. The increase in IL-6, TNF-α and IL-1β higher in the lungs than in the liver (Figure 3A vs 3B), which could explain the decrease in lung colonization after 18 h of infection and the maintenance of liver colonization (Figure 2). A decrease on IL-10 production was detected in the liver between the 1 h and 3 h post-infection, although the levels remained statistically similar to the controls throughout the infection. In lungs, an increased in IL-10 production was only detected 24 h post-infection, probably in response to the pro-inflammatory cytokines production.

Another important feature of the innate immune response is the cell recruitment to infected organs. We first analyzed chemokine production in the lungs and liver of infected mice. As observed with cytokines, until 3 h after *E. coli* infection, no statistically significant production of any analyzed chemokine was observed (Figure 4). MIP-2 was preferentially produced in lungs, whereas in the liver, an early and higher production of KC was detected. Although 30 min post-infection high levels of KC were detected in

the lungs of some animals, the average of KC production is not statistically different from controls.

Next, we analyzed macrophage and neutrophil recruitment to liver and lungs of infected mice. Erythrocytes and epithelial cells were excluded from analysis by gating CD45 positive cells [39]. The number of leukocytes in the liver of infected animals remained at similar levels of controls at all timepoints, with a tendency to decrease in the first hour and increase until the 12 h of infection (Figure 5). The percentage of macrophages in the liver increase in proportion after 3 h of infection, but differences in number of cells are only detected 12 h post-infection. Surprisingly, the number of neutrophils, the first immune cells to be recruited to infected organs, is maintained in levels that are not statistically different to the observed in control, although a tendency to increase between the first and third hour of infection is clearly observable.

In lungs, the number of CD45 positive cells increases after 6 h of infection. Both macrophages ($F4/80^+$) and neutrophils ($Ly6G^+$) increased in number 12 h post-infection (Figure 6A). Unexpectedly, a population double-positive for F4/80 and Ly6G marker was detected in the lungs of infected mice after six hours of infection (Figure 6A and B). As observable in Figure 6B, this population increased overtime, with a decrease in number of single positive Ly6G cells. Dot plot analysis suggests that neutrophil population acquire the expression of F4/80 marker post-infection (Figure 6B). This population was observable in flow cytometry analysis of neonatal lungs in mice in another study, but the authors neglected its presence [40]. Double positive $F4/80^+Ly6G^+$ cells were also detected in peritoneal lavages of adult mice intraperitoneally injected with heat-killed *Pseudomonas aeruginosa* [41]. In this paper, the authors designate this population as double-positive myeloid cells and identified a similar ability to produce myeloperoxidase in response to *P. aeruginosa* as single positive Ly6G cells [41]. In our model, this double positive population decreases in the lungs after 18 h of *E. coli* oral infection (Figure 6A).

Blocking IL-10 signalling does not reduce mortality in *E. coli*-infected pups

IL-10 has been associated with neonatal susceptibility to GBS-infection [42,43]. In *E. coli* K1 neonatal infections, IL-10 production was been associated with host protection [24,44,45]. To determine the importance of this anti-inflammatory cytokine to *E. coli*-induced mortality in our model, IL-10 signalling was blocked before infection. As shown in Figure 7, anti–IL-10R mAb treatment did not confer resistance to newborns challenged with *E. coli*. Although the mice treated with anti-IL10R mAb started to die sooner than controls, the mortality rate at the end of the experiment was similar (Figure 7). Moreover, in a sub-cutaneous model of neonatal *E. coli* K1 lethal infection, both the lack of expression of IL-10 (in IL-10 KO mice) and antibody blocking of IL-10R in WT mice did not have any influence on the outcome of infection (data not shown).

The importance of the pro-inflammatory cytokine, IL-1β, in *E. coli* neonatal infection is not yet well characterized. To decipher the role of this pro-inflammatory cytokine in our model, newborn mice were treated with anti-IL1R antibody 12 h prior to the *E. coli* K1 challenge. In orally *E. coli* K1-infected pups, anti-IL-1R increased the neonatal mortality from 47% (controls) to 93% (Figure 7). Therefore, IL-1β conferred protection against *E. coli* infection.

Contrary to what was observed in neonatal GBS infection, IL-10 has no role on the susceptible of neonates to *E. coli* K1 infection. Moreover, IL-1β production seems to has a protective role during *E. coli* K1 neonatal infection

DISCUSSION

E. coli is the second most common cause of neonatal infections with mortality rates that reach the 40% and morbidity in survivors that surpass the 40% [5,7,9,10]. In *E. coli* infections antibiotic-resistance is particularly serious, a recent report, showed that 75% of obstetric and 95% of pregnant women vaginal *E. coli* isolates were resistant to, at least, one of the antimicrobial agents tested, with 65% of all isolates being resistant to ampicillin [15]. Therefore, there is a clear need for more efficient preventive measures against *E. coli* neonatal infections. The understanding of the pathogenesis of *E. coli* and its interaction with the neonatal immune system is essential for the generation of new therapeutics and prophylactics. Here we proposed a mouse model of studying the neonatal immune response to the *E. coli* infection that is similar to the human route of infection. Gastric and oral neonatal *E. coli* infection in rodent models has been developed in the last decades but in rat [25,30-34]. The development of an oral mice model for the study of neonatal *E. coli* K1 sepsis and meningitis was already tried, but was unsuccessful in confer bacteremia and mortality in conventional laboratory mice strains [35]. In our mice model, an age-dependency to susceptibility to *E. coli* infection was observed similarly to observed in human neonatal [13]. A twenty-four hour difference in the age of the animals was enough to abrogate the 43% mortality rate. This result could explain the lack of mortality and bacterial recovery on Balb/c mice infected orally with *E. coli* described in Pluschke et al study, where 3 to 5 days old animals were used [35]. The strong age dependency in *E. coli* infections has been associated with microbiota gut colonization [29] and with the level of the maturation of the intestinal mucus barrier [37]. A study with bioluminescent *E. coli* K1 oral infection, showed also an age dependency on bacterial colonization on upper organs of intestinal track in neonatal rats, as in non-keratinized esophageal tissues and in the oral cavity [32]. Neonates, and particularly preterms, exhibit reduced esophageal motility and luminal clearance compared to adults [46], which can contribute to the vulnerability to colonization and invasion of *E. coli* K1 in newborns. Moreover, despite the existence of a strong correlation between systemic *E. coli* K1 infection and gastrointestinal tract colonization, the aspiration of the contaminated fluid into the lungs and other regions of respiratory tract may provide an alternative entrance to the bacteria into the systemic circulation [32]. In this regard, the majority of the articles with non-invasive *E. coli* K1 neonatal infection in mice models use intranasal

inoculation [24,27,47,48]. In our model, both gastrointestinal and respiratory tract colonization was achieved. Indeed, *E. coli* was detected in the stomach and small intestine of all infected animals, 30 and 60 min post-infection (data not showed) and, during infection, it was observed the formation of small bubbles in nostrils, which indicates that part of the inoculum reached the respiratory tract. That was further confirmed by the high colonization of the lungs of infected mice as soon as 30 min post-infection. Systemic colonization of all pups was observed after half hour of infection. Blood colonization was maintained at similar levels from the first to the last timepoint (30 min to 24 h) (although a tendency to decrease after 18 h was observable) and, in the liver, *E. coli* colonization increased over time. The spleen was the last analysed organ to be colonized, but reached statistically significant values as soon as 12 h post-infection. *E. coli* K1 is strongly associated with sepsis and meningitis and we found brain colonization 3 h post-infection, with viable bacteria being detected in the brain of some animals as soon as 30 min post-infection. We cannot exclude that the bacteria present in the blood could account for some of the bacteria found in other tissues. The fragility of the organs of the newborn mice and the small timepoints tested make impossible the *ex vivo* perfusion for blood removal. Nevertheless, we observed an increase in colonization in all organs, while blood colonization was maintained constant throughout the study.

Pathogen recognition by immune cells and the generation of an immune response is initiated as soon as the bacteria enter the host. However, despite the elevated colonization observed in lungs and liver at 30 min post-infection, an increase in the production of pro-inflammatory cytokines IL-6, IL-1β and TNF-α and chemokines KC and MIP-2 was detected only after 6 h of infection (Figure 3). This delay in the induction of inflammatory mediators had, as consequence, a delay on the recruitment of cells to infected organs. As soon as an increase of cytokines and chemokines was detected, an increase in the number of macrophages and neutrophils was also observed (Figure 4 and 5). Furthermore, in lungs, at 6 h post-infection a double positive population for Ly6G and F4/80 appeared that, at 18 h timepoint, accounted for more than 60% of CD45 positive cells it this organ. This population was already described in a study with *P. aeruginosa* infection and was identified as having the capability of producing the same amount of myeloperoxidase as neutrophils in response to this gram-negative bacterium [41]. Extra-intestinal pathogenic *E. coli* has the ability to survive inside

neutrophils and macrophages [26,27,49] and, in a neonatal mice model of intranasal *E. coli* infection, neutrophil depletion was associated with resistance to infection [27]. In other studies, however, a decrease in neutrophil and macrophage recruitment was associated with an increase in *E. coli* colonization and susceptibility to infection [23,37]. The role of IL-1β in *E. coli* infections is not consensual. The treatment of 10-day *E. coli* i.p. infected mice with recombinant IL-1 conferred resistance to the bacterial-induced mortality [35]. In an adult model of *E. coli* pneumonia, however, IL-1β production in lungs was associated with an increase in susceptibility to infection [50]. In our study, at 6 h post-infection a significant IL-1β production in lungs and a small production in liver were observed. We showed that the production of this pro-inflammatory cytokine had a protective effect, once the blocking of its signaling increased the mortality of infected pups up to 90%.

In neonatal GBS infection, the induction of an early IL-10 production by the bacteria leads to an inhibition of the recruitment of protective neutrophils to infected organs [42,43]. In *E. coli* infections, IL-10 has been associated with host protective responses [24,44,45]. In our model of *E. coli* infection, IL-10 levels in the liver were maintained similar to controls throughout the experiment. In lungs, IL-10 production was detected only at 24 h, probably in response to pro-inflammatory cytokine levels. Moreover, blocking of IL-10 signaling by anti-IL-10R antibodies did not alter the neonatal susceptibility to *E. coli* infection. Therefore, *E. coli* K1, despite the similarities with GBS in mode of transmission and neonatal manifestations of neonatal diseases, does not rely on an early IL-10 production to evade the neonatal immune system. Additional studies will be required to determine which immune mediators and bacterial factors are responsible for the delay in the immune response to *E. coli* K1 in our model and for the neonatal susceptibility to this bacterium.

Overall, this study opens the possibility to the use of our mice model to study the key features of *E. coli*-induced neonatal sepsis and meningitis. We shed some light on the kinetics of colonization and on innate immune response to this bacterium. We found an unexpectedly quick systemic colonization that it is not followed by a promptly innate immune response. We believe that a protective immune response should be mounted in the first hour of infection in order to prevent systemic dissemination that led to sepsis and to inhibit the brain colonization and consequent meningitis sequelae.

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Figure Legends

Figure 1. Age dependency of *E. coli* K1-induced neonatal mortality. Balb/c pups with less than 24 or 48 h of life were orally infected with $2x10^6$ CFU of *E. coli* IHE3034 in 10 µL. Littermate controls received the same amount of PBS. Newborns were kept with their mothers during the experiment and were evaluated twice a day. All pups were sacrificed if they were in moribund state due to ethical reasons. Survival curves were determined over a 9-d experimental period. Log-rank (Mantel-Cox) test *p<0.05 between <24 h old infected and <48 h old infected groups.

Figure 2. *E. coli* K1 rapidly systemically colonize neonatal orally infected mice. (A) Lung, (B) blood, (C) liver, (D) spleen and (E) brain *E. coli* K1 colonization at specific timepoint post-infection. In this and in the following figures, neonatal (< 24 h old) Balb/c mice were infected orally with $2x10^6$ CFU of *E. coli* IHE3034 in 10µL of PBS, the littermate controls received the same amount of PBS (0h) and newborns were kept with their mothers during the experiment. Blood was collected by facial vein puncture after anesthesia with isoflurane (IsoFlo® Esteve). Liver, spleen, lungs, and brain of infected pups were aseptically removed at indicated time points and homogenized in PBS. Blood and organs were serial diluted in PBS, plated on Todd–Hewitt agar and incubated overnight at 37˚C. 30 min (n=6); 1 h (n=10); 3 h (n=13); 6 h (n=15); 12 h (n=12); 18 h (n=14); 24 h (n=14); 48 h (n=5). One-way ANOVA with Tukey's multiple comparisons test between timepoints and one sample t-test for comparison with control (theoretical mean = 0) *p<0.05; **p<0.01; ***p<0.001.

Figure 3. Kinetics of neonatal cytokine production after *E. coli* K1 oral infection. (A) Lung and (B) liver IL-1β, TNF-α, IL-6 and IL-10 levels at specific timepoint post *E. coli* oral infection. Liver and lung were removed at indicated time points, homogenized in PBS and lysis buffer was added to allow protein recovery. Cytokine production was evaluated by ELISA. 0 h (n=28); 30 min (n=6); 1 h (n=10); 3 h (n=13); 6 h (n=15); 12 h (n=12); 18 h (n=14); 24 h (n=14); 48 h (n=5). One-way ANOVA with Tukey's multiple comparisons test *p<0.05; **p<0.01;

***p<0.001. Statistic on the top of each timepoint represents a comparison with controls (0h).

Figure 4. Kinetics of neonatal chemokine production after *E. coli* K1 oral infection. (A) Lung and (B) liver MIP-2 and KC levels at specific timepoint post *E. coli* oral infection. Liver and lung were removed at indicated time points, homogenized in PBS and lysis buffer was added to allow protein recovery. Chemokine production was evaluated by ELISA. 0 h (n=28); 30 min (n=6); 1 h (n=10); 3 h (n=13); 6 h (n=15); 12 h (n=12); 18 h (n=14); 24 h (n=14); 48 h (n=5). One-way ANOVA with Tukey's multiple comparisons test *p<0.05; **p<0.01; ***p<0.001. Statistic on the top of each timepoint represents a comparison with controls (0h).

Figure 5. Cell recruitment to the liver of pups infected with *E. coli* K1. FITC anti-mouse CD45 Ab was used for leukocyte detection, neutrophils were identified by biotin anti-mouse Ly6G plus streptavidin-PECy5 staining and macrophages were identified by PE anti-mouse F4/80 staining. Cell number was evaluated using micro particle size standard. Fluorescence was analyzed using an Epics XL cytometer, and data were analyzed with FlowJo software. 0 h (n=28); 30 min (n=6); 1 h (n=10); 3 h (n=13); 6 h (n=15); 12 h (n=12); 18 h (n=14); 24 h (n=14); 48 h (n=5). One-way ANOVA with Tukey's multiple comparisons test *p<0.05; **p<0.01; ***p<0.001. Statistic on the top of each timepoint represents a comparison with controls (0h).

Figure 6. Cell recruitment to the lungs of pups infected with *E. coli* K1. FITC anti-mouse CD45 Ab was used for leukocyte detection, neutrophils were identified by biotin anti-mouse Ly6G plus streptavidin-PECy5 staining and macrophages were identified by PE anti-mouse F4/80 staining. Cell number was evaluated using micro particle size standard. Fluorescence was analyzed using an Epics XL cytometer, and data were analyzed with FlowJo software. (A) Graphic representation of number of CD45 cells, and number and percentage of macrophages, neutrophils and double positive cells of all animals. (B) Representative dot plot of Ly6G F4/80 analyses on CD45 gated leukocytes. 0 h (n=28); 30 min (n=6); 1 h (n=10); 3 h (n=13); 6 h (n=15); 12 h (n=12); 18 h (n=14); 24 h (n=14); 48 h (n=5). One-way ANOVA with Tukey's multiple comparisons test

*p<0.05; **p<0.01; ***p<0.001. Statistic on the top of each timepoint represents a comparison with controls (0h).

Figure 7. IL-10 and IL-1R signaling in the susceptibility to *E. coli* K1-induced death. Balb/c pups with less than 12 h of life were injected i.p. with 60 μg of anti–IL-1R mAb, anti–IL-10R mAb, or control IgG 12 h prior to oral infection with $2x10^6$ CFU of *E. coli* IHE3034. Newborns were kept with their mothers during the experiment and were evaluated twice a day. All pups were sacrificed if they were in moribund state due to ethical reasons. Survival curves were determined over a 9-d experimental period. Log-rank (Mantel-Cox) test *p<0.05

Figure 1

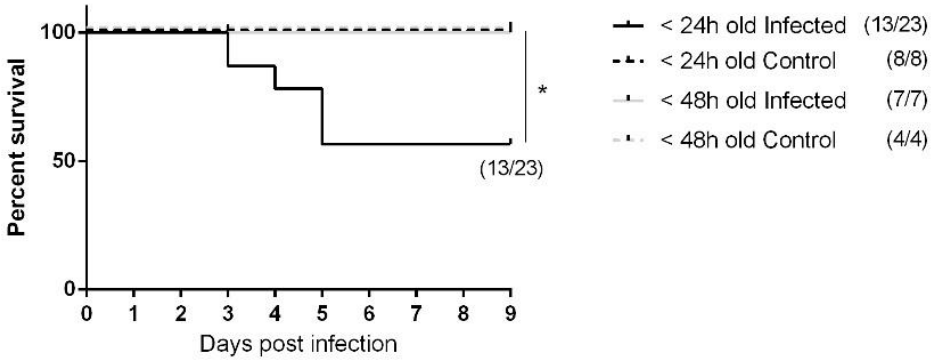

100
50
0
Percent survival
0 1 2 3 4 5 6 7 8 9
Days post infection
*
(13/23)
< 24h old Infected (13/23)
< 24h old Control (8/8)
< 48h old Infected (7/7)
< 48h old Control (4/4)

Figure 2

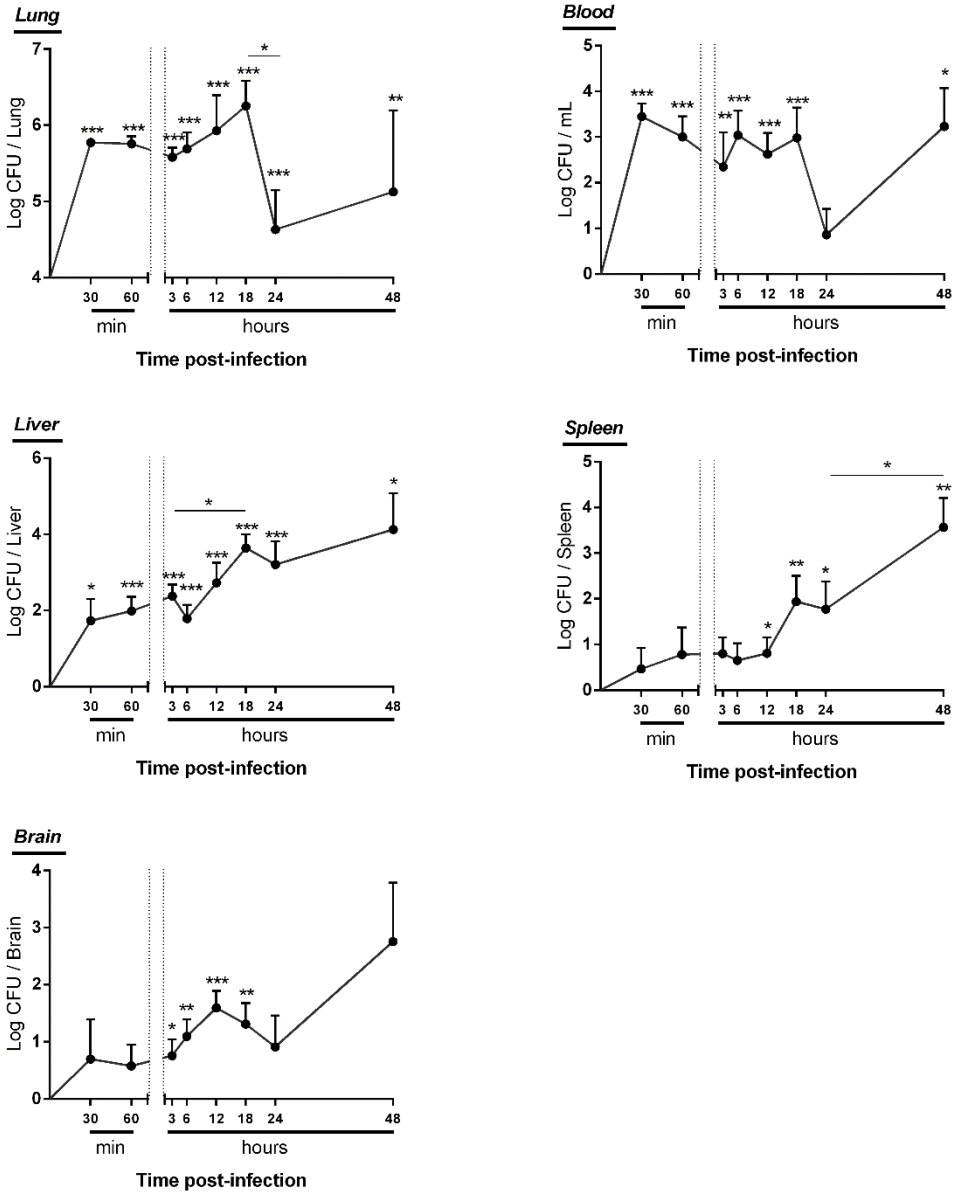

Lung
Log CFU / Lung
Time post-infection
Blood
Log CFU / mL
Time post-infection
Liver
Log CFU / Liver
Time post-infection
Spleen
Log CFU / Spleen
Time post-infection
Brain
Log CFU / Brain
Time post-infection
min
hours
30 60 3 6 12 18 24 48

Figure 3

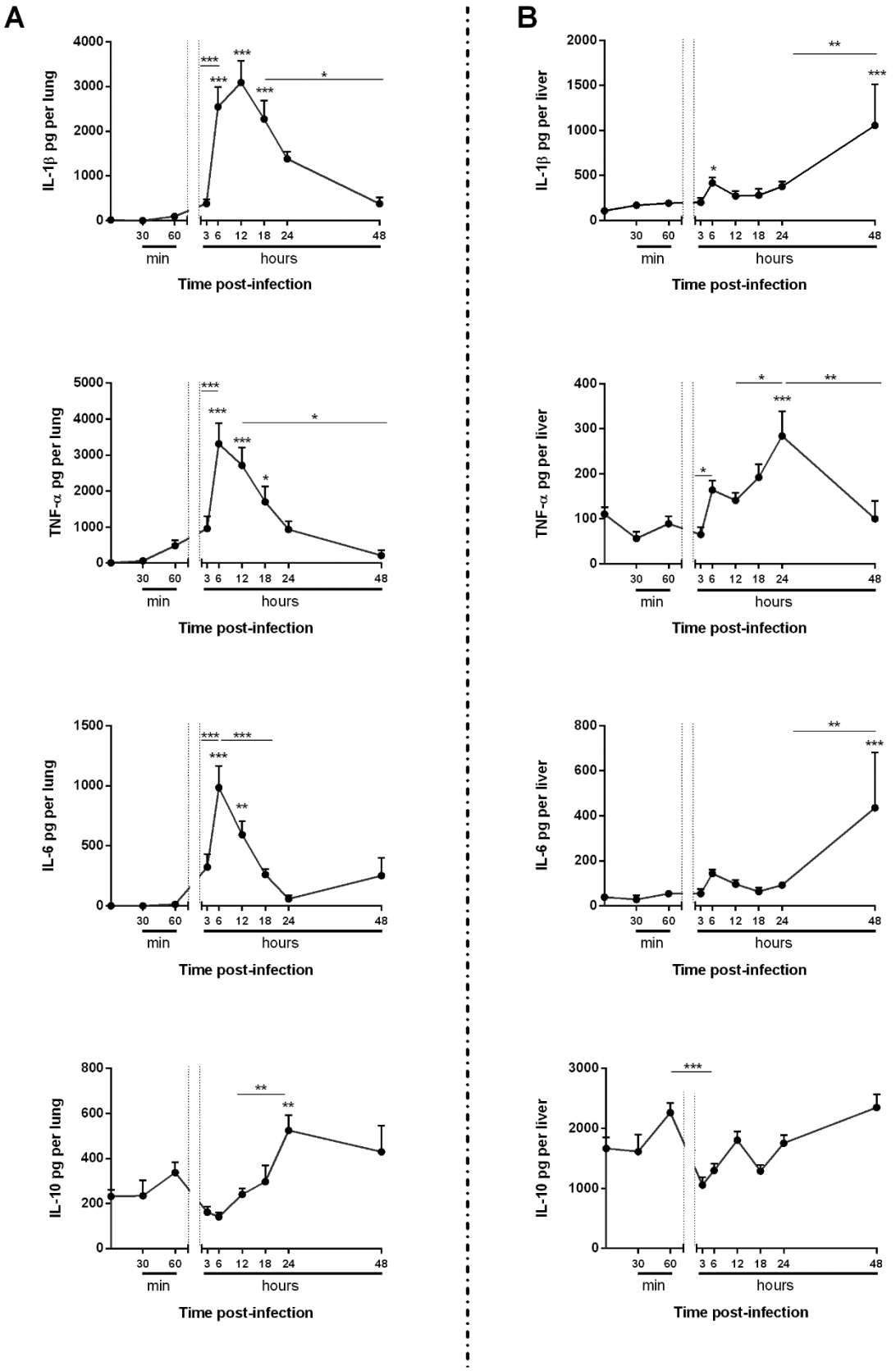

A
B
IL-1β pg per lung
IL-1β pg per liver
TNF-α pg per lung
TNF-α pg per liver
IL-6 pg per lung
IL-6 pg per liver
IL-10 pg per lung
IL-10 pg per liver
min
hours
Time post-infection

Figure 4

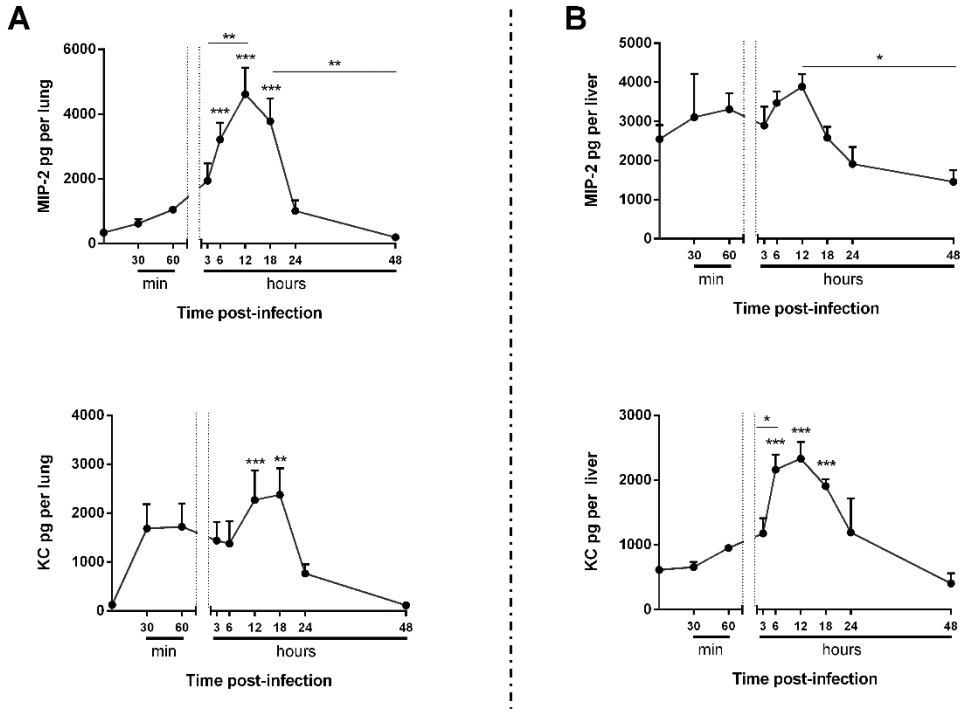

A
B
MIP-2 pg per lung
MIP-2 pg per liver
KC pg per lung
KC pg per liver
min
hours
Time post-infection

Figure 5

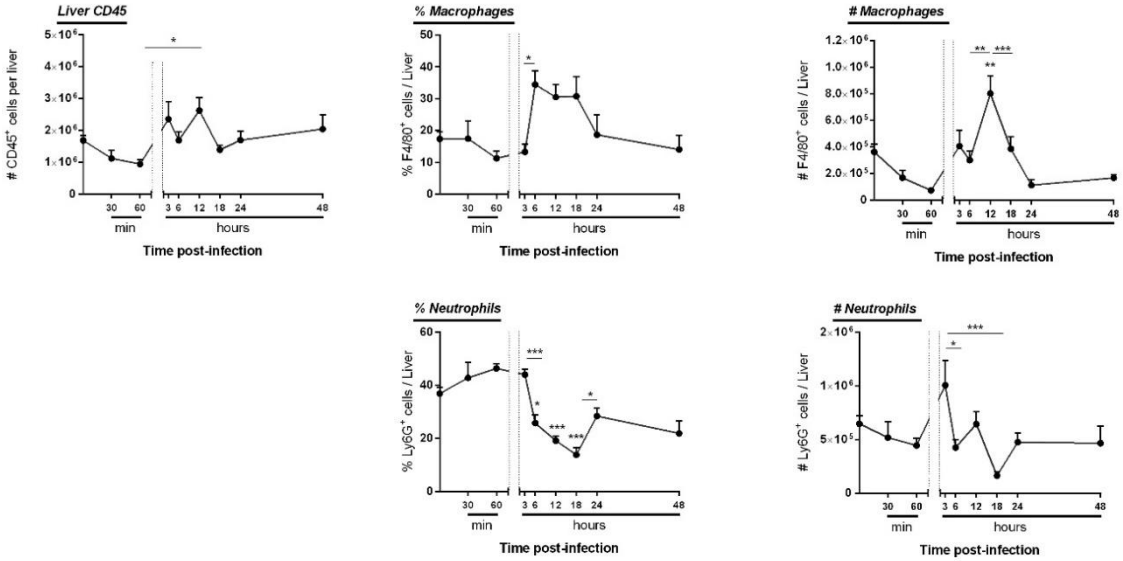

Liver CD45
CD45+ cells per liver
% Macrophages
% F4/80+ cells / Liver
# Macrophages
# F4/80+ cells / Liver
% Neutrophils
% Ly6G+ cells / Liver
# Neutrophils
# Ly6G+ cells / Liver
min
hours
Time post-infection

Figure 6

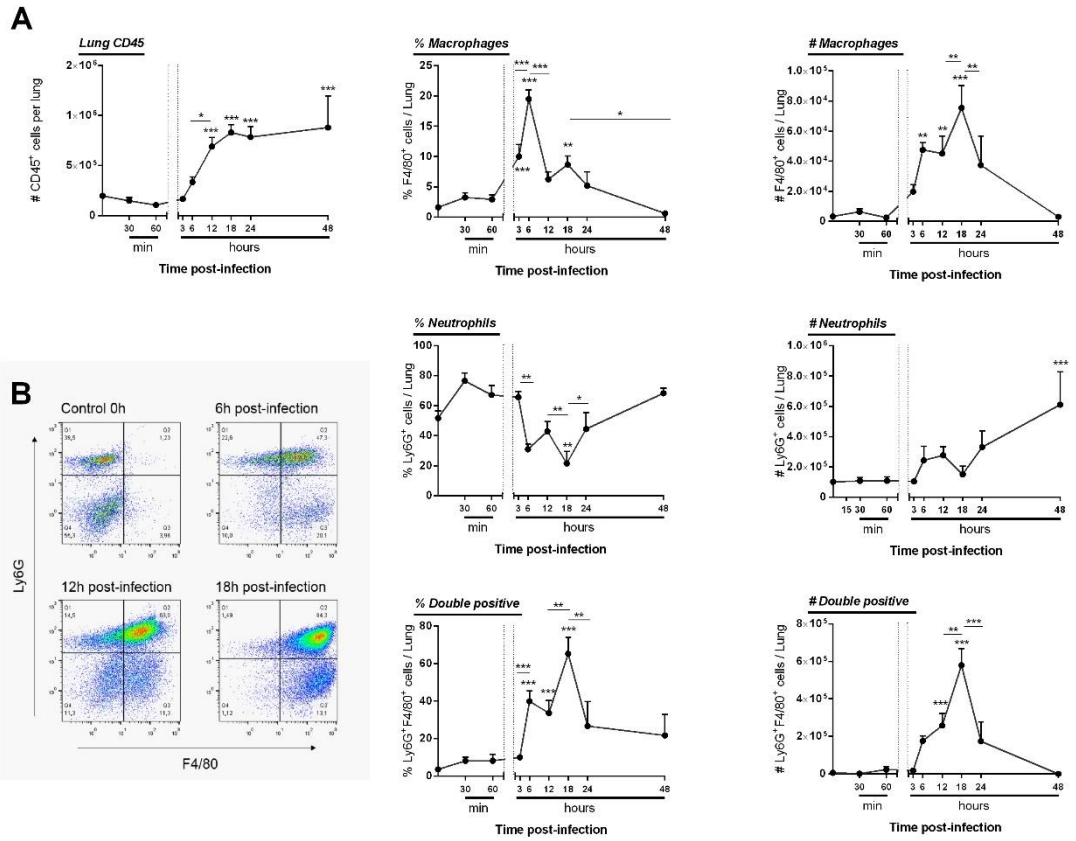
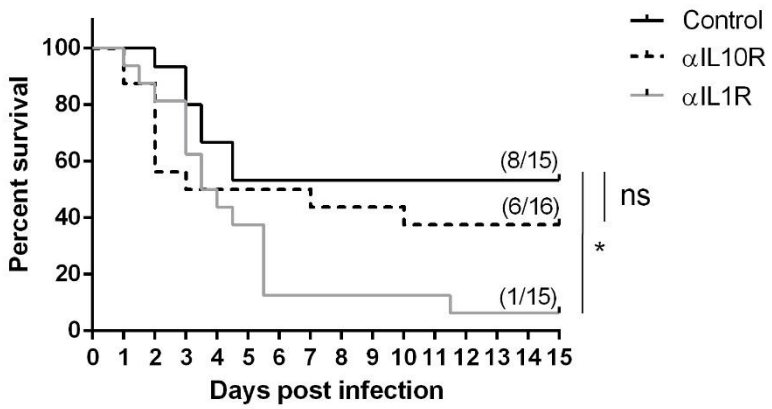

A
Lung CD45
CD45+ cells per lung
% Macrophages
% F4/80+ cells / Lung
# Macrophages
# F4/80+ cells / Lung
% Neutrophils
% Ly6G+ cells / Lung
# Neutrophils
# Ly6G+ cells / Lung
% Double positive
% Ly6G+F4/80+ cells / Lung
# Double positive
# Ly6G+F4/80+ cells / Lung
min
hours
Time post-infection
B
Control 0h
6h post-infection
12h post-infection
18h post-infection
Ly6G
F4/80

Figure 7


Control
αIL10R
αIL1R
Percent survival
100
80
60
40
20
0
(8/15)
(6/16)
(1/15)
ns
*
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days post infection

CHAPTER III.
FINAL DISCUSSION

DISCUSSION

In the 21st century, despite all the medical advances, infections remain one of the top causes of mortality worldwide being children and immunocompromised adults the main risk populations. This thesis attempts to shine a small, but hopefully significant, light on two of the infectious agents, Group B *Streptococcus* (GBS) and *Escherichia coli,* that continuously affect the life of newborns and immunocompromised adults.

GBS is a known leading cause of life-threatening bacterial infection in newborns since the 60's. Guidelines for screenings and *intrapartum* antibiotic prophylaxis (IAP) to GBS-colonized mothers have successfully reduced early-onset GBS disease (EOD; that occur in the first week of life). In the last years, however, the rates of EOD were maintained stable and GBS continues to be the most frequent cause of neonatal infections [1-3]. The IAP treatment shows severe limitations in inhibiting the rates of GBS late-onset manifestations of the disease (LOD; that occur between the first week and the first month of life) and the rates of stillbirths and prematurity caused by this bacterium [2,3]. Currently, several health entities expressed serious concern regarding the use and misuse of antibiotics on a global scale [4,5]. The risk of antibiotic overuse, especially during pregnancy, may surpass the benefits associated with the prophylaxis [5]. One of the most relevant and well known consequences of antibiotic use is the emergence of antibiotic-resistant strains [6]. IAP use during pregnancy has been shown to, not only have an effect on the emergence of GBS-resistance strains, but also to have been associated with the shift to *Escherichia coli* neonatal infections [7-9]. Another important aspect of antibiotic use during pregnancy is the alteration of the neonatal gut colonization. It is now known that the colonizing microorganisms co-evolved with host innate immune system, resulting in an elaborate interdependency and feedback mechanisms by which microbiota drive postnatal maturation of the gut and development of the immune system [10,11]. IAP treatment reduces the diversity and composition of maternal intestinal and vaginal microbiota, influencing the appearance of beneficial bacteria in the child [12,13]. These alterations are linked to an increased susceptibility to the development of several diseases [14,15] and may have an influence on emotional

responses (like stress, anxiety and depressive-like behavior) and brain neurochemistry (altered expression of neurotransmitters or theirs receptors) [16-19].

Vaccination could not only abolish the contraindications of antibiotic use, but also prevent the appearance of more cases of GBS neonatal infections and the prevention of preterm labor, stillbirths and late-onset GBS infections [20-22]. This therapeutic strategy would also allow the prevention of GBS infections in low and middle-income countries where IAP, due to its cost, is inaccessible [23].

Several GBS vaccines have been developed in the last decades. A major effort and hope is being placed on capsular polysaccharide (CPS) conjugated vaccines. A trivalent vaccine, with CPS from serotypes Ia, Ib, and III, developed by Novartis, is already in Phase II of clinical studies [24,25]. GBS, however, is divided into 10 different serotypes, based on their CPS, and each CPS induces antibodies with low cross-reactivity with the remaining CPS [26]. The pattern of GBS serotypes responsible for invasive infections varies with time and geographical location and serotypes that are not contemplated on trivalent CPS vaccine are gaining ground on GBS invasive infections [27-30]. Moreover, this glycoconjugated vaccines directed against GBS CPS will not protect against infections by non-typeable GBS isolates that are increasingly being reported [31-33].

Our group developed a vaccine based on GBS' glycolytic enzyme glyceraldehyde-3-phosphate dehydrogensase (GAPDH) [34,35]. This protein is responsible for catalyzing one of the central reactions of glycolysis and is, thus, essential for both anaerobic and aerobic cells. On GBS, this protein was found extracellularly on clinical invasive strains from all serotypes [35-37], which suggests that antibody binding should not be impaired by surface capsular polysaccharides. We showed that maternal vaccination with the recombinant form of GAPDH was highly effective in protecting the offspring against a lethal infection with GBS [35] (Figure 1). The effectiveness of the GBS' GAPDH vaccine, associated with the essentiality of GAPDH to bacterial growth in blood and universality of the protein among GBS serotypes, makes it an attractive target for the development of a human vaccine. To move a candidate vaccine from the

laboratory to the clinic, it has to pass through several mandatory stages and preclinical tests on animals to assure its safety [14, 48]. Therefore, one of the first questions addressed on **Manuscript 1** was:

i) Is recombinant (r)GAPDH$_{GBS}$ vaccine safe?

To answer this question, we evaluated a series of toxicological parameters on Balb/c mice after repeated administrations of rGAPDH-Alhydrogel formulations, using three different doses that showed similar immunogenicity. This is of great importance, since antibodies are key mediators of host immune responses against GBS [35,38,39]. During the toxicological studies the animals were followed closely but none of the animals, in any of the groups, presented any clinical signs or histological abnormalities in organs and tissues. Moreover, the panel of biochemical and acute inflammation parameters tested confirmed the harmlessness of the vaccine. Although preclinical studies must be performed in independent laboratories under Good Laboratory Practices (GLP) certification, our results point to a favorable outcome of that tests and increases the interest of a financial investment in that direction. Another important aspect of the vaccine to support clinical trial approval is its stability [40]. In **Manuscript 1** we also showed that the rGAPDH vaccine conserves its potency and safety, without significant alteration, at 4°C for at least 12 months. This feature is especially significant for the use of this vaccine in low-income countries where it is often difficult to provide appropriate storage conditions [41].

In the last decades, a remarkable increase in GBS invasive infections in non-pregnant adults has been observed, especially among the elderly and patients with underlying medical conditions [42-46]. These infections are associated with substantial morbidity and mortality, reaching fatality ratios higher than the ones associated with neonatal infections [44,45]. In non-pregnant adults, serotypes V and Ia are the most commonly associated with GBS invasive disease [29,46-48]. Serotype V, is highly associated with antibiotic-resistance and, in some reports, more than 50% of the invasive isolates of this serotype had at least one antibiotic resistance mechanism [46,49]. Thus, an alternative treatment to antibiotics is required against GBS infections in non-pregnant adults. For this reason we

investigated the potential of rGAPDH$_{GBS}$ vaccine this group, by answering the second question addressed on **Manuscript 1:**

ii) Can rGAPDH$_{GBS}$ vaccination confer protection against GBS infections in non-pregnant adult mice?

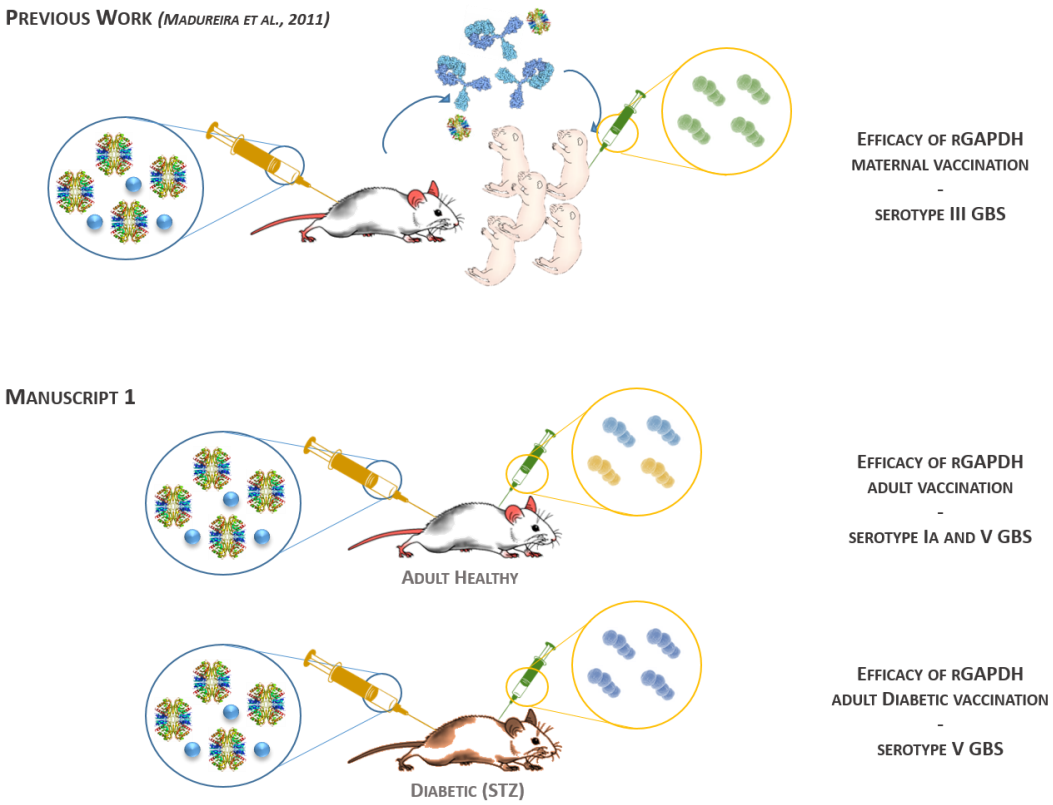


Figure 1. rGAPDH vaccination confers protection against neonatal and adult GBS infection. Previous work of *Madureira et al*, proved that maternal vaccination with rGAPDH-vaccine protected the progeny against a lethal infection with GBS from serotype III (top). In Manuscript 1 we proved that rGAPDH-vaccination also protected adult susceptible mice against GBS serotype Ia and V (middle) and diabetic mice against GBS serotype V (bottom).

Vaccination promoted an increase in resistance to GBS by abolishing mortality and decreasing bacterial colonization in several organs (Figure 1). The decrease of bacterial colonization on heart and brain of vaccinated animals was of

particular significance since endocarditis and meningitis are the two clinical presentations of GBS invasive infections with the worst prognosis in terms of morbidity and mortality in non-pregnant adults [38, 39]. Endocarditis and meningitis pathogenesis are complex processes, but the ability of GBS to bind to host components, such as fibrinogen, fibronectin and plasminogen has been associated with both adhesion and disease progression [50-52]. GBS GAPDH interaction with host extracellular matrix components, such as plasminogen, actin and fibrinogen was already described [37,53]. Thus, a decrease of $GAPDH_{GBS}$ association with host molecules could be, in part, contributing to the protective effect of anti-$GAPDH_{GBS}$ antibodies in GBS infection.

We also observed an inflammatory response mediated by production of pro-inflammatory cytokines associated with protection. This inflammatory response, when induced early post-infection, has also associated with GBS clearance in adult mice model [54]. Therefore, the protection observed in adults susceptible mice by rGADH-vaccination is, probably, a consequence of an increased complement activation and phagocytic-activity dependent on antibodies. We intend to abrogate the signaling of each pro-inflammatory cytokine to determine their direct or indirect role in the protection that we observed.

An important aspect of vaccination of non-pregnant adults that should be taken into account is the fact that the majority of GBS infections in this group occurs in elderly adults [42,55,56]. The existence of underling medical conditions, hospitalization and bedridden states added to a decreased of integrity of the anatomical barriers and immune senescence, make this group particularly susceptible to GBS invasive infections [56]. The profound dysfunction in immune responses, especially in cell-mediated immunity and neutrophil activity, could render vaccination ineffective in this group [57,58]. Nevertheless, pneumococcal polysaccharide vaccine is recommended for adults with more than 65 years of age [59] and the administration of serotype V CPS conjugate vaccine in healthy adults with ages comprised between 65 and 85 years old was able to elicit specific antibodies as strongly as those in the 18–50 years old group [60]. Moreover, neutrophils from elderly individuals, in the presence of specific antibodies against type V CPS, were able to mediate the opsonophagocitic killing

of the serotype V GBS strain *in vitro* [61]. These data validates the possibility of eliciting an effective immune response against GBS by vaccination of the elderly.

From the adult populations usually considered at at-risk for invasive GBS disease, individuals with diabetes mellitus stand-out [42,45,62,63]. In fact, the rising of the incidence of GBS infections in non-pregnant adults has been associated not only with the aging of the population, but also with the increasing prevalence of individuals with diabetes [45]. Diabetes mellitus is present in 20 to nearly 50% of non-pregnant adults infected with GBS [45,64-66] and, although the reason for the propensity of adult diabetics to develop some infections is not yet well defined, it appears to be due to a combination of immune dysfunction, angiopathy, neuropathy, gastrointestinal and urinary dysmotility [67]. Glycemia influences the host defense mechanisms such as wound healing, cellular immunity and complement functions [68]. Enhanced susceptibility to GBS infection in diabetic mice has already been described for serotypes II and IV [69,70]. Having in mind the importance of diabetes in susceptibility to GBS infection, in **Manuscript 1** we also determined the efficacy of the rGAPDH$_{GBS}$ vaccine in a mouse model of diabetes. In this study we used a GBS strain that belongs to serotype V, since this is serotype is the most frequent on diabetic patients [45]. The experiments on diabetic mice were only performed with males, since females are considerably more resistance to the drug used to induce the diabetes, streptozotocin [71], probably due to estradiol's ability to protect pancreatic β cells from apoptosis induced by oxidative stress [72]. rGAPDH$_{GBS}$-vaccinated mice presented lower colonization levels in all analyzed organs, when compared with Sham-immunized animals. These results proved that vaccination with rGAPDH$_{GBS}$ also protects adult diabetic mice against GBS serotype V infection.

Overall, GAPDH proved to be a promising target antigen for vaccination against GBS infections in the groups of risk: neonates and susceptible adults.

Since GAPDH is ubiquitously expressed in all type of cells, and several microorganisms use this enzyme to bind to extracellular matrices or modulate the host-immune response [73-77], we and others use the GAPDH as a target for vaccination [78-84]. Having in mind the central role of GAPDH$_{GBS}$ in modulating

the neonatal immune system to allow the establishment of GBS bacteremia [35], we hypothesized whether other microorganisms use the same mechanism to induce a neonatal infection. Due to the growing importance of *E. coli* as a neonatal pathogen [85], and that $GAPDH_{E.coli}$ has already been described as an extracellular virulence factor of this Gram-negative bacterium [86], in **Manuscript 2** we addressed the question:

iii) Can an rGAPDH-based vaccine confer protection against *E. coli* neonatal infections?

Bacterial GAPDH share a high degree of similarity, which hints to the possibility that a vaccine based on the GAPDH protein from one microorganism could protect against the others unrelated pathogens. This was already observed for the fish pathogens *Aeromonas*, *Edwarsiella* and *Vibrio* species, where cross protection (ranging from 62% to 78%) was achieved against five pathogens [87]. Although $GAPDH_{GBS}$ shares a 50% amino acids homology with $GAPDH_{E.coli}$, antibodies specific for each GAPDH are not able to recognize the GAPDH from the other bacteria. This was also described with the GAPDH from the two mastitis pathogens (*Streptococcus uberis* and *Streptococcus dysgalactiae*) that a difference in few amino acids also resulted in lack of cross protection [83].

$GAPDH_{GBS}$, in addition to the ability to interact with host extracellular matrix proteins [53], shows a B cell stimulatory effect *in vitro* [34], promotes IL-10 production [34,35] and induces apoptosis in murine macrophages [88]. In **Manuscript 2**, we investigated if $GAPDH_{E.coli}$ had the same properties of $GAPDH_{GBS}$ on host immunity. However, the obtained results showed that $GAPDH_{E.coli}$ is unable to stimulate B cell activation or induce cytokine production and, unlike $GAPDH_{GBS}$, induces splenic cell death. These results proved that, notwithstanding the similarities between the amino acid sequences of the two GAPDH, their effects on host immune system are distinct. Furthermore, the capacity of $rGAPDH_{GBS}$ to induce cell death appears to be dependent on the cell type used since it was already described that $rGAPDH_{GBS}$ induces apoptosis in murine macrophages but we did not detect cell death in splenic cells stimulated with this protein.

The surprising result, however, came from the $GAPDH_{E.coli}$ immunization assays. Maternal vaccination with $rGAPDH_{E.coli}$-Alhydrogel formulations not only did not confer protection but also increased the neonatal susceptibility to oral *E. coli* K1 infection. A similar tendency, although not statistically significant, was also observed with passive immunization with anti-$rGAPDH_{E.coli}$ IgG antibodies to pups. This is, to our knowledge, the first time that a negative effect is associated with GAPDH-based vaccines. An increase in neonatal susceptibility to an infection, especially to a normal colonizer of the gastrointestinal tract, highlights the need for precaution in the generalization of the development of GAPDH-based vaccines. The reason for this phenomenon is not yet clear. An increased bacterial accessibility to bloodstream facilitated by antibodies is not excluded and will be further investigated. Nevertheless, in our model of oral *E. coli* infection (characterized in **Manuscript 3**), the bacteria reached the bloodstream very soon upon infection, without the need of facilitating antibodies. Moreover, a tendency to an increased susceptibility to *E. coli* infection was also observed when antibodies against $rGAPDH_{E.coli}$ were administered i.p., suggesting that the negative effect of the antibody administration probably occurs after the bacteria reaches the bloodstream. The mechanism by which these antibodies are conferring susceptibility in a neonatal model of *E. coli* infection will be further investigated.

The negative effect that was observed with GAPDH immunization on *E. coli* neonatal infection could not be exclusive to the use of antibodies against this used antigen. Indeed, despite several *E. coli* K1 virulence factors having been already identified, there is not any effective vaccine being developed against *E. coli* neonatal infections. It is possible that IgG opsonization of *E. coli* K1 facilitates the internalization of the bacteria by macrophages and neutrophils in a way that favors the bacterial survival. The interaction of the bacterium with FcγRI (mediated by *E. coli* K1 outer membrane protein A (OmpA)) is associated with bacterial invasion and survival in macrophages [89]. OmpA interacts with FcγRI α-chain and avoids the association of the γ-chain (crucial for inducing the anti-microbial activity of macrophages [90]), even in the presence of IgG2a opsonization [89] (Figure 2 and 3). Therefore, it is possible that the antibodies IgG binding to FcγRI could enhance the *E. coli* K1 internalization and intracellular

survival in an environment where phagocytic killing is inhibited by OmpA (Figure 3). We intend to explore this hypothesis with *in vivo* studies with F(ab)2 specific to $rGAPDH_{E.coli}$ to determine the role of the Fc binding to the FcγRI in the susceptibility of $rGAPDH_{E.coli}$ vaccination to *E. coli* K1 neonatal infection. In parallel we plan to perform *in vitro* opsonophagocytic studies with *E. coli* K1 WT and $OmpA^-$ to determine the effect of the antibodies on bacterial invasion and survival within phagocytes is dependent of this outer membrane protein A. Moreover, if *E. coli* K1 associates with CR3 instead with FcγRI and TLR2 on phagocytes, bacterial clearance is achieved [89,91]. IL-10 administration during *E. coli* K1 neonatal bacteremia shifted the intracellular uptake of bacteria towards CR3 [91] (Figure 2). Therefore, we also intend to induce CR3 expression on phagocytes, by IL-10 administration, in anti-GAPDH treated and *E. coli* K1 infected mice. This experiment will allow us to understand if the type of bacteria association with phagocytes interferes with the neonatal susceptibility induce by antibody administration in our model.

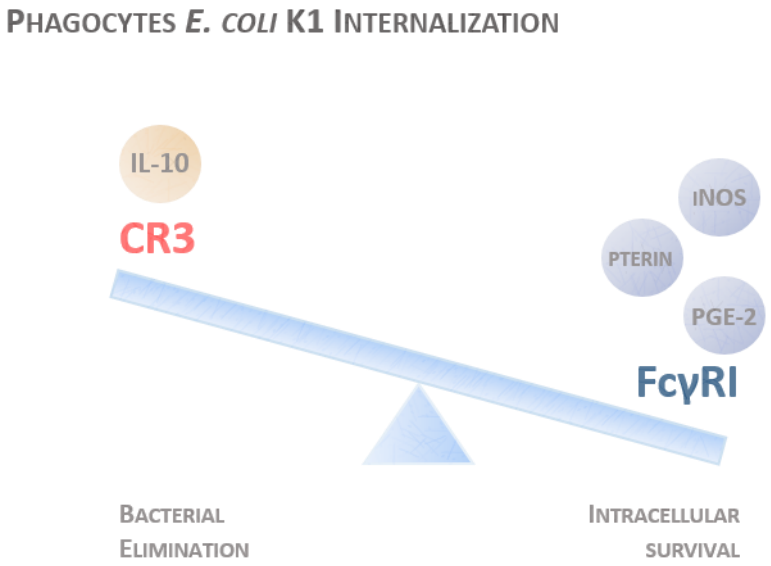


Figure 2. *E. coli* K1 elimination or survival inside phagocytes is dependent by which receptor the bacteria is internalized. *E. coli* K1 interaction with FcγRI leads to bacterial uptake and intracellular survival of the bacteria [89]. Pterin, prostaglandin E-2 (PGE-2) and NOS production are associated with a balance towards FcγRI expression on phagocytes during *E. coli* K1 infection [91-93]. On the other side of the balance, is the *E. coli* K1 interaction with CR3. This association leads to bacterial uptake and clearance

inside phagocytes [89,91]. The expression of CR3 on phagocytes is amplified by IL-10 production/administration during infection [91].

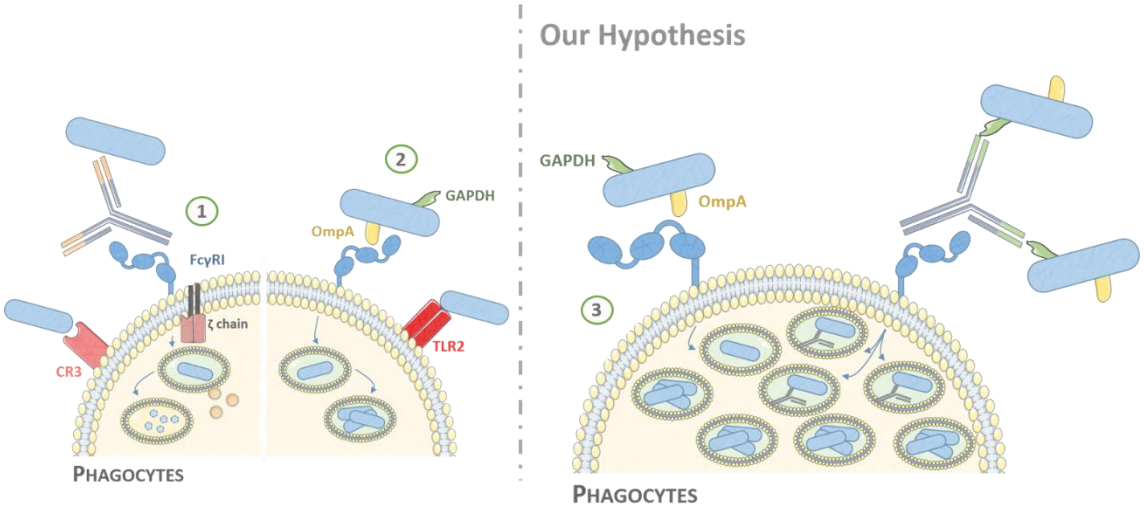


Figure 3. **Representation of association with *E. coli* K1 with phagocytes receptors and possible mechanism of susceptibility conferred by anti-GAPDH antibodies.** 1) In the absence of OmpA, *E. coli* K1 associates with CR3 leading to bacterial clearance. Also in this case, when IgG antibody interacts with FcγR, it leads to recruitment of ζ-chain and the consequent intracellular activation of microbicidal mechanisms of phagocytes. 2) *E. coli* K1 OmpA interacts with FcγRI, inducing the bacterial internalization without activation of ζ-chain-dependent mechanisms. In this scenario, TLR2 recognition of *E. coli* K1 also increases the intracellular survival. 3) We speculate that, in our mice model, since the microbicidal intracellular cascade of FcγR is inhibited by OmpA, the anti-GAPDH antibodies are facilitating de *E. coli* K1 internalization and accelerating bacterial dissemination.

Due to increasing interest on GAPDH as a vaccine target for multiple human and veterinary vaccines [84] it is possible that, in the next years, other GAPDH-based vaccine could be considered against human pathogens. Having in mind the negative effect of GAPDH-vaccination in our *E. coli* K1 infection model, the cross-reactivity of the antibodies induced by GAPDH-based vaccines with *E. coli'* GAPDH should be tested, especially if they are intended to be used in women. It

is possible that a GAPDH-vaccine, especially against a Gram-negative bacteria, could lead to a recognition of the *E. coli'* GAPDH and, therefore, induce susceptibility to *E. coli* K1 in neonates, despite the efficacy against the bacteria to which they were designed.

Additionally, there is the possibility that the antibodies against the GAPDH of a pathogen recognize homologous protein of probiotic strains and, therefore, interfere with microbiota colonization. This possibility, regarding the $GAPDH_{GBS}$ vaccine, is being investigated by our group.

We showed that GAPDH, despite being a virulence factor for intestinal-pathogenic *E. coli* [86], is not relevant for neonatal *E. coli* K1 infection. However, how this Gram-negative bacterium survives and colonizes the neonates? This question was addressed in **Manuscript 3.**

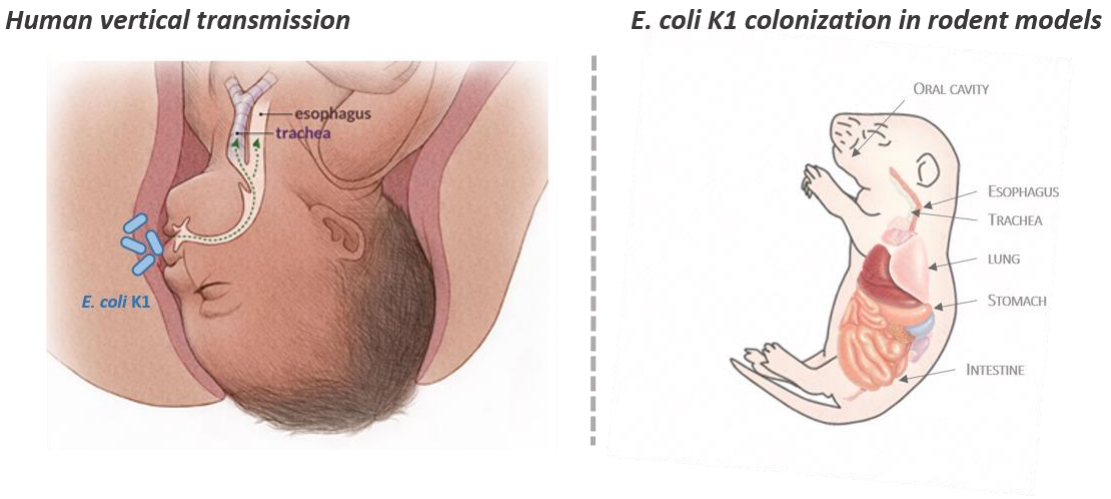


Figure 4. *E. coli* K1 bacterial dissemination in humans and in rodent models of neonatal infection. During birth, the newborn aspirates the *E. coli* K1 contaminated vaginal fluids. The bacteria can assess both the respiratory and the digestive system. In a rat model of *E. coli* K1 infection, the bacteria has been shown associated with the oral cavity, esophagus tissue and intestine [94]. Mice models with intranasal *E. coli* K1 infection, prove that this bacteria as the ability to transverse the respiratory tract [89]. In our model of oral *E. coli K1* infection, we achieved both respiratory and digestive tract infection. *Newborn - illustration of Amanda Montanez.*

E. coli, as GBS, are bacteria that the newborns can acquire perinatally from contaminated vaginal fluids [95] (Figure 4). Therefore, first, we selected a model that allowed to mimic of that type of infection and, at the same time, enabled the characterization of the kinetic of the neonatal immune response. Oral *E. coli* neonatal infections had already been employed in rats, with positive results, and shed some insights about the complex and dynamic patterns of *E. coli* K1 colonization and dissemination [94,96]. However, an oral neonatal mice model has not yet developed and the use of mice is preferable to study the host-*E. coli* K1 interactions since the majority of immunological studies with other neonatal pathogens are performed on mice (allowing therefore the comparison of results) and rat models limit the use of reagents and transgenic animals. Although in 1988, an oral *E. coli* infection was tried in 3-5 days old mice, the results showed that it was unable to induce bacteremia or dead in WT mice strains and was, therefore, set aside [97]. We change the time of oral inoculation, and administrated the bacteria at the day of birth to Balb/c mice. The results showed a 43% of mortality rate and a 100% bacteremia at the first hours post-infection, but, if the infection was performed after on day of life (>24 h but <48 h), the mortality was completed abolished. This age-dependency of *E. coli* K1 infection explains the lack of mortality observed in the 1988 study, where mice with 3-5 days old were used [97].

The strong age-dependency in *E. coli* infections can be associated with external or internal factors. During the birth and soon thereafter, bacterial colonization of a previously germfree intestinal tract begins, in a dynamic way, as the host develops. The hypothesis that the evolution of the microbiota profile with neonatal age could contribute to a decrease to the neonatal susceptibility to *E. coli* K1 should not be excluded. In this regards, changes in the composition of gut microbes in neonates mice by antibiotic exposure during birth, led to a deregulation of neutrophil homeostasis and increased the susceptibility to *E. coli* K1 infection [98]. Furthermore, in a study of the human infant intestine, an initial colonization with *E. coli* was detected in an infant that disappeared as soon as the colonization of bifidobacteria was detected [99]. Moreover, despite that at the anatomical level, the gastrointestinal tract is fully formed at birth it undergoes significant morphological and cytological differentiation and maturation in the

postnatal period. Some of this physical “immaturity” can contribute to the neonatal susceptibility to *E. coli* K1 infection. A study with bioluminescent *E. coli* K1 oral infection showed that this bacterium adheres to non-keratinized esophageal tissues and to the oral cavity of neonatal rats [32]. A reduced esophageal motility and luminal clearance, characteristic of neonates (particularly preterms) [46], can contribute to the vulnerability to colonization and invasion of *E. coli* K1 when compared to adults. Moreover, the incomplete development of the intestinal barrier during early neonatal life, that includes deficits in α-defensins and mucin, can provide a permissive environment for translocation of the intestinal colonizing-*E. coli* K1 into the bloodstream [38]. The respiratory tract may also provide an alternative entrance to the bacteria into the systemic circulation [32]. The majority of studies with non-invasive *E. coli* K1 infections in neonatal mice use an intranasal inoculation and, even in this model, the bacterium showed the ability to translocate the epithelial barrier and reach the bloodstream to induce meningitis [89,91,100,101].

In our model, *E. coli* colonization was observed in both gastrointestinal and respiratory tract. The bacterium was detected in the stomach and small intestine of all infected animals (data not shown) and in the lung as soon as 30 min post-infection, consistent with the observation of the entry of inoculum to the respiratory tract during infection.

Interestingly, as shown in **Manuscript 3,** the bacterium delivered by a non-invasive way quickly reached the bloodstream and established a systemic infection. Indeed, as soon as 30 min post–infection, the bacterium was detected in the blood, in levels that are maintained during the first 18 h of infection. Which endothelial barrier is first being surpassed by *E. coli* K1 in our model is yet to be known, but it is possible that the bacterium is reaching the bloodstream through more than one location and at different timepoints. The use of fluorescent *E. coli* K1 and intravital imaging could help us dissect this question.

In the liver, spleen and brain the *E. coli* colonization increased over time. Since *E. coli* K1 is strongly associated with neonatal meningitis in humans, brain colonization is essential for a model intended for translational studies of human *E. coli* K1 neonatal diseases. At 12 h post-infection, 76% of the infected pups

presented brain *E. coli* K1 colonization. It is thus likely, that part of the *E. coli* K1 survivors of our model will present neurological sequelae. Animal models of *E. coli* K1 meningitis are important to the characterization of pathogenesis of disease and to the understanding of the cellular and molecular immune mechanisms involved. In humans, long-term sequelae in patients who had neonatal *E. coli* meningitis are frequent, particularly neurosensorial sequelae, like deafness and blindness, and neurodevelopmental sequelae, like learning impairment [102]. Ventriculitis with intraventricular haemorrhage frequently accompanies neonatal *E. coli* meningitis (27%) [103]. A characterization of the neurodevelopment of the survivors in our model, by analysis of their performance on behavioural studies, and histological analysis of the brain could help us understand if the *E. coli* K1 oral infection mice model does, in fact, mimic not only the mortality, but also the morbidity associated with *E. coli* K1 infection in humans.

Another interestingly result also shown in **Manuscript 3**, was a delay in triggering the immune response after oral infection. It is known that an immune response is initiated as soon as the pathogen has contact with an immune cell and its pathogen associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs). Several PRRs have been associated with the immune response to *E. coli* K1, including Toll-like receptors (TLR) 4 and 2 [100,104] and the β2-integrin CD11b/CD18 (complement receptor 3 (CR3)) [91]. CR3 expression on neonatal neutrophils is decreased during the first month of life and it is further reduced in preterm neonates [105]. On the other hand, basal expression and cellular distribution of TLRs is similar in blood monocytes and T cells of newborns and adults (with exception of preterms that have diminished TLR4 expression) [106-109]. In the case of bacteremia, peripheral blood cells from both preterm and term neonates showed the capacity to up-regulate TLR2 and TLR4 during Gram-positive and Gram-negative bacteremia, respectively [110]. Furthermore, the expression of the lipopolysaccharide-binding protein (LBP) and CD14 (co-receptor with TLR4 and MD2 for LPS detection) is normally up-regulated during neonatal sepsis [111-113]. Nevertheless, in our model, despite the elevated colonization of lungs and liver 30 min post-infection, an increase in the production of pro-inflammatory cytokines like IL-6, IL-1β and TNF-

α and the chemokines KC and MIP-2, as well as a recruitment of neutrophils and macrophages was only detected after 6 h of infection.

A similar delay in the neonatal immune response to a pathogen has already been described by our group. In the case of GBS neonatal infections, the bacterium induces an early IL-10 production that inhibits a protective neutrophil recruitment to infected organs [35,114]. In light of these results, we hypothesized that neonates are at risk of rapidly developing immunoparalysis after bacterial-induced sepsis, due to their commitment towards IL-10 production. In *E. coli* K1 infection, however, the blocking of IL-10R signaling by antibodies did not alter the outcome of infection. Moreover, in a neonatal model of *E. coli* K1 intranasal infection, IL-10 production was associated with host protective responses since it prevented the development of hyperinflammatory immune responses and induced bacterial clearance [91]. Consequently, the “irresponsiveness” that we observed in our model is not dependent on an early and increased IL-10 production.

One of the key aspects of early life immunity is tolerance, first, to maternal antigens, then to the world antigens, coming from microbiota or food. Therefore, the innate immune response is shifted to the production of immunossupressive/regulators mediators. As described above, IL-10 is one of those mediators, but it is not the only one. Newborns and infants have high levels of plasma adenosine, an endogenous purine that accumulates extracellularly as a result of its cellular production and transport via membrane nucleoside transporters or by extracellular metabolism of ATP by the ecto-nucleotidases CD39 and CD73 that are expressed by leukocytes and other host cells [115-117] (Figure 5). In newborns, adenosine is associated with the inhibition of TLR-mediated TNF-α production and consequent polarization of innate immune responses towards IL-6 and IL-10 [118,119]. The induction of adenosine production has already been associated with *E. coli* [120,121]. The stimulation with *E. coli* up-regulated the CD39 expression in both murine and human macrophages [120] and, in a model of *E. coli* peritonitis, both CD39 and CD73 were up-regulated while adenosine-degrading enzymes were down-regulated [121]. It is possible that, similarly to what happens with *Trypanossoma cruzi* and *Legionella pneumoniae* [122,123], *E. coli* K1 induces CD39 and CD73 expression

to generate an adenosine-rich environment which allows it to escape the immune surveillance. This hypothesis will be tested using inhibitors of CD39 and CD73 as well as antagonists of adenosine receptors *in vivo*.

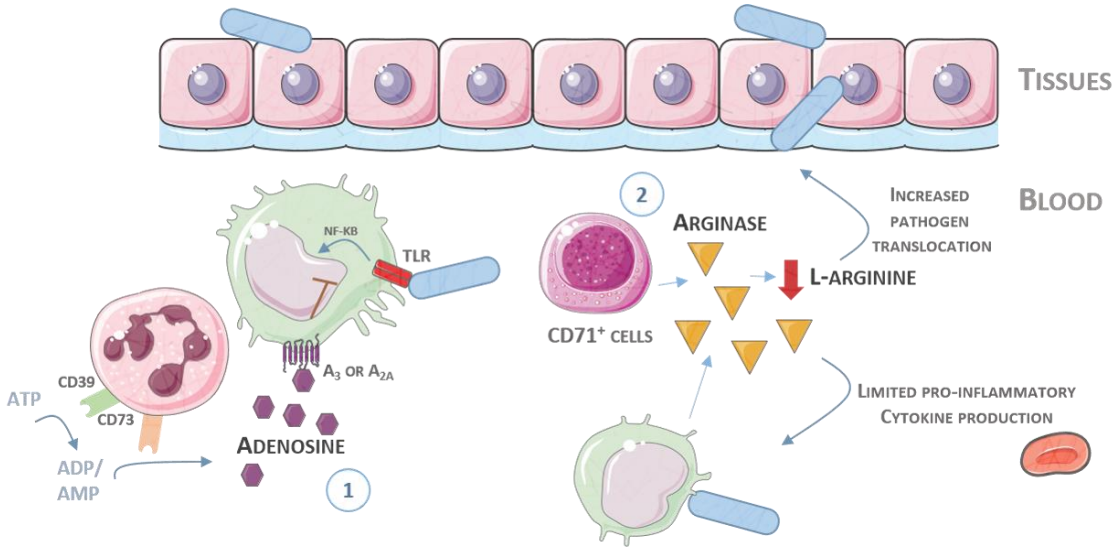


Figure 5. Possible mechanisms for the neonatal immunosuppression observed after *E. coli* K1 oral infection. 1) Elevated levels of adenosine in neonatal blood act through adenosine receptors on neonatal cells and inhibit TLR-mediated pro-inflammatory cytokines. CD39 expression is upregulated in murine and human macrophages stimulated with *E. coli* [120]. 2) Neonates possess an enriched population of immunosuppressive $CD71^+$ cells. These cells compromise the neonatal host defenses against *E. coli* K1 [124]. $CD71^+$ cells as well as infected immune cells produce high levels of arginase that degrade L-arginine and, consequently, limit pro-inflammatory cytokine production and increase pathogen translocation. (*Adapted from [125]*).

Recently, a unique population of $CD71^+$ erythroid precursor cells was identified as a key element to facilitate the colonization of the neonatal intestine by commensal microbiota in a non-inflammatory manner [124]. The depletion of these cells in neonatal mice, or natural postnatal age-related decline in their numbers, was correlated with an enhanced production of pro-inflammatory mediators and increased the neonatal resistance to i.p. infection of *Listeria monocytogenes* and *E. coli* [124] (Figure 5). It is therefore possible that these cells are responsible for the immunosuppressive state that we observed during

E. coli K1 oral infection. It is known that the anti-inflammatory property of these cells is mediated by arginase-2, since molecular inhibition of this enzyme or supplementation with L-arginine outweighs its effects [124]. Small clinical trials have shown the benefits of enteral L-arginine supplementation in prevention of necrotizing enterocolitis in premature infants [126-128]. We intend to test, at least in an initial phase, the effect of L-arginine supplementation in the outcome of *E. coli* K1 neonatal infection.

After the initial "irresponsiveness" to *E. coli* K1 infection, we observed a sustained production of pro-inflammatory cytokines IL-6, TNF-α and IL-1β and chemokines MIP-2 and KC, as well as phagocyte recruitment to infected organs. IL-1β production proved to be essential to the host immune response to *E. coli* K1 oral infection, since the blocking of its signaling with antibodies increased neonatal mortality. This is another aspect in which the neonatal protective immune response to *E. coli* differs from GBS. Treatment with IL-1R blocking antibodies delayed (and slightly decreased) the GBS-induced mortality in neonatal mice [114], an effect we did not observe in *E. coli* K1 infection. The role of other pro-inflammatory immune mediators was not yet addressed. We also intend to quantify the colonization levels in treated animals, to understand if an increase in susceptibility is associated with bacterial dissemination.

The role described for macrophages and neutrophils in some studies of *E. coli* K1 neonatal infection is quite unconventional. In models with intranasal *E. coli* inoculation, these phagocytes have shown to have a more detrimental than protective effect [89,101,129]. As previously mentioned, the type of phagocyte activation, however, more than its presence, seems to determine the outcome of infection [89,91]. In our model, we only observed cell recruitment to the organs after the establishment of infection, indicating that the systemic invasion of the organs (at least of the liver) is not dependent on the bacterium that is reaching the organs inside recruited phagocytes. Nevertheless, we cannot exclude that a "Trojan-horse"-like mechanism is not being used by bacteria to evade the immune response. We intend to deplete neutrophils and macrophages, prior to infection, in order dissect the role and the timing of their importance for the susceptibility (or resistance) in our neonatal model of *E. coli* K1 infection.

We detected, in lungs of infected mice, a double positive population for Ly6G and F4/80. It is, to our knowledge, the first time that these cells have been described in a neonatal infection. Very little is known about this population. In a study with *Pseudomonas aeruginosa* infection these cells were described as having the capability of producing the same amount of myeloperoxidase as neutrophils in response to infection [130] and were designated as immature myeloid cells. Since at 18 h post-infection, these cells accounted for more than half of CD45 positive cells in the lung of *E. coli* K1 infected pups, I believe that it is important to characterize this population and understand its role in this neonatal infection. It is possible, that the protective role of the depletion of neutrophils and macrophages during intranasal *E. coli* K1 neonatal infection could be, in part, mediated by the elimination or modulation of this cell population. This hypothesis will be tested in the future.

In conclusion, the data presented in this thesis, re-enforces the efficacy of $rGAPDH_{GBS}$ immunization, now in a model of adult GBS infection, and provides evidence of the safety and stability of this vaccine. The results presented here, also highlight the need of precaution in extrapolating the efficacy of GAPDH-based vaccines to all microorganisms since the maternal vaccination with this antigen increases the susceptibility of neonate mice to oral infection with *E. coli*. Moreover, in this thesis, a murine model of *E. coli* K1 infection that uses a relevant route of inoculation was established opening new possibilities to further characterize and explore the cellular and molecular immune mechanisms involved in the pathogenicity of *E. coli* K1 neonatal infections.

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ANNEX