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UNIVERSIDADE DO PORTO

Unravelling the role of IFN-γ in the immunopathology observed in GAPDH vaccinated mothers

Ana Isabel Pereira Mestre

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Supervisors

Paula Ferreira, Associate Professor, ICBAS

Elva Bonifácio Andrade, Researcher, ICBAS

Author

Ana Isabel Pereira Mestre,

up201506222@fc.up.pt

Rua do Campo Alegre, s/n, 4169-007 Porto (FCUP)

+351 916140598

Supervisors

Paula Maria das Neves Ferreira da Silva, Associate Professor,

pauferr@icbas.up.pt

Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto (ICBAS-UP)

+351 220528215

Elva Bonifácio Andrade, Researcher,

ebandrade@icbas.up.pt

Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto (ICBAS-UP)

+351 963253992

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Ana Isabel Mestre

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Resumo

O estreptococos do grupo B (EGB) é o principal agente responsável por infeções neonatais, nomeadamente pneumonia, meningite e septicemia. A colonização materna do sistema gastrointestinal e geniturinário são os principais fatores de risco que levam ao aparecimento de doenças neonatais causadas pelo EGB. A administração de antibióticos intraparto em mulheres que se apresentem colonizadas com EGB é atualmente a medida de prevenção utilizada. No entanto, devido ao aumento do número de bactérias resistentes a antibióticos e evidência que estes levam a um atraso na colonização por parte de bactérias benéficas, a vacinação materna contra o EGB surge como uma estratégia atrativa para a prevenção da doença causada pelo EGB. O nosso grupo desenvolveu uma vacina materna baseada na forma extracelular da enzima glicolítica gliceraldeído 3-fosfato desidrogenase (GAPDH), que provou ser eficaz em ensaios pré-clínicos em que foi utilizado um modelo experimental em que os recém-nascidos eram infetados após o nascimento. No entanto, resultados preliminares mostraram que quando a vacina foi testada num modelo de ratinho que reproduz a fisiopatologia da infeção neonatal for EGB, esta não conferia proteção e levava ao desenvolvimento de patologia mortal nas mães que tinham sido vacinadas após o parto. Adicionalmente, estudos feitos relativamente à resposta imune materna sugeriam que pudesse estar a ocorrer uma mudança para um perfil mais pró-inflamatório da resposta celular, caracterizada por uma produção elevada de IFN-γ sistemicamente. Portanto, o objetivo deste trabalho é descobrir quais os mecanismos por detrás da imunopatologia e mortalidade materna observada em fêmeas vacinadas, que foram intravaginalmente colonizadas com EBG.

Para tal, ratinhos BALB/c foram ativamente imunizados com a forma recombinante da GAPDH (rGAPDH) juntamente com o adjuvante Alhydrogel (*rGAPDH-vaccinated*) ou apenas com o adjuvante (*Sham-vaccinated*). Fêmeas gestantes foram intravaginalmente inoculadas com a estirpe hipervirulenta BM110. Foi confirmada a ineficácia da vacina da rGAPDH em proteger a progénie e a resposta imune dos progenitores a nível sistémico e local (sistema reprodutor feminino) foi analisada. Também foi feita uma avaliação histopatológica das progenitoras vacinadas com a rGAPDH. Os nossos resultados mostram que a imunização ativa de futuras mães com a rGAPDH contribui para a disseminação sistémica do EGB em 50% das progenitoras vacinadas com a rGAPDH, apresentando colonizações altas em órgãos como o útero, pulmão, rins e fígado. Este grupo de progenitoras que desenvolveu patologia foi designado como *rGAPDH-vaccinated* doentes. Análise histopatológica mostrou que os

pulmões destas progenitoras apresentavam uma maior incidência de enfisema, comparativamente com os pulmões de progenitoras *Sham-* ou progenitoras *rGAPDH-vaccinated* saudáveis. A vacinação materna com a rGAPDH também revelou que esta pode ser prejudicial durante a gravidez, visto que uma progenitora desenvolveu corioamnionite, e os fetos sucumbiram possivelmente devido à infeção por EGB *in utero*, confirmado pela coloração de Gram feita às placentas da progenitora. Adicionalmente, anticorpos contra a rGAPDH do EGB não levaram ao aumento da sua infecciosidade, visto que nenhuma morte foi observada quando progenitoras foram passivamente imunizadas durante a gravidez e antes de serem inoculadas.

De maneira a determinar se os esplenócitos de progenitoras *rGAPDH-vaccinated* doentes mostravam uma produção de citocinas alterada, esplenócitos de fêmeas *Sham-* e *rGAPDH-vaccinated,* doentes e saudáveis, foram removidos e estimulados *ex vivo* com GAPDH (do EGB e do ratinho), LPS, Concanavalina A, ou o EGB. Os resultados obtidos mostraram níveis elevados de IFN-γ, tal como uma tendência para níveis elevados de IL-17A em fêmeas *rGAPDH-vaccinated* doentes após estimulação dos esplenócitos com EGB. A expressão relativa de IL-17A e IFN-γ foi analisada localmente no útero através de RT-qPCR. Foi observada uma diminuição da expressão de IFN-γ em progenitoras doentes vacinadas com a rGAPDH, quando comparado com o grupo *Sham-vaccinated*. Observou-se também uma tendência para o aumento da expressão de IL-17A em progenitoras doentes vacinadas com a rGAPDH, quando comparadas com progenitoras pertencentes ao grupo *Sham-vaccinated*.

Estes resultados levaram-nos a estudar o papel destas duas citocinas, IFN- γ e IL-17, na colonização por EGB em animais vacinados, mas fora do período de gravidez. No entanto, nestas fêmeas observou-se que uma infeção persistente de EGB leva a um aumento da produção de IFN-γ por células T CD4+ no aparelho reprodutor feminino de animais vacinados com a rGAPDH, comparativamente ao grupo *Sham-vaccinated*. Estes resultados levam-nos a acreditar que o IFN-γ pode ser importante para a diminuição da colonização por EGB, no entanto a diminuição da expressão desta citocina no contexto da gravidez pode levar a que esteja a ocorrer uma disseminação sistémica da bactéria e consequentemente uma resposta inflamatória exacerbada com produção elevada de IFN-γ.

Os nossos resultados realçam a importância do uso correto de modelos animais em fases de testes pré-clínicos, de maneira a que menos terapias inovadoras falhem aquando da sua entrada nas fases iniciais de testes clínicos.

Abstract

Group B *Streptococcus* (GBS) is the leading causative agent of neonatal infections, including pneumonia, meningitis and septicaemia. Maternal colonization in the gastrointestinal and genitourinary tracts is the primary risk factor for neonatal disease. The prevention of GBS infections relies on *intrapartum* antibiotic prophylaxis (IAP) to GBS-colonized women. With the increasing bacterial resistance to antibiotics, as well as the delay in colonisation of beneficial bacteria, maternal vaccination against GBS is the most attractive strategy to prevent GBS disease. Our group developed a maternal vaccine targeting the extracellular form of the glycolytic enzyme glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), that proved effective in pre-clinical trials using an experimental model where the offspring were infected after delivery. However, preliminary results had shown that when the vaccine was tested in a mouse model that reproduces the pathophysiology of neonatal GBS infection, it did not confer protection and caused lethal pathology to the vaccinated mothers upon birth. Moreover, studies on the maternal immune response were suggestive of a possible shift towards a pro-inflammatory cellular response, characterized by high systemic production of IFN-γ. Therefore, the aim of this work was to uncover the mechanisms behind the maternal immunopathology and mortality observed in vaccinated females, intravaginally colonized with GBS.

For that purpose, BALB/c mice were actively immunized with recombinant GAPDH (rGAPDH) and the adjuvant Alhydrogel (rGAPDH-vaccinated group) or only with the adjuvant (Sham-vaccinated group). Pregnant mice were intravaginally inoculated with the hypervirulent GBS strain BM110. The inefficacy of rGAPDH vaccine in protecting the offspring was confirmed, and the progenitors systemic and local [female reproductive tract (FRT)] immune response were analysed. Moreover, histopathology evaluation of rGAPDH-vaccinated mothers was also performed

Our results revealed that maternal active immunization with rGAPDH was contributing to a systemic spread of GBS in 50% of the rGAPDH-vaccinated progenitors, with high colonization rates in organs such as the uterus, lungs, kidneys and liver. This group of females that developed pathology was designated as sick rGAPDH-vaccinated. Histopathological analysis of the lungs revealed that sick rGAPDH-vaccinated progenitors had higher incidence of emphysema, than progenitors from the Sham- or healthy rGAPDH-vaccinated groups. Maternal vaccination with rGAPDH also revealed to be detrimental during pregnancy, as one progenitor developed chorioamnionitis, and the foetus possibly succumbed to *in utero* due to GBS infection, as confirmed by the

Gram staining of the placentas. Antibodies against GBS rGAPDH were not leading to a higher GBS infectivity, as no death was observed when pregnant females were passively immunized during pregnancy, before bacterial inoculation.

To address whether splenocytes from sick rGAPDH-vaccinated progenitors had altered cytokine production, splenic cells from uninfected, Sham- or rGAPDH-vaccinated females were removed, and *ex vivo* stimulated with GAPDH (from GBS and mouse), LPS, concanavalin A, or GBS. The obtained results showed high levels of IFN-γ, and a tendency towards higher levels of IL-17A in sick rGAPDH-vaccinated females upon GBS stimulation. The relative expression of IL-17A and IFN-γ was also evaluated locally, in the uterus by RT-qPCR. A significant downregulation of IFN-γ was observed in rGAPDH-vaccinated females, when compared with the Sham-vaccinated group. Moreover, and a tendency towards overexpression of IL-17A was found in the sick progenitors, when compared with the sham-vaccinated ones.

These results prompted us to study the role of IFN- γ and IL-17 for GBS vaginal colonisation in vaccinated animals, in non-pregnant females. However, in non-pregnant females, persistent GBS infection leads to a higher production of IFN-γ by $CD4^+$ T cells on the FRT of rGAPDH-vaccinated animals, when compared with the Sham-vaccinated group. Therefore, IFN-γ might be important for GBS clearance, but its local downregulation in the context of pregnancy could be leading to GBS systemic dissemination and consequently heightened inflammatory responses with high production of IFN-γ.

These results emphasize the utmost importance of the use of the correct animal model in a preclinical testing setting, so that less innovative therapies fail upon entering early stages of clinical trials.

Keywords

GBS, vaccine, GAPDH, maternal immunopathology, IFN-γ, IL-17.

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Abbreviations List

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ACOG - American College of Obstetricians and Gynecologists

APP – American Academy of Pediatrics

AV – Aerobic Vaginitis

B

BBB – Blood Brain Barrier

BSA – Bovine Serum Albumin

BV – Bacterial Vaginosis

C

CC – Clonal Complex

CD - Cluster of Differentiation

CDC - Centers for Disease Control and Prevention

cDNA - Complementary DNA

CFU – Colony-forming units

CNS – Central Nervous System

CPS – Capsular polysaccharide

CST - Community state type

CVF – Cervical vaginal fluid

D

DAP- Diaminopimelic acid

DGAV – Direção Geral de Alimentação e Veterinária

DPBS - Dulbecco's Phosphate-Buffered Saline

E

E. coli - *Escherichia coli*

EDTA - Ethylenediamine tetra acetic acid

ECM – Extracellular matrix

ELISA – Enzyme-Linked Immunosorbent Assay

EOD – Early-onset disease

EMMPRIN - Extracellular matrix metalloproteinase inducer

F

FcRn – Neonatal Fc receptor

FRT – Female reproductive tract

G

G – Gestational day

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GBS – Group B *Streptococcus*

H

H&E - Haematoxylin and eosin

HvgA - Hypervirulent GBS adhesin

h – hour

I

i.vag – intra-vaginally

IAP - *Intrapartum* antibiotic prophylaxis

IFN – Interferon

Ig – Immunoglobulin

IL - Interleukin

J

K

L

LOD – Late-onset disease

Ly6G - Lymphocyte antigen 6 complex locus G6D

M

MAPK - Mitogen-activated protein kinase

min – minutes

MLST – Multilocus sequence typing

MMP-8 - Matrix metalloproteinase-8

N

NETs - Neutrophil extracellular traps

NF-kB - Nuclear factor-κB

NGAL – Neutrophil gelatinase-associated lipocalin

NK – Natural Killer cell

NOD - Nucleotide-binding oligomerization domain

NLRs - NOD-like receptors

O

P

PBS – Phosphate-buffered saline

PI – Pilus Island

Plg - Plasminogen

PND – Postnatal day

PRRs - Pattern recognition receptors

Q

R

rGAPDH – Recombinant GAPDH

RNA - Ribonucleic acid

ROS – Reactive oxygen species

RT – Room Temperature

S

s.c – subcutaneously

SLPI - Secretory leucocyte protease inhibitor

Srr - Serine-rich-repeats

ST – Sequence type

T

Th – T helper cell

TH – Todd-Hweitt

TLR - Toll-like receptors

U

UK – United Kingdom

ULOD – Ultra-late onset disease

V

VIP - Virulence-immunomodulatory protein

W

X

Y

Z

Introduction

Group B *Streptococcus*

Group B *Streptococcus* (GBS), a common designation for *Streptococcus agalactiae*, is a β-haemolytic, encapsulated, Gram-positive bacterium that colonizes the gastrointestinal and genitourinary tract of more than 50% of the healthy adult population [1]. Despite being considered a commensal in adults, this bacterium is the main cause of mortality and morbidity amongst newborns [2, 3]. Moreover, it can also negatively affect pregnant women, the elderly [1], diabetics and immune-compromised adults [3]. It is estimated that nearly 20% of pregnant women worldwide present colonization in their vaginal mucosa [2-5], and 40-75% of their born children present GBS colonization [3, 4]. Risk factors for neonatal colonization include heavy maternal colonization, lack of *intrapartum* antibiotic chemoprophylaxis (IAP) during delivery, intrapartum fever and African ethnicity [3]. Incidence of GBS disease amongst newborns ranges between 0.49-0.53 per 1000 live births and the mortality rate ranges between 8.4-9.6% [6, 7]. The high mortality rate observed in newborns can be explained by the complications derived from GBS infection since it can lead to the development of bacteraemia, sepsis, pneumonia, encephalopathy, and meningitis.

GBS neonatal disease can be divided into early-onset disease (EOD), when the disease arises in the first 7 days of life, or late-onset disease (LOD), when it occurs between 7 and 90 days of life [5]. EOD is typically associated with vertical transmission from the mother to the neonate, either by ascending infection *in utero* or through the aspiration of vaginal fluids during delivery [2]. LOD can also result from vertical transmission of the bacterium, but has also been associated with the horizontal transmission of GBS [5], originating from external sources like the mother through ingestion of infected human milk [8], nosocomial or the surrounding community [3]. GBS disease in infants aged over 90 days is very uncommon and is defined as ultra-late onset disease (ULOD), with data suggesting an even higher association between prematurity and development ULOD meningitis (45%) than prematurity and LOD derived meningitis (21%) [9].

Both types of neonatal GBS disease present differences regarding the case fatality rate and their specific clinical manifestations. EOD presents the highest case fatality rates (4-10%), contrasting with the 2-6% mortality rate of LOD [10]. Sepsis and pneumonia are characteristic of GBS EOD, while LOD is typically associated with

bacteraemia and meningitis, thus presenting a higher morbidity rate than EOD, of approximately 50%. Infants who survive meningitis, are at high risk of developing permanent sequelae [5, 11], such as blindness, hearing loss, seizures [9] and learning impairment [1].

Incidence of GBS maternal disease is also of great concern as there is an increased risk of GBS invasive disease during pregnancy, which could also impact on the outcome of pregnancy. The incidence of GBS invasive disease in pregnant women is 0.38 cases per 1000 pregnancies, the double when compared to nonpregnant woman, and presents a case fatality of 0.2% [5, 12]. The majority of these cases are detected during delivery (66.7%), however, some are also identified during the postpartum period (32.5%) [12]. Unlike neonatal GBS disease, maternal disease is mostly associated with preterm birth, stillbirth, and neonatal colonization, being maternal sepsis quite rare [2]. Despite a diminish in maternal deaths caused by sepsis by the 1980s (4.6%), it has been re-emerging as a prominent cause of maternal death in the United Kingdom (UK), being responsible for almost 25% of deaths between 2009 and 2012, becoming the second most common cause of death among pregnant women [12]. This tendency, of an increase in incidence and severity of maternal sepsis, has also been registered in Europe and in the United States [13]. Several factors can be accounted for this increase, such as modifications in the virulence of organisms, changes in maternal risk factors for sepsis, and clinical signs of sepsis can be hidden by the physiological changes that happen during pregnancy [12].

GBS infection can also give rise to postpartum maternal disease in the form of asymptomatic and symptomatic urinary tract infections, pyelonephritis, bacteraemia or sepsis [14]. According to the Centers for Disease Control and Prevention (CDC), about 2% of pregnant women in the united states who contracted invasive group B streptococcal infections developed GBS pneumonia [15]. GBS derived meningitis during pregnancy or in the postpartum period is rare but can also occur [16, 17]. A case of postpartum endocarditis of the tricuspid valve and mitral valve due to GBS infection has also been reported [18, 19].

GBS Classification and Virulence Factors

GBS was first isolated from other Streptococci in 1887 by two French veterinarians, from cows with mastitis, being later designated as *Streptococcus agalactiae, agalactiae* meaning “no milk [20, 21]. Thereafter, in the 1930s Rebecca

Lancefield studied haemolytic streptococci, and developed a grouping classification that distinguishes *Streptococcus* species according to antigenic carbohydrates present on the cell wall [22, 23]. In this classification *S. agalactiae* is the only species belonging to the serogroup B [24]. This has led to a synonymous use of the term “Group B Streptococcus”.

Several additional methods can be used to classify GBS isolates. Serotyping based on the sialic acid-rich capsular polysaccharide (CPS) is commonly used for epidemiological purposes [25]. CPS is an important virulence factor that contributes to GBS persistence and survival within the host, through immune evasion and interference with the host’s immune defences [1, 25-27]. Thus far, ten variants of CPS have been described (Ia, Ib, III, IV, V, VI, VII, VIII and IX) , with CPS IX being the most recently proposed serotype [3, 26, 28]. This type of classification is highly valuable to understand the worldwide and regional distribution of each serotype, as well as its prevalence in specific risk groups, such as pregnant women and newborns. Serotypes I-V have been demonstrated to be the causative agents of 97% of invasive GBS cases and serotype III to be responsible for almost half the EOD (43%) and 73% of LOD cases in newborns [5].

Another form of GBS classification is according to its sequence type (ST). STs are obtained using multilocus sequence typing (MLST), a technique that involves sequencing of internal fragments of seven housekeeping genes. Variations among these genes lead to different sequence types (STs), which can then be clustered into clonal complexes (CCs) if they share six or seven matching alleles [25, 26]. Most human GBS isolates belong to ST-1, ST-17, ST-19 or ST-23 [3]. Capsular serotypes are not strictly associated with a specific ST [27], as different serotypes can belong to the same ST. However, there is a correlation between CPS III and ST-17 [27], CPS Ib and ST-12 [26, 29]. CC17 strains mostly belonging to serotype III are responsible for most neonatal invasive infections and are strongly associated with meningitis [26], accounting for more than 80% of GBS LOD cases [25].

Virulence factors allow bacteria to persist more efficiently inside a host and thrive in harsh environments, like the vagina. Many of these factors are involved in adherence and invasion of host epithelial cells but can also be implicated in immune evasion and immunosuppression [30]. The first step necessary for GBS colonization is successful adhesion to the host epithelial cells, through adhesion factors that are present on the bacterial cell surface [31]. These factors interact with proteins present in the extracellular matrix (ECM) and/or the cellular membrane of epithelial cells, thus resulting in biofilm formation [31]. Additionally, they can also contribute to invasion through modulation of

the epithelial cytoskeleton or disruption of the epithelial cell layer [31]. Some adhesins from GBS that have shown to mediate adherence and/or invasion to human endothelial cells are the group B streptococcal C5a peptidase (ScpB), GBS immunogenic bacterial adhesin (BibA) and the streptococcal fibronectin-binding protein A (SfbA) [25]. SfbA has been shown to be important for GBS establishment in the vagina as it contributes to invasion of both vaginal and cervical epithelial cells [32]. Pili are surface-protruding structures anchored in the cell wall of GBS that provide adhesion to vaginal cells [33], aid in *in vitro* biofilm formation [34] and are involved in the invasion of the central nervous system (CNS) of newborns by promoting crossing of the blood-brain barrier (BBB) [25]. Three pilus islands (PI) named PI-1, PI-2a and PI-2b were identified in GBS strains, with PI-2b described to be essential for GBS virulence in neonatal mice [35] and was shown to be conserved in ST-17 strains [36]. Its haemolytic activity due to the presence of the virulence factor β-hemolysin/cytolysin (β-H/C) is also a contributing factor in GBS infection and immune evasion as strains with increased haemolytic expression display increased GBS invasion [31] of the amniotic cavity, placenta [37] uterine and fetal tissues during gestation [30]. Additionally, this pigment has been shown to promote bacterial dissemination and invasion across the epithelial and endothelial layers of the BBB [38].

With the availability of whole genome-sequencing, two virulence factors restricted to the hypervirulent GBS strain ST-17 were identified. The surface-anchored hypervirulent GBS adhesin (HvgA) and the allele Serine-rich-repeats (Srr) 2, are LPXTG-surface proteins anchored to the bacterial cell wall by sortase A and both mediate GBS adherence to various epithelial cells [39] through the binding of host fibrinogen [31]. HvgA has been shown to lead to increased GBS intestinal colonisation and be important for crossing of both the intestinal barrier and BBB, through the vascular endothelium and the choroid plexus respectively [31], contributing to the development of meningitis as observed in an animal mice model [39, 40]. Srr2 also contributes to enhanced GBS virulence in mice [41] through a high-affinity interaction between this glycoprotein and fibrinogen [42], contributing to colonization the of the vaginal tract [33].

GAPDH as a virulence factor

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an enzyme involved in the 6th step of glycolysis, converting glyceraldehyde-3-phosphate to D-glycerate 1,3-bisphosphate. This enzyme is considered a moonlighting protein as it can be involved in different functions beyond its role in glycolysis, such as cell signalling, control of gene expression, interaction with other proteins and also a microbial virulence factor [43].

Acquirement of this functional diversity is related to the existence of multiple binding sites, the location of the protein, complex formation, oligomerization, chemical modifications, among others [44].

Our group identified GBS GAPDH as a novel virulence factor [45]. Additional research showed that when present at the cell surface, GBS GAPDH can bind to human plasminogen (Plg), and be converted into plasmin by host-derived activators [46]. This mechanism contributes to GBS infectiveness as plasmin is a proteolytic enzyme that mediates the activation of metalloproteases and collagenases and the degradation of fibrin and other extracellular matrix proteins such as fibronectin [46]. Highjack of this system by GBS leads to an enhanced ability to cross the BBB and consequently the development of meningitis [47].

GBS GAPDH is also considered a virulence-immunomodulatory protein (VIP) due to its ability to induce the production of the immunosuppressive cytokine interleukin (IL)-10 in both adult and neonatal mice [45, 48]. Studies have shown that production of IL-10 during early stages of infection, inhibits the development of an effective pro-inflammatory response and neutrophil trafficking to infected sites, thus contributing to pathogen immune evasion [48, 49]. Maternal vaccination with a recombinant GAPDH (rGAPDH) was shown to abrogate IL-10 production upon GBS infection and therefore confer immunity against GBS infection in neonatal mice [48]. Additional assays showed that rGAPDH did not present cross-reactivity against both mouse and human GAPDH, and it was safe and stable in toxicological (pre-clinical) studies performed in adult mice [50].

GBS Prevention and Therapeutics

Currently, the only preventive measure against GBS invasive disease in neonates and parturients is the administration IAP prior to delivery [26]. In 1996 the first consensus guidelines for the prevention of perinatal Group B Streptococcal disease were published by the Centers for Disease Control and Prevention (CDC) in collaboration with the American Academy of Pediatrics (APP), the American College of Obstetricians and Gynecologists (ACOG), and other organisations [25, 51, 52]. These guidelines have been updated in 2002 and 2010. Mostly recently, these guidelines are being gradually updated in regards to detection and identification of GBS strains [53], prevention of EOD in newborns [54] and management of infants at high risk for GBS disease [51].

According to the given guidelines, pregnant women who present an antepartum positive rectovaginal culture, performed between the 35th and 37th weeks of gestation,

alongside with women who present additional risk factors for GBS transmission to the newborns, are given IAP 4 hours before delivery in order to prevent GBS disease transmission to the progeny [55, 56]. Some countries such as the UK and the Netherlands use only risk-based factors to determine the need for IAP [55]. These risk factors include a previous infant with GBS disease, preterm labour (<37 weeks), premature or prolonged rupture of membranes and maternal fever (temperature >38ºC) [5, 52]. Beta-lactam antibiotics such as penicillin and ampicillin [51] are the most used for GBS IAP, but if the parturient has a history of penicillin allergy antibiotics such as cefazolin, vancomycin and clindamycin can also be used [57].

Adoption of such measures has resulted in a significant decrease of EOD rates [26], from 1.7 per 1000 live births in the 1990s [52] to between 0.23 and 0.37 per 1000 live births in 2019 in the United States [56]. However, LOD rates did not change with this practice, continuing to be the main cause for neonatal mortality and morbidity [25]. Reports estimate that more than 40% of pregnant women in the United States receive antibiotics 4 hours before delivery [58]. These strategies used to lower the incidence of GBS disease in newborns and postpartum infections after caesarean section [58], raise a concern regarding the overuse of antibiotics and early exposure of the fetus to antibiotics while in utero. Overuse of beta-lactam antibiotics lead to GBS strains with increased resistance to penicillin, erythromycin, clindamycin and vancomycin [5, 51]. Lack of data on long-term effects of IAP administration raises other concerns regarding the acquirement of healthy microbiota in the newborns [26], both passed by the mother or naturally after birth [59]. Reports show that infants up to 3 months of age exposed to IAP have lower bacterial diversity and consequently less beneficial commensals on their intestinal microbiota when compared to infants born from mothers that did not receive IAP [60]. Moreover, such changes at an early age can have an impact on the development of several systems, including the immune, the endocrine, the metabolic and the central nervous system [61]. It has been shown that alterations in the gut microbiota can lead to long term health problems such as asthma, enteric dysbiosis, allergies, diabetes and inflammatory bowel disease [57, 58, 62, 63].

Furthermore, therapeutics for maternal disease are the same used for the prevention of GBS disease in newborns born from GBS colonized mothers, in which the antibiotics aforementioned are administered 4 hours prior to delivery [14]. IAP administration lead to a 21% reduction of invasive GBS disease among pregnant women [64]. Although IAP lead to a successful reduction of EOD cases and GBS induced maternal disease, an alternative strategy to prevent LOD in newborns and avoid the use of antibiotics is urgently needed.

Maternal Vaccination against GBS

Maternal vaccination has been proposed to be the best alternative to prevent both forms of GBS disease in newborns, while avoiding the emergence of novel GBS antibiotic resistant strains. This approach relies on the basis that IgG can cross the placenta via the neonatal fragment crystallizable (Fc) receptor (FcRn), from 13 weeks of gestation, with an exponential increase during the third trimester, and also after birth via breastmilk [65]. Due to the long half-life of IgG antibodies, passive immunity arises as an opportunity to deliver protection to the infant during the first 3 months of life, the period of highest susceptibility to disease [28]. This approach to reduced neonatal disease, has been further sustained by data from countries which have achieved successful reductions in neonatal cases of tetanus and pertussis as a result of maternal vaccination against these diseases [65]. Regarding GBS, it has been estimated that a successful vaccine with 80% efficacy and 90% coverage would be able to prevent 107,000 stillbirths and infant deaths [65].

Design of a vaccine able of preventing GBS infection dates to the 1930s when Rebeca Lancefield demonstrated that CPS-specific antiserum could provide immunity to mice [25, 66]. In 1976, Baker and Kasper established an association between low levels of maternal antibodies against serotype-specific CPS and the occurrence of GBS infection in newborns as well as the occurrence transplacental transference of these antibodies in healthy newborns [26, 67]. Since then, several vaccines against GBS have been developed, however, none have been approved for human use yet and are still under trial [26].

Several vaccines that use CPS as an antigen coupled to an immunogenic carrier have been developed. Monovalent CPS-conjugate vaccines have undergone phase I and II clinical trials and demonstrated to be safe and immunogenic in healthy women [25]. However, multivalent CPS-conjugate vaccines are a better option already in development as they have a wider coverage of serotypes and avoid possible capsular switching [25]. One trivalent CPS-conjugate (Ia, Ib, III) by Novartis/GSK is in consideration for phase III clinical trials [25, 68], and a pentavalent vaccine (Ia, Ib, II, III and V) that would cover more than 90% of the serotypes responsible for invasive neonatal disease, started phase I clinical trials in healthy volunteers [26, 65, 69]. Although promising, these CPS based vaccines do not cover uncommon serotypes, non-typable GBS strains [48] and have the potential for future serotype replacement in the treated populations and capsular switching [26], as previously observed in a vaccine against *Streptococcus pneumoniae* [30].

Vaccines using surface proteins are currently the alternative to CPS based vaccines, as these proteins can be found on a larger variety of GBS strains. MinervaX completed phase I trials for a vaccine consisting of the N-terminal domains of the surface proteins alpha-C and Rib [68-70], as studies have shown an association between low levels of antibodies against these proteins and invasive neonatal disease [26, 71]. Other possible vaccine candidates using surface proteins that are still on preclinical studies include the surface immunogenic protein (SIP) [72], leucine-rich repeat protein LrrG [73], ScpB [74] and Lmb [25, 75].

The optimum vaccine should be easy to implement in low income and developing countries, where screening isn't easily implemented. Also, it should be directed to structurally conserved antigens that are either essential for survival and/or virulence of the organism, to avoid selection of mutations or escape of immune recognition by the host [50].

Our group developed a maternal vaccine against GBS targeting its glycolytic enzyme GAPDH. This vaccine was shown to be effective on a mouse model in which pups were born from mothers actively immunized with the recombinant protein (rGAPDH), or mothers passively immunized with anti-rGAPDH IgGs or $F(ab')_2$ fragments [48]. Protection was achieved through lowering of IL-10 levels and restoration of neutrophil trafficking to infection sites, which consequently lead to higher survival rates and less organ colonization in pups born from rGAPDH-vaccinated mothers when compared to the control groups [48]. Despite the obtained results showing to be very promising, subcutaneous and intraperitoneally infection routes were used, which do not mimic the natural course of infection in humans [48], hence the need for a more reliable model to study GBS disease and test this vaccine in newborns.

Novel mouse model of GBS pathogenesis

Animal models are key contributors for developments made in biomedical research *i.e* discovery of important biochemical and physiological processes, translational research of candidate drugs and elucidation of disease mechanisms [76]. According to a report published by the European Union regarding animal use for scientific research, 9.39 million animals were used in 2017, 81% being mice [77]. Mice present several advantages to other mammals specifically it's easy handling, small size, docile behaviour, breeding speed and yield [76]. Furthermore, mouse models are more exact

than *in vitro* models, as the last fails to replicate the complexity of cross-interactions that occur between the complex systems of mammals [78].

Despite wide usage of the mouse as a model to study human infectious diseases, it's capability to closely reproduce human disease has been put into question as mouse and human present slight differences in their immune responses to certain stimuli [79, 80]. Additionally, lack of proper experimental design, failure to accurately represent the human disease [81] and poor reporting in peer-reviewed literature also contribute to its translational limitations as roughly 85% of early clinical trials fail after success in preclinical testing [76, 82].

Animal models to study GBS disease have been developed in mice, rat, rabbit, pig, drosophila, rhesus monkey and zebrafish and all have provided important knowledge [83-87]. However, these are either difficult to translate into clinic or focus on specific steps of the pathophysiology of the disease (organ colonization, septicaemia, meningitis) [83-87]. Notably, the use of non-natural routes of infection, such as intraperitoneal, subcutaneous, intracerebral or intra-cerebrospinal fluid injection of GBS [50, 86, 87], that disregard mother-to-newborn transmission and the normal course of the disease in the newborn (bacteraemia-meningitis sequence), can lead to misleading information. This highlights the importance of a careful choice of the animal model, inoculation route of administration and bacterial strain to be used in all studies.

In order to better recapitulate the pathophysiology of GBS disease, our group developed a novel mouse model which allows vertical transmission of the bacterium from mother to child during gestation and/or vaginal delivery. This model focus on intravaginally inoculating pregnant females on two gestational days (G), 17 and 18, with a hypervirulent strain BM110, which belongs to serotype III and the clonal complex CC-17 [88]. With this model we are able to overcome limitations present in other models used to study the disease. We verified that using this model neonates presented colonization in the liver, lungs, gut and brain, and those who didn't succumb to GBS infection experienced long-term neurological sequelae [88].

Preliminary results from our group using this model to assess maternal rGAPDH vaccination, showed that pups born from vaccinated mothers were not protected [89]. Moreover, 50% of vaccinated mothers developed disease after delivery and died, when vaginally colonized during pregnancy [89].

Vaginal mucosal immunity and microbiome

The vaginal mucosa, which comprises the ectocervix and the vagina, is constituted by a stratified squamous non-keratinized epithelium overlaid by a mucosal layer continuously lubricated by cervicovaginal fluid (CVF) [90]. CVF is an acidic media that is able to confine exogenous molecules and organisms and contains antimicrobial molecules such as antibodies (IgA and IgG), β-defensins, mucins, neutrophil gelatinase-associated lipocalin (NGAL), secretory leucocyte protease inhibitor (SLPI), among others [91]. This system is the first line of immunological and physical defence against potential pathogens, preventing them from contacting subsequent layers of the vaginal tissue [90, 91].

Immune cells from both the innate and adaptive immunity are also present in the vagina and cervix distributed throughout the lamina propria, such as neutrophils, macrophages, classic dendritic cells (DCs), Langerhans cells, natural killer (NK) cells, B and T lymphocytes [90, 92]. Trafficking and activation of immune cells are mediated by pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and NOD-like receptors (NLRs), and is also regulated by endocrine signalling [93, 94]. Recognition of microbial ligands through PRRs leads to a signalling cascade that results in the release of chemokines and cytokines, which are activated and regulated via a nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK) signalling pathway, resulting in the development of an immune response. These inflammatory responses can result in clearing of infection but in some cases, it can disrupt the mucosal surface and promote other infections [91, 95].

Another key component of the vaginal ecosystem is its natural microbiota that exists in a regulated mutualistic relationship with the host which results in enhanced immunity through the production of specific gene products and competition with pathogenic organisms [91]. The dominant genus present in the vaginal microbiota is *Lactobacillus*, specifically *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*, although it can be also colonized by other anaerobes such as *Gardnerella*, *Atopobium*, *Mobiluncus*, *Megasphoera, Prevotella*, *Streptococcus*, *Mycoplasma*, *Ureaplasma*, *Dialister*, *Bacteroides*, etc [96, 97]. Different vaginal microbiotas can be classified into community state types (CSTs), with CST-I, -II, -III and -V dominated by *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*, respectively, while CST-IV is comprised by mixed microbial communities with prevalence of anaerobic species [91, 98]. Species present in CST-IV have the ability to cause infection when the percentage of Lactobacilli is low and are associated with vaginal dysbiosis that can manifest as bacterial vaginosis (BV) [90, 99]

or aerobic vaginitis (AV) [91]. Both conditions arise from a decrease in *Lactobacillus* dominance leading to a decreased lactic acid concentration and a higher vaginal pH [100-102]. Different race/ethnic groups and biogeographical locations can present more predisposition to be colonized with anaerobes belonging to CST-IV, such as Hispanic and Black women, and present a vaginal higher pH in the presence or absence of clinical infection [97]. Increased microbial diversity in the vagina due to lost Lactobacillus dominance leads to changes in the immune and epithelial homeostasis, which is associated with negative reproductive outcomes, such as pregnancy loss and preterm delivery, and increased susceptibility to infectious disease [99]. Additionally, vaginal microbial communities dominated by non-lactobacilli, apart from *L. inners*, are associated with greater pro-inflammatory responses [91, 96].

Maintenance of high levels of *Lactobacillus* is seen as a marker of good vaginal health [96] as the presence of these commensals as part of the vaginal microbiota is itself a mechanism of protection to the host given that these organisms compete for nutrients and adhere to vaginal epithelial cells, thus haltering growth, and adhesion of other microbes with invasive potential [99, 103]. Lactobacilli (apart from *L. inners*) produce antimicrobial peptides such as hydrogen peroxide (H_2O_2), inhibiting the growth of catalase-negative anaerobes [104], bacteriocins, bacteriocin-like substances and biosurfactants [105]. Furthermore, Lactobacilli modulate the local immune response [95] and promote autophagy facilitating the elimination of virus, bacteria, or protozoa on infected cells [106, 107]. The normal acidic pH (3.5-4.5) observed during a woman's reproductive age is driven by the levels of estrogen, glycogen and Lactobacilli [101, 108, 109].

It has been shown that certain Lactobacilli can impair GBS from adhering to vaginal epithelial cells [110, 111] and *in vitro* studies have demonstrated antimicrobial activity of these commensals against GBS [112] as well as reduction of colonization *in vivo* [113, 114]. Recent studies described a relative reduction of Lactobacilli populations in GBS-positive women, whereas others failed to observe such changes [103, 115, 116], therefore more research in how the vaginal commensal population can affect GBS colonization is needed.

Hormonal changes in a women's life, such as puberty and menopause, can lead to modifications in one's vaginal microbiome. During pregnancy, the vaginal microbiota is predominantly composed by Lactobacilli and gets increasingly more stable as pregnancy progresses [117-120] due to heightened estrogen-stimulated glycogen deposition and subsequent breakdown into lactic acid [91]. Besides the protective roles

of D-lactic acid mentioned above, it is especially important during pregnancy as it inhibits the synthesis of the enzyme matrix metalloproteinase-8 (MMP-8) by modulating the action of extracellular matrix metalloproteinase inducer (EMMPRIN; CD147) produced by vaginal epithelial cells [107]. This results in a decreased possibility of an ascending intrauterine infection as it prevents cervical tissue digestion. A study performed by Scholl et. al that compared vaginal fluids between GBS-positive and GBS-negative pregnant women, revealed that GBS-positive women presented higher concentrations of EMMPRIN and MMP-8, and as mentioned above, both crucial for the disruption of the integrity of the cervix and, thereby, increasing the possibility of GBS infection to the upper genital tract [121]. Thus, a dysbiosis during pregnancy can lead to serious implications to maternal and especially neonatal health, as the first lines of defence against opportunistic pathogens are impaired.

Vaginal immune response to GBS

GBS colonization on the vaginal tract can either be asymptomatic or lead to invasive disease [96] resulting in inflammation, leukocyte and parabasal cell infiltration [91]. Animals models of GBS disease demonstrated that vaginal immune responses to GBS are mostly mediated by neutrophils, mast cells and macrophages [30]. Vagina-resident mast cells are activated upon vaginal GBS colonization through the haemolytic activity of GBS, leading to degranulation and release of inflammatory mediators (IL-6, IL-8, TNF-α, and histamine), thus resulting in the recruitment of neutrophils and macrophages to aid in GBS clearance [122]. Neutrophils participate in GBS clearance through the formation of neutrophil extracellular traps (NETs) and phagocytosis [123]. Similarly, to neutrophils, macrophages aid in GBS clearance through phagocytosis. In response to phagocytosis, GBS activates the NLRP3 inflammasome, in both neutrophils [124] and macrophages [37], through membrane permeabilization by pigment, resulting in secretion of IL-1β and other pro-inflammatory cytokines by neutrophils. Its hemolytic pigment can prevent GBS phagocytic death through sequestration of reactive oxygen species (ROS), resulting in pyroptosis of GBS infected cells [124] and avoidance of killing by NETs [125]. The role of NK cells and dendritic cells in GBS colonization is not known [30].

Studies have shown that IL-17 and IL-17^{+} cells are important for the elimination of hyper adherent and invasive GBS strains from the vaginal epithelia [126], suggesting that the Th17 pathway is important for riddance of GBS colonization [30]. Moreover, IL-23, an important cytokine for maintenance and expansion of the T helper (Th) 17 (Th17)

subset, has been identified as important for the reduction of GBS vaginal colonization [126]. The mucosal T-cell response to GBS colonization is not well understood, as cytokines involved in Th1, Th2 and Th17 pathways have been identified as important for GBS clearance, but T cells were not directly classified as important [123]. Although, one study demonstrated that B cell-deficient mice vaginally inoculated with GBS took longer to eliminate GBS colonization in comparison to wild-type mice, the mucosal humoral response to GBS is poorly understood [127].

In the light of our preliminary results with the GAPDH vaccine and maternal inoculation with GBS, we must confirm the inefficacy of a GAPDH-based vaccine to prevent GBS disease in newborns and understand the mechanisms by which GAPDH-vaccinated mothers colonized with GBS develop lethal pathology after delivery. Moreover, to better understand the possibility of a dysregulated vaginal immune response against GBS during perinatal period, it is crucial that a more thorough analysis is made, to identify all the intervening parts and how each one contributes to GBS clearance or disease.

Objectives

Previous results obtained by our group demonstrated that when rGAPDH vaccine was tested in a mouse model that reproduces the pathophysiology of neonatal group B streptococcal infection it did not confer protection and nearly 50% of the vaccinated progenitors developed disease after birth [89]. These results led us to hypothesize that the disease observed in females intravaginally colonized with GBS is linked to a proinflammatory immune cell shift with high systemic production of IFN-γ caused by rGAPDH vaccine.

Therefore, the goal of this project is to test our hypothesis in order to uncover the mechanisms behind the immunopathology and mortality observed in females vaccinated with rGAPDH and intravaginally colonized with GBS.

To accomplish this, the following tasks were performed:

1. To confirm that the vaccine does not confer protection to neonates;
2. To do a histopathology evaluation of rGAPDH-vaccinated mothers;
3. To determine if antibodies are contributing to maternal disease;
4. To characterize the vaginal immune response to GBS colonisation;
5. To determine if IFN-γ has a pivotal role on the development of maternal disease.

This study will elucidate why GBS goes from an asymptomatic colonizer to lethal pathogen in mothers immunized with rGAPDH and if IFN-γ is a contributor to this shift.

Materials and Methods

1. Animals and Ethics Statement

Six weeks old female and male BALB/c mice were purchased from Charles River Laboratories and kept at ICBAS animal facilities, under a 12 hour (h) cycle of light/dark, with access to food and water *ad libidium*. Animal experiments were approved by the national authority *Direção Geral de Alimentação e Veterinária* (DGAV), and by the ICBAS Animal Ethical Committee (Nº 113/2015). The study was conducted in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123), the Directive 2010/63/EU and the Portuguese rules (DL 113/2013). Researchers working directly with animals are officially accredited to do animal experimentation. All efforts possible were made to minimize animal suffering and also the number of animals used throughout all experiments.

2. Bacterial strains and growth conditions

For the following work, the GBS strain BM110, belonging to the capsular type III and the hypervirulent clonal complex 17 (CC-17) was used [88]. The strain was kindly given by Prof. Patrick Trieu-Cuot from the Institut Pasteur. The bacterium was cultured in Todd-Hewitt (TH) broth or agar (Difco Laboratories) containing 5 µg/mL of colistin sulphate and 0.5 µg/mL of oxalinic acid (Streptococcus Selective Supplement, Oxoid), at 37ºC.

3. Active Immunization

Female BALB/c mice were randomly distributed into two groups and were injected subcutaneously (s.c) three times, with a 3-week interval between each immunization. Each immunization was performed with 100 µL of a preparation containing 25 µg of rGAPDH in a 1:40 phosphate-buffered saline (PBS) / Alhydrogel suspension (Aluminium hydroxide Gel) (rGAPDH-vaccinated group). The Sham-immunized group received 100 µL of 1:40 PBS / Alhydrogel suspension. Seven days after last injection, peripheral blood was collected, and serum levels of anti-rGAPDH IgG antibodies were determined by Enzyme-Linked Immunosorbent Assay (ELISA). Upon confirmation of high levels of anti-rGAPDH IgG antibodies in the vaccinated group, female mice were mated.

4. Passive Immunization

Female BALB/c mice were passively immunized during pregnancy, 1 day prior to infection, with 100 µg of anti-rGAPDH IgG antibodies, previously isolated from sera from rGAPDH immunized animals. Control animals received 100 µg of non-specific IgG antibodies.

5. Antibody Purification

To obtain anti-rGAPDH or control IgG antibodies, sera from both rGAPDH-vaccinated and non-vaccinated progenitors were pooled and applied on a Protein G HP affinity column (HiTrap, GE Healthcare Bio-Sciences AB), according to the manufacturer's instructions. The obtained product was dialyzed against NaCl 0.9% (Braun), concentrated using an Amicon Ultra Centrifugal Filter (Merck), and quantified using a Pierce™ BCA Protein Assay Kit (Thermo Scientific). Bovine serum albumin (BSA) was used as a standard (0.01–0.5 mg mL^{-1}). 10 µL of the samples and BSA standards were loaded into wells in duplicate and 200 µL of reagent diluent was added into each well. After a 30 minute (min) incubation at 37ºC, the plate was read at 562 nm using a Thermo Multiskan Ex Spectrometer.

6. Pregnancy monitorization

Two to three females were matted and examined daily for the presence of a vaginal plug. Observation of the vaginal plug was set as the first day of gestation (G1), and when observed, the female was separated from the other mice and housed individually until delivery.

7. Vertical model of GBS infection

The GBS strain BM110 was pre-inoculated in 10 mL of TH Broth and incubated overnight at 37ºC. In the following morning, 250 µL of the pre-inoculum was transferred into 25 mL of warm TH Broth (1:100 dilution). The bacterial culture was grown at 37ºC for approximately 3 hours (h) until it reached mid-log phase (OD_{600} ~ 0.800). The culture was washed twice with sterile PBS to discard growing media, and its concentration was set to an OD600nm of 0.450, using a Jenway 6300 Spectrophotometer, in order to obtain a culture with a known concentration.

Pregnant BALB/c mice were intra-vaginally (i.vag) inoculated with $8x10^4$ CFU of GBS BM110 in a 40 µL volume with a micropipette, on gestational days 17 and 18. The day of the delivery was defined as postnatal day (PND) 0, pregnant mice were allowed

to delivery spontaneously and the pups were kept with the mothers during the whole protocol.

Vaginal lavages to access GBS vaginal colonization were performed on days 1 and 5 after delivery. These were performed by vaginally introducing 50 µL of sterile PBS using a micropipette, and this method was repeated 10 times with up and down movements for each 50 µL, prefacing a total of 150 µL. To count GBS colonisation, vaginal lavages were serially diluted in sterile PBS, posteriorly plated onto selective medium (*CHROMagar, StrepB agar*) and incubated at 37ºC overnight.

Survival curves of neonates were determined during 21 days, for actively immunized mothers during 30 days, and both passively immunized mothers and pups during 7 days. To access organ bacterial colonisation by GBS, both in pups and progenitors, organs were aseptically removed at the defined timepoints and posteriorly homogenized in PBS. Serial dilutions of each organ were prepared and plated for CFU counts.

The female reproductive tract (FRT) of infected mice was collected for different experiments: histological analysis, CFU assessment, RNA cytokine quantification and local cytokine production. The regions collected for each analysis were the same for all animals and the scheme followed is represented in Figure 1. Additionally, the same region was used to determine both CFU and quantify cytokines.

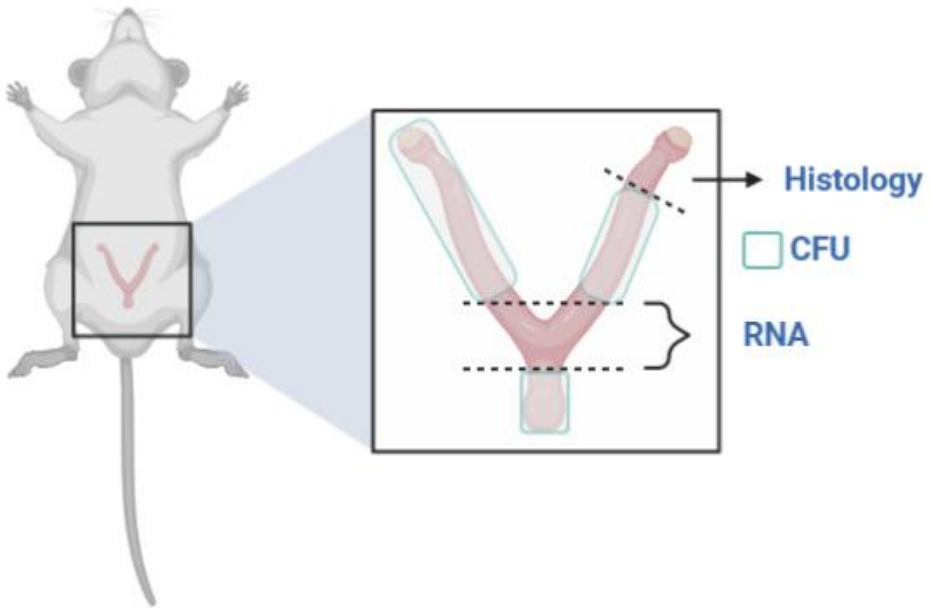

Figure 1. Scheme of FRT sections collected for histology, CFU count, local cytokine quantification and RNA cytokine analysis.

8. Histopathology

The lungs, liver, kidney, heart, reproductive tract and placentas were collected on day 5 after delivery, fixed in 10% buffered formalin, routinely processed and embedded in paraffin. Sections of the reproductive tract of mice were cut according to Kittel et. al [128]. Histologic 4-5 µm-thick sections were cut for staining. Hematoxylin and eosin (H&E) and Gram staining were made according to the standard protocols. H&E and Gram images were analysed with the help of a pathologist (Augusto Faustino).

9. Lung semiquantitative scoring

Semiquantitative scoring systems are extensively used in biomedical research to incorporate histological data and usually comprise several parameters which are separately quantified [129]

The slides containing the lung tissue were scanned using the image acquisition OlyVIA software (Soft Imaging Solutions, Olympus) and analysed for atelectasis, emphysema and haemorrhage. The choice of pathologies for the histological score was made *a priori* after a preliminary comparison of lungs from all treatment groups. In a first analysis, each pathology was given a percentage it occupied in that section of the lung. This was made for all 3 pathologies analysed. To ensure consistent and accurate results, each analysis was performed 2 times for emphysema and atelectasis and 4 times for haemorrhage. Since haemorrhage results were the ones who showed less consistency between the first two analysis, two extra analysis were made for this parameter. Given the maximum and minimum percentage found in each pathology, three representative classes were created for each pathology.

	Emphysema	**Atelectasis**	**Haemorrhage**
Class 1	0-13%	0-30%	0-10%
Class 2	>13-25%	>30-59%	>10-19%
Class 3	≥26%	≥60%	≥20

Absolute values were given to each class, 1 for class 1, 2 for class 2 and 3 for class 3 which comprised the most severe cases observed. The lung scoring methodology was developed by both the student and an experience pathologist (Augusto Faustino).

10. Immunohistochemistry

Histologic 4-5 µm-thick sections were cut for immune detection. Briefly, slides were deparaffinized, hydrated in decreasing alcohol concentrations and washed in deionized water.

Antigen retrieval was performed using 10 mM Sodium Citrate buffer (Dako) in a 37ºC water bath for 25 min, and cooled thereafter for 10 min. The slides were washed 2 times for 5 min with PBS 1x, followed by endogenous peroxidase blocking with 3% hydrogen peroxide in methanol for 5 min. The same washing process as referred previously was repeated. Slides were blocked with 100 µL of 10% normal serum with 5% BSA for 30 min. Without rising, blocking was followed by incubation with the primary antibody (anti-Ly6G, Biolegend) diluted 1:1000 in 5% BSA in PBS 1x, overnight at 4ºC. The negative control did not receive the primary antibody and was incubated only with 5% BSA in PBS 1x. After this step, the negative control slide was washed separately throughout the rest of the protocol, to avoid cross contamination. The slides were rinsed 3 times for 5 min in PBS 1x and incubated for 30 min at room temperature with the secondary antibody Anti-Rat IgG diluted from the VECTASTAIN Elite® ABC Kit, Rat IgG (Marvai LifeSciences), diluted 1:200 in 5% BSA in PBS 1x. Each section was washed the same way as referred before and was incubated for 30 min with the VECTASTAIN Elite® ABC Kit, Rat IgG (Marvai LifeSciences) 1:100 of A and 1:100 of B. Another washing was carried out and the 3,3-Diaminobenzidine (DAB) (Leica Biosystems) liquid substrate was applied for 30 seconds (s). The reaction was stopped in running tap water for 10 min.

The obtained slides were counterstained with hematoxylin for 1 min and washed one more time in running tap water for additional 10 min. Slides were then dehydrated in increasing alcohol solutions (70%, 95% and 100% alcohol), incubated twice in xylene and finally mounted in Enttelan mounting medium (Merck).

11. Organ cytokine concentration

Liver and uterus from adult females were homogenized in sterile PBS and posteriorly diluted 1:2 in lysis buffer [EDTA free mix of protease inhibitors (7x) diluted in 1,5 mL of sterile H2O with Tween 20 (0,1%)] that contained an EDTA free mix of protease inhibitors (Roche) and Tween 20 at 0,1%. The obtained solution was incubated on ice for 30 min and vortexed every 10 min. Next, samples were centrifuged for 10 min, at 13000 rpm, at 4ºC to remove the pellet and fat of the organ. This step was repeat until the supernatant was clear and presented no fat.

12. Cytokine detection

ELISA was used to quantify cytokines in sera, vaginal lavages, culture supernatants and uterine tissue. The following cytokines and respective kits were used: IL-4, BioLegend; IFN-γ and IL-10, R&D Systems; IL-1β, IL-6 and IL-17A, Invitrogen. Each cytokine was assayed according to the manufacturer's instructions for each kit. Microplates were read in a Thermo Multiskan Ex Spectrometer, using a wavelength of 450 and 570 nm. GraphPad was used to interpolate cytokine values from samples.

13. Isolation of splenic cells

Spleens were removed and gently dissociated in 10 mL of RPMI, by pressing through a 70 µm nylon mesh cell strainer using a 1-mL syringe plunger, to yield a single cell suspension. The obtained cells were washed by centrifugation for 5 min, at 500 g, at 4ºC, and counted.

14. Splenic cell *in vitro* re-stimulation

For *ex vivo* cytokine production, splenocytes were collected, and $5x10^5$ cells were seeded per well in a 96 U-shaped plate. Cells were incubated with $5x10^7$ CFU of fixed GBS, 20 µg/ml of rGAPDH (GBS and mouse), 6 µg/ml of Concanavalin A (ConA), 20 ng/ml of LPS or left without stimulation with RPMI media only (negative control). Triplicates were made for each condition and cultures were kept at 37ºC with 5% of CO_2 for 72 h, except for ConA which was only cultured for 48 h. The supernatants were collected and stored at -80 ºC until analysis.

15. RNA Extraction

All fragments for RNA were carefully extracted, snap-frozen immediately and stored at -80ºC. RNA from the cervix and adjacent areas was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Additionally, all samples were weighted before the RNA extraction protocol. The RNA extraction kit used requires the sample to be mechanically homogenized in lysis buffer. Ethanol is then added to the lysate to provide ideal binding conditions of the RNA to the silica membrane in the column provided. The obtained RNA is then eluted in 30 µL of RNAse free water. RNA quality was evaluated through UV spectroscopy (NanoDrop 1000, Thermo Fisher Scientific) and samples were kept at -80 ºC until further use.

16. Real-Time qPCR

RNA was converted into complementary DNA (cDNA) through a polymerase chain reaction (PCR) on a TProfessional Thermocycler (Biometra). For this, the iScriptTM cDNA Synthesis Kit was used and the manufacturer's instructions were followed.

For the real-time PCR reaction, the SsoFast Evagreen Supermix Kit (Biorad) was used to amplify and track gene expression. The reaction was carried out in a Rotor-Gene 6000 Real-Time PCR Machine (Corbett Research) and the following parameters were used for IL-17A and β-actin: initial denaturation at 95 ºC, 40 annealing cycles of 60 ºC, with fluorescence read at the end of each cycle. The annealing temperature for IFN-γ was 55 ºC, but the number of cycles was the same.

The following primers were used: *Il17*, sense, 5'- GCTCCAGAAGGCCCTCAGA-3', and anti-sense, 5'- AGCTTTCCCTCCGCATTGA-3'; *Ifng*, sense, 5'-CCTCAAACTTGGCAATACTC-3', and anti-sense, 5'-CACACCTGATTACTACCTTCT-3'; *β-actin*, sense, 5'- AGAGGGAAATCGTGCGTGAC-3', and anti-sense, 5'-CAATAGTGATGACCTGGCCGT-3'.

The gene expression for each cytokine was determined by using the comparative Ct method ($2^{-\Delta\Delta Ct}$). This method compares the Ct value of the target gene with the Ct value of a reference gene. β-actin was used as reference gene.

17. β-estradiol experiment

Non-pregnant female BALB/c, from both Sham- or rGAPDH-vaccinated groups, were given 100 µL β-estradiol diluted 1:100 in sesame oil as previously described [126]. Two days after β-estradiol administration, females were intra-vaginally inoculated with $2x10^8$ CFU of GBS BM110 WT.

18. Isolation of leukocytes from the vaginal tissue, cervix and uterus

Vaginal tissue, cervix and uterus were collected and cut into small segments for easier digestion. Tissue was incubated in digestion solution, containing Roswell Park Memorial Institute (RPMI, Sigma Aldrich) 1640 Medium, 20 mM HEPES, 10% FBS and 2 mg/mL of collagenase D, for 2 h at 37 ºC, with agitation. During the digestion all samples were vortexed to help break down tissue and disaggregate cells. Samples were pushed through a 70 µm cell strainer with the help of a 1-mL syringe plunger to obtain a single-cell suspension, and 10 mL of RPMI media was added to wash the cell strainer.

The obtained suspension was centrifuged at 400 g during 5 min. The obtained supernatant was discarded, and the cell pellet was resuspended in 10 mL of ice-cold washing solution (ice-cold PBS with 2% FBS). Cells were centrifuged 5 min at 400 g, at 4 ºC and the supernatant was discarded. This last step was repeat one more time. Finally, the last pellet was resuspended in 200 µL of complete cell culture media and posteriorly counted.

19. Intracellular Cytokine Staining

For this protocol, $1x10^6$ of splenic or vaginal cells were stimulated with 10 ng/well PMA (phorbol. 12-myristate 13-acetate), 100 ng/well ionomycin and 2 µg/well Brefeldin A (BFA), at 37 ºC and 5% of CO_2, for 4 h and 30 min.

Surface Staining

The plate was centrifuged at 1200 rpm, for 2 min, at 4ºC and the supernatant was removed by flicking the plate.150 µL of PBS-2% FBS buffer was added to each well, and the last step, of centrifugation and supernatant removal, was repeated. 30 µL of a mix containing the surface antibodies were added to each well and the plate was incubated for 30 min on ice, protected from light. The following antibodies were used: CD3 FITC (Clone 17A2), CD45 PerCP-Cy5.50 (Clone 30-F11), CD4 Pacific Blue (Clone HK1.4), and γ/δ Pe-Cy7 (Clone GL3). After this incubation, the plate was washed two more times with PBS-2% FBS buffer as mentioned above.

Cell Fixation and Permeabilization

After the last wash, 150 µL of PBS-formaldehyde 2% was added to each well and incubated for 20 min, at room temperature. Next, the plate was centrifuged and washed with PBS-2% FBS buffer as referred above. Each well received 150 µL of Permeabilization (Perm) Buffer (Saponin 10%, PBS-2% FBS) which was left incubating for 10 min, at room temperature. The plate was centrifuged at 1200 rpm for 2 min at 4ºC and the supernatant was discarded.

Intracellular Staining

For the intracellular staining of cytokines, 50 µL of a mix containing the intracellular antibodies [IFN-γ PE (Clone XMG1.2), and IL-17A APC (Clone TC11-18H10.1) in Perm Buffer was added to each well, and incubated for 20 min in the dark, at room temperature. Next, 150 µL of Perm Buffer were added to each well and mixed gently. The plate was centrifuged as mentioned above and the supernatant was discarded. This step was repeat one more time. Finally, the plate was washed 2 times with 150 µL PBS-2% FBS

buffer and cells suspended in ice-cold FACS buffer (1% BSA, 0.01% NaN_3 in DPBS) FACS. Data were acquired on a FACSCanto II flow cytometer (BD Biosciences). Post-acquisition analysis was performed using FlowJo software v10 (Tree Star). All steps of the analysis are specified in the results section.

20. Statistical Analysis

All data obtained was analysed using GraphPad Prism software (v. 8, GraphPad software Inc. CA). Survival curves were analysed using the log-rank (Mantel-Cox) test. CFU data was log10 transformed. Normalized values were analysed by Student's *t* test where two experimental groups were compared or by one-way ANOVA three groups were analysed, together with a Sidak's Multiple Comparison Test. Lung semiquantitative scoring data are non-parametric, and non-parametric statistical analyses were used. Statistics were analysed using Kruskal-Wallis test and Dunn's multiple comparisons test. Significance was represented by the following symbols: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and n.s = not significant.

Results and Discussion

Maternal GAPDH-vaccination does not prevent neonatal GBS disease

Previous results obtained by our group showed that maternal rGAPDH-vaccination confers protection to neonates against GBS infection [48]. However, these results were obtained using an animal model that did not mimic the route of GBS infection in humans, as GBS was injected intraperitoneally or subcutaneously in newborns mice [48]. To better understand the pathophysiology of neonatal GBS disease, our group developed a novel mouse model that reproduces the route of infection as it occurs in humans [88]. Our preliminary results showed that rGAPDH-maternal vaccination did not confer protection to their offspring. We repeated the experiment increasing the total number of animals. As shown in Figure 2, rGAPDH maternal vaccination does not confer protection to newborns with rGAPDH-vaccinated group having a higher mortality percentage (approximately 74%) than the control group (approximately 50%), although not reaching statistical significance. The last death was recorded on PND8.

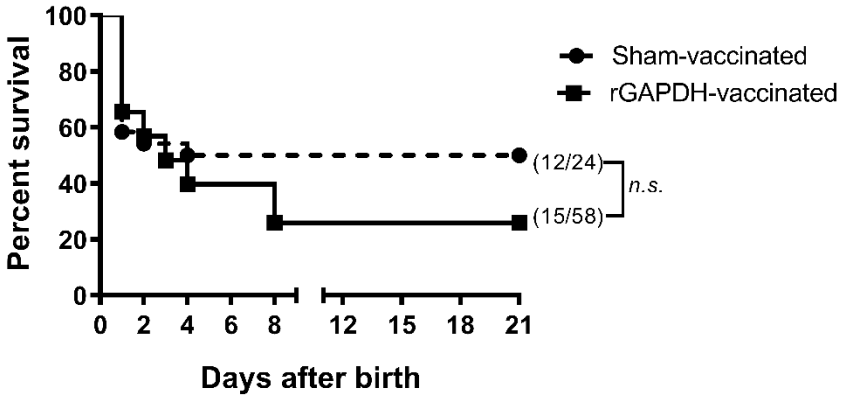

Figure 2. Maternal vaccination with GBS rGAPDH results in neonatal mortality. Female BALB/c mice were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). Pregnant BALB/c mice were intra-vaginally inoculated with $8x10^4$ CFU of GBS BM110 at gestational days 17 and 18. Kaplan-Meier survival curve monitored for 21 days. Numbers in parenthesis represent surviving pups versus the total number of pups. N.s., not significant.

This data shows that the rGAPDH vaccine does not protect newborns from GBS infection presenting a lower percentage of survival than infected control pups. Previous unpublished data obtained from our group showed that the observed mortality was not due to an absence of antibody transfer from mother to newborn as anti-rGAPDH IgG antibodies were detected in the serum of pups born from rGAPDH-vaccinated mothers [89].

Next, to confirm whether the high mortality was associated with an inability of neonates to control GBS infection, bacterial load was quantified in different organs of pups born from Sham- or rGAPDH-vaccinated mothers, at post-natal day (PND) 1, PND3 and PND5. Since the main route of GBS transmission from mother to child is during natural parturition, where inspiration of contaminated vaginal fluids can occur [130], we assessed the bacterial load in the lungs of pups born from both groups. We found that pups born from Sham- or rGAPDH-vaccinated mothers presented approximately the same level of colonization that equally decreased thereafter (Fig. 3A). One of the organs analysed was the liver and similarly to the lung, colonization levels did not vary between groups, decreasing after PND1 (Fig. 3B).

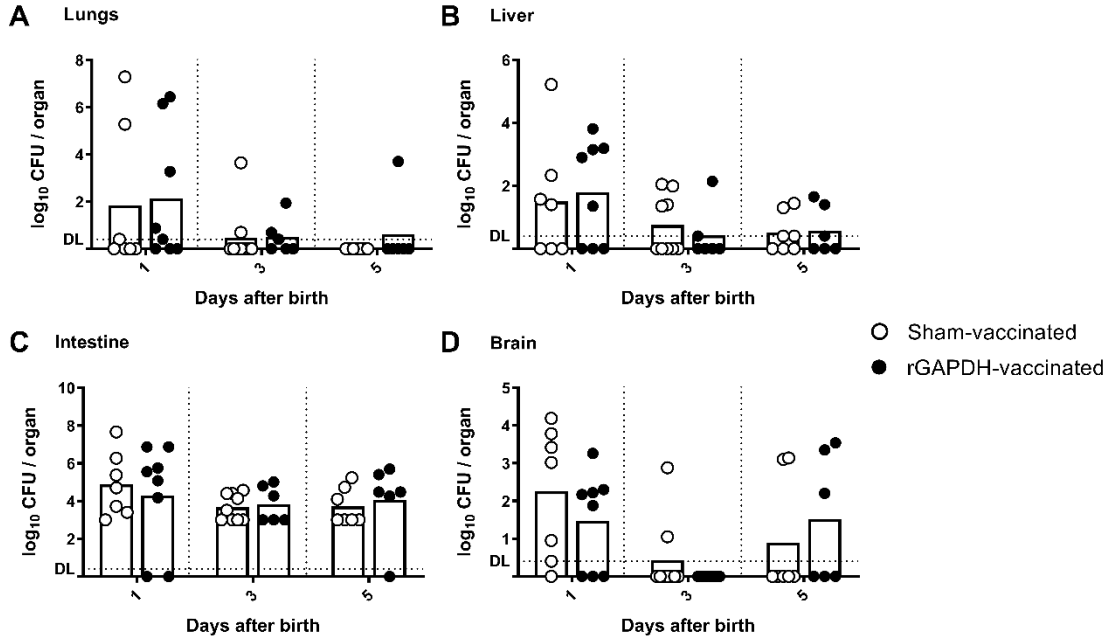

Figure 3. Organ colonization of pups in different time points. Female BALB/c mice were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). Pregnant BALB/c mice were intra-vaginally inoculated with $8x10^4$ CFU of GBS BM110 at gestational days 17 and 18. GBS colonization in the (A) Lungs, (B) Liver, (C) Gut and (D) Brain at postnatal day (PND) 1, 3 and 5. Each symbol represents data from one animal [mean, n=7-9 (Sham-vaccinated), n=6-8 (rGAPDH-vaccinated)]. DL, detection limit.

Ingestion of vaginal fluids during parturition is also another route of GBS infection in newborns [131] thus we also analysed the GBS colonization in the intestine. No significant differences were observed between the two groups, and colonization remained stable throughout the time points studied (Fig. 3C).

Meningitis is a known complication from GBS infections [2], therefore we also analysed the brain. At PND1, pups born from Sham-vaccinated mothers presented a tendency for higher colonization in their brains, when compared to pups born from

rGAPDH-vaccinated mothers, however, this difference did not reach statistical significance (Fig. 3D). The opposite was observed at PND5, where pups born from rGAPDH-vaccinated mothers present higher colonization of the brain, despite not statistically significant (Fig. 3D). At PND3 only two animals born from Sham-vaccinated mothers were colonized, and no colonization was found in the rGAPDH-vaccinated group (Fig. 3D).

Altogether, these data validated our preliminary results confirming that maternal vaccination with rGAPDH does not confer protection to newborns in a vertical model of GBS infection and that newborns born from rGAPDH-vaccinated mothers are unable to control GBS infection.

rGAPDH vaccination leads to maternal death upon birth

Previous unpublished work from our group had already shown that maternal rGAPDH vaccination lead to mortality of nearly 50% of the progenitors after delivery [89]. However, the number of animals used was low and increasing it was mandatory. Confirming the preliminary results, maternal vaccination with rGAPDH leads to a mortality rate of 50% in the rGAPDH-vaccinated progenitors (Fig. 4). Whereas in the Sham-vaccinated group no death was recorded, in the rGAPDH-vaccinated 9 out of 17 progenitors developed clinical signs of distress and dyed or were euthanized, due to ethical reasons when we could notice the animal was severely sick (Fig. 4).

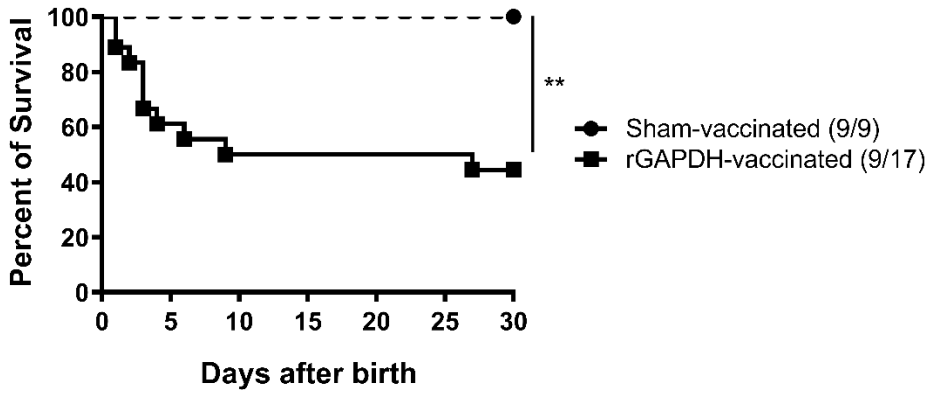

Figure 4. Maternal rGAPDH vaccination leads to maternal death. Female BALB/c mice were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). Pregnant BALB/c mice were intra-vaginally inoculated with $8x10^4$ CFU of GBS BM110 at gestational days 17 and 18. Kaplan-Meier survival curve of progenitors registered over 30 days. Numbers in parenthesis represent surviving adults versus the total number of adults in the experiment. $^{**}P < 0,01$.

Such result was unforeseen as pre-clinical studies performed by our group had demonstrated that rGAPDH vaccine was safe and stable, showing no signs of systemic toxicity and local reactogenicity [50]. Furthermore, in a model where the progenitors were not intravaginally colonized with GBS, rGAPDH maternal vaccination did not show any side effects in the progenitors [48]. However, in none of those pre-clinical studies the females were intravaginally colonized with GBS during gestation.

To understand if the observed mortality could be associated with an overall dissemination of GBS beyond the vaginal mucosa, organs such as liver, lungs, kidney and uterus were collected 5 days after birth, and the bacterial burden was quantified. The time point chosen was consistent with the onset of clinical signs of distress. Progenitors belonging to rGAPDH-vaccinated group were divided into two sub-groups: animals who showed signs of disease were classified as sick rGAPDH-vaccinated and the remaining animals who did not show signs of disease were designated as healthy rGAPDH-vaccinated. Unless otherwise stated, this division was used in all the remaining experiments presented in this thesis.

Sick progenitors from rGAPDH-vaccinated group presented significantly higher bacterial loads in the liver than Sham-vaccinated progenitors (Fig. 5). Although significance between healthy and sick progenitors wasn't observed a tendency for higher colonization in sick progenitors was observed (Fig. 5). Similarly, to what was found in the uterus, only 2 out of 5 healthy rGAPDH-vaccinated progenitors presented colonisation in the liver, contrasting with the 4 out of 5 sick progenitors that were colonised. Regarding the lungs, sick progenitors presented significantly higher colonisations when compared to both Sham-vaccinated and healthy rGAPDH-vaccinated groups (Fig. 5). No differences between Sham and healthy rGAPDH-vaccinated progenitors were observed in the lungs (Fig. 5). In the kidney, a tendency for increased bacterial levels was found in the sick rGAPDH-vaccinated progenitors, when compared with healthy rGAPDH-vaccinated (P = 0.0789) or Sham-vaccinated (P = 0,1367) progenitors (Fig. 5). Curiously, some females from the healthy rGAPDH-vaccinated group presented higher levels of GBS colonization in the analysed organs than their counterparts, especially in the uterus. This could be a sign of GBS systemic dissemination which may eventually culminate in the development of disease, since we observed maternal mortality until PND28 (Fig. 5).

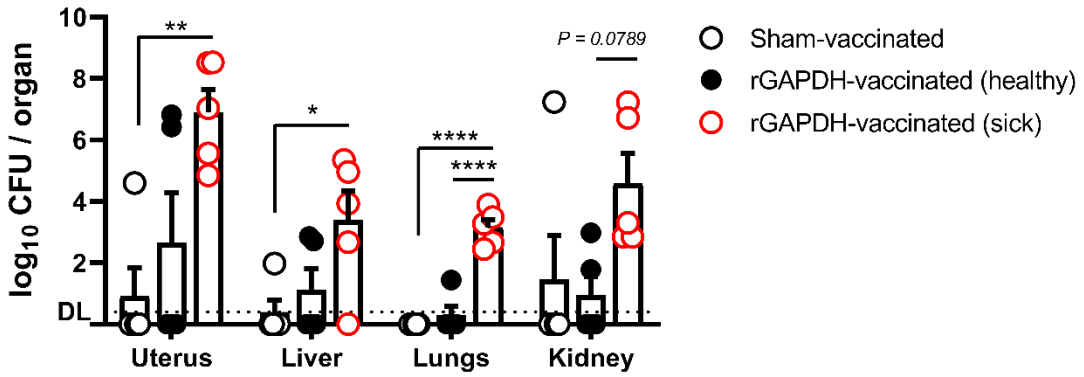

Figure 5. Maternal immunization with GBS rGAPDH leads to bacterial dissemination. Female BALB/c mice were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). Pregnant BALB/c mice were intra-vaginally inoculated with $8x10^4$ CFU of GBS BM110 at gestational days 17 and 18. Organs were collected on day 5 after delivery. Data is referent to GBS counts in the liver, lungs, kidney and uterus. Each symbol indicates data from a single progenitor and columns the mean + SEM (n=5, Sham-vaccinated, healthy and sick rGAPDH-vaccinated). DL, detection limit. *P < 0.05, **P < 0.01, ****P < 0.0001.

This data show that maternal immunization with rGAPDH contributes to systemic spreading of the bacterium, from the vagina to systemic organs, some of these not directly connected to the urogenital tract.

To gain further insight in the disease aiming at uncovering the cause of rGAPDH-vaccinated progenitors death, histopathological analysis was performed. For comparison, organs from Sham-vaccinated, sick and healthy rGAPDH-vaccinated infected progenitors were collected. As controls, organs from uninfected progenitors belonging to the Sham- or rGAPDH-vaccinated groups were also collected and analysed.

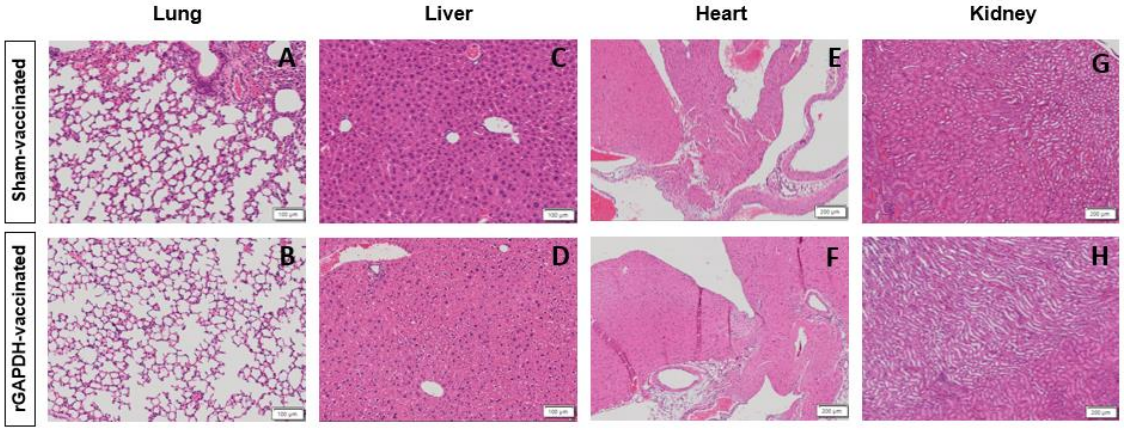

Figure 6. Histological images of uninfected progenitors. Female BALB/c were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH plus Alum (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). Each organ was collected on day 5 after delivery. Sections of the lung (A-B), liver (C-D), heart (E-F) and kidney (G-H) were subjected to H&E staining. Representative sections are shown. Scale bar, 100 µm.

Organs from uninfected progenitors showed no signs of pathology or bacterial colonization as expected (Fig. 6). Histological cuts from uninfected progenitors were used for comparison in the subsequent analysis performed in infected progenitors. Additionally, the images selected for each group are representative of all cases analysed. Exceptions to the pattern observed are referred throughout the chapter.

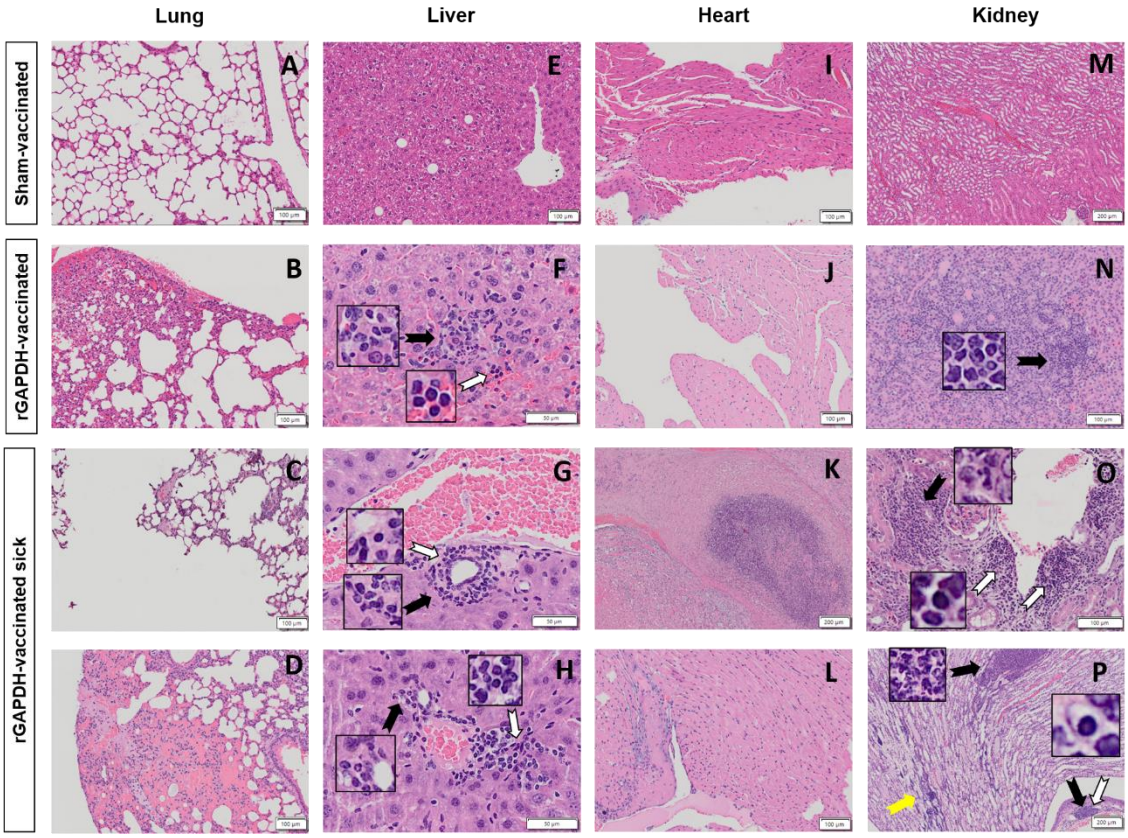

Figure 7. Histological images of infected progenitors. Female BALB/c were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH plus Alum (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). On gestational days 17 and 18, pregnant BALB/c mice were intra-vaginally inoculated with $8x10^4$ CFU of GBS BM110. Progenitors who presented external signs of disease are included in the sick rGAPDH-vaccinated group. Each organ was collected on day 5 after delivery. Sections of the lung (A-D), liver (E-H), heart (I-L) and kidney (M-P) were subjected to H&E staining. Representative sections are shown. Scale bar, 50 and 100 µm. Black arrows indicate the presence of neutrophils, white arrows indicate lymphocytes and yellow arrows mark bacterial colonization.

Histopathologic analysis of the lung revealed a higher occurrence of emphysema, defined by an enlargement of the alveoli with destruction of the alveolar walls, on sick rGAPDH-vaccinated progenitors (Fig. 7C) when compared to Sham- and healthy rGAPDH-vaccinated progenitors (Fig 7. A-B) which is in alignment with the respiratory distress observed in sick progenitors. Moreover, 1 out of 4 sick rGAPDH-vaccinated progenitors presented a large haemorrhage in the lung portion analysed (Fig. 7D). Additionally, two sick rGAPDH-vaccinated progenitors presented purulent pneumonia. (Fig. 8).

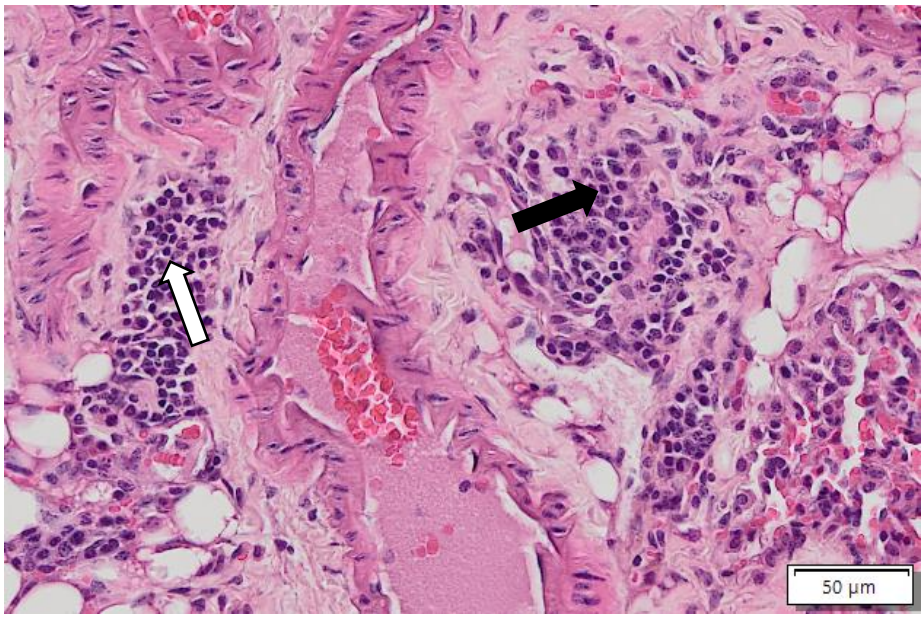

Figure 8. Purulent pneumonia in a sick rGAPDH-vaccinated progenitor. Female BALB/c were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH plus Alum (rGAPDH-vaccinated). On gestational days 17 and 18, pregnant BALB/c mice were intra-vaginally inoculated with $8x10^4$ CFU of GBS BM110. Progenitors who presented external signs of disease are included in the sick rGAPDH-vaccinated group. The organ was collected 5 days after delivery and subjected to H&E staining. Representative section is shown. Scale bar, 50 µm. Black arrows indicate the presence of neutrophils and white arrows indicate lymphocytes.

To better compare lung pathologies between the different groups, a semi-quantitative lung score was made regarding emphysema, atelectasis and haemorrhage. Despite non-significant, there is a tendency for more emphysema on sick rGAPDH-vaccinated progenitors comparatively to Sham-vaccinated and healthy rGAPDH-vaccinated progenitors (Fig. 9A). Additionally, 3 out of the 4 progenitors from the sick rGAPDH-vaccinated group presented more emphysema than progenitors from the sham-vaccinated group (Fig. 9A). Lung atelectasis and haemorrhage were always higher on both rGAPDH-vaccinated groups, however, this increase did not reach statistical significance (Fig. 9B and C).

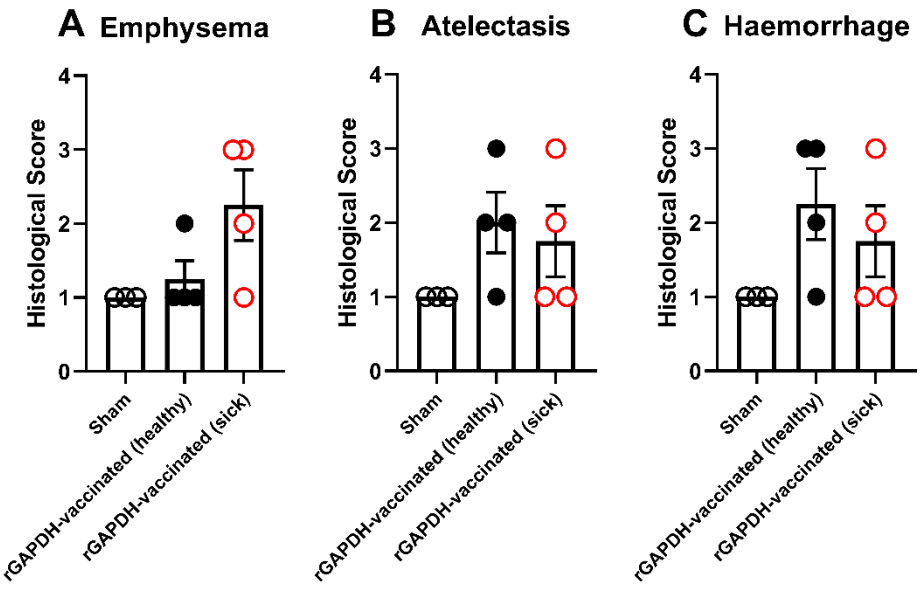

Figure 9. Histological semi-quantitative scoring of the lung. Female BALB/c were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH plus Alum (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). On gestational days 17 and 18, pregnant BALB/c mice were intra-vaginally inoculated with $8x10^4$ CFU of GBS BM110. Progenitors who presented external signs of disease are included in the sick rGAPDH-vaccinated group. The organ was collected 5 days after delivery. Data is referent to lung (A) Emphysema, (B) Atelectasis and (C) Haemorrhage. Each dot represents data from a single progenitor. Columns represent the mean ± SEM of each group, n=3 (Sham-vaccinated) and n=4 (healthy and sick rGAPDH-vaccinated).

The liver exhibited the presence of focal hepatitis in the rGAPDH-vaccinated progenitors, both healthy and sick, mainly of lymphocytic origin (Fig. 7F-H), which was not observed in the Sham-vaccinated group (Fig. 7E). Regarding the histopathology observed in the heart, only in one sick rGAPDH-vaccinated mother a sceptic thrombus, as well as endo and myocarditis was present (Fig. 7K). All the remaining hearts from all groups presented no lesions. Analysis of the kidneys showed focal nephritis in healthy rGAPDH-vaccinated progenitors (Fig. 7N) and extensive focal pyelonephritis in sick rGAPDH-vaccinated progenitors (Fig. 7O-P), whereas none of these was present in Sham-vaccinated kidneys (Fig. 7M). Furthermore, in one of the progenitor's kidneys from the sick rGAPDH-vaccinated group, it was possible to observe bacterial colonization with coccus shaped bacteria. To further investigate if it could be due to GBS ascension, we performed a Gram staining of the kidney, confirming it to be a Gram-positive bacterium which disseminated to medulla and cortex of the kidney (Fig. 10).

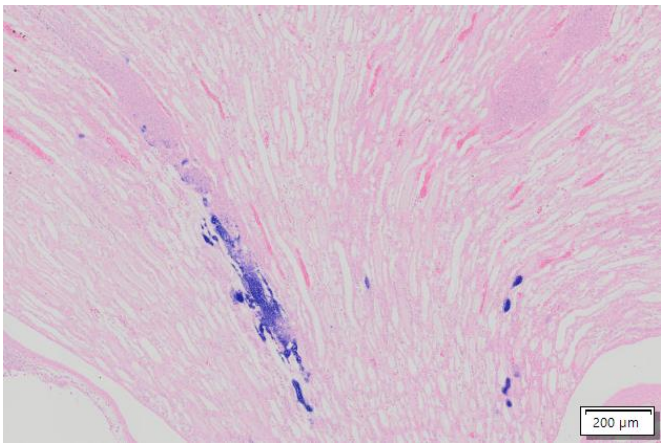

Figure 10. Gram colouration of a kidney slide from a sick rGAPDH-vaccinated progenitor. Female BALB/c were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH plus Alum (rGAPDH-vaccinated). On gestational days 17 and 18, pregnant BALB/c mice were intra-vaginally inoculated with $8x10^4$ CFU/mL of GBS BM110. Progenitors who presented external signs of disease are included in the sick rGAPDH-vaccinated group. The organ was collected 5 days after delivery. Sections of the kidney were subjected to Gram-staining. Blue/violet colouration indicates the presence of Gram-positive bacteria. Representative section is shown. Scale bar, 200 µm.

In a more severe case, a rGAPDH-vaccinated female developed clinical signs of disease during labour and was not able to give birth. Due to ethical reasons, the parturient female was euthanized. Placentas were collected for histopathology analysis, to determine if uterus ascension of the bacteria was associated with a complicated birth and the clinical symptoms presented by the parturient.

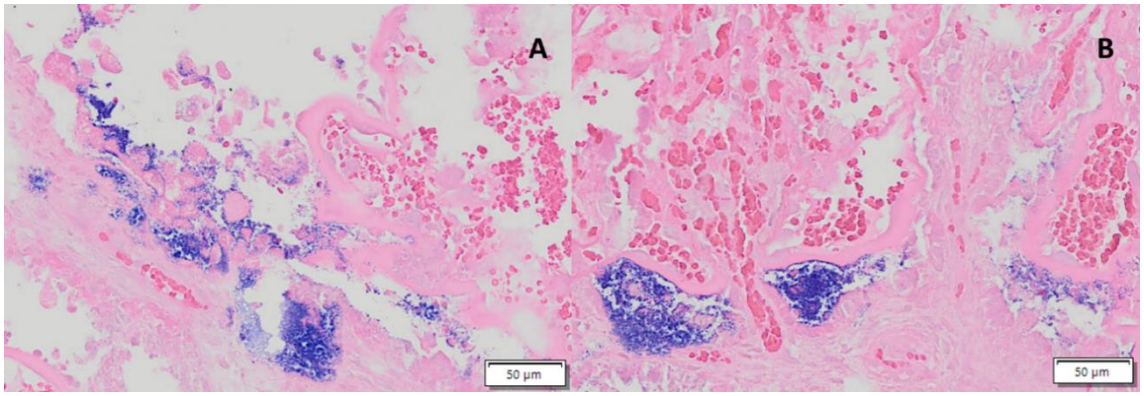

Figure 11. Gram colouration of placentas from a sick rGAPDH-vaccinated progenitor. Female BALB/c were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH plus Alum (rGAPDH-vaccinated). On gestational days 17 and 18, pregnant BALB/c mice were intra-vaginally inoculated with $8x10^4$ CFU of GBS BM110. Placentas were collected from a dying parturient mouse. Sections of each placenta were subjected to Gram-staining. Blue/violet colouration indicates the presence of Gram-positive bacteria. Representative section is shown. Scale bar, 50 µm.

Gram staining of the placentas revealed that bacterial infiltration was located on the edges of the placenta and very often close to blood vessels (Fig. 11A). One of the placentas shows colonization on the interior of the placenta (Fig 11B.). This data shows that there is a vagina to uterus ascension of the bacteria and that it can reach important

organs, such as the placenta, involved in the development of the foetus and suggesting the breaking of the protective interface that the placenta has during pregnancy. The progenitor from which the placentas were collected carried 3 pups that according to the placental histology possibly died due to GBS infection *in utero*. Studies have already confirmed that GBS can successfully cross the placenta and invade the amniotic fluid due to the presence of β-hemolysin/cytolysin, a pluripotent toxin present in GBS that contributes for its virulence [37]. This invasion of the placenta leads to the development of chorioamnionitis, which is characterized by neutrophilic infiltrates in the placental membrane and the chorionic membrane [132, 133]. Although chorioamnionitis is a less frequent manifestation of GBS disease, it is estimated that 3% of GBS positive women develop chorioamnionitis [134]. This pathology leads to severe outcomes such as fetal cerebral white matter injury, infection, sepsis and death [135]. It can also affect severely the mother potential causing wound infection, bacteraemia, endomyometritis and postpartum haemorrhage [135].

Altogether, our results suggest that rGAPDH-vaccination can lead to puerperal sepsis in 50% of vaccinated progenitors. Moreover, although less frequent, a possible impact in pregnancy, with poor outcomes for the progenitor and the fetus, can also occur in rGAPDH-vaccinated animals and it cannot be disregarded.

rGAPDH antibodies are not involved in the immunopathology observed

Antibody-mediated enhancement of infection has largely been shown for viral mediated diseases [136-138]. Regarding bacteria, one study has shown that antibodies can aggravate infection caused by *Staphylococcus aureus,* by increasing the susceptibility of the host to infection [139]. Preliminary data from our group, with only two animals per group, suggested that antibodies against GBS rGAPDH were not contributing to vaccine-induced death [140]. Thus, to confirm that antibodies against GBS rGAPDH were not leading to a higher GBS infectivity and thus causing maternal death, we established a group of passively immunized animals, using anti-rGAPDH IgGs purified from the peripheral blood of animals immunized with rGAPDH. The same amount of non-specific IgGs (Isotype control) isolated from non-vaccinated animals were used as control. Passive vaccinations were administered to BALB/c mice during pregnancy, one day prior to GBS intravaginal inoculation.

Passive immunization with anti-rGAPDH IgG antibodies reduced the neonatal mortality to 40%, whereas nearly all mice in the control group died during the first 2 days after birth (2/15, approximately 87% of mortality) (Fig. 12).

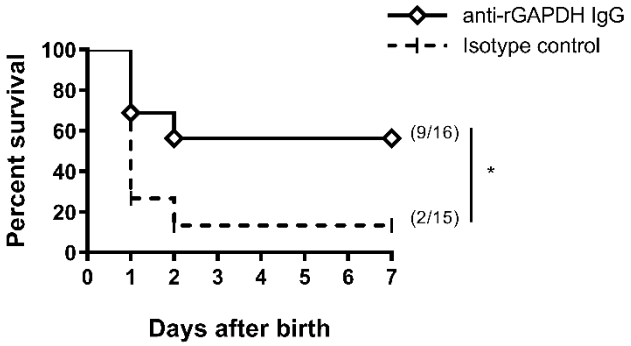

Figure 12. Passive immunization can confer some protection against neonatal GBS infection. Pregnant female BALB/c were passively immunized with IgG antibodies anti-rGAPDH (anti-rGAPDH IgG), or with non-specific IgGs (Isotype control), at gestational day 16. Pregnant BALB/c mice were intra-vaginally inoculated with $8x10^4$ CFU/mL of GBS BM110 at gestational days 17 and 18. Kaplan-Meier survival curve monitored for 7 days. Numbers in parenthesis represent surviving pups versus the total number of pups. **P* < 0.05.

The vaccinated progenitors were also monitored for 7 days and no deaths were observed in neither of the two studied groups (Fig. 13A), suggesting that antibodies are not mediating the maternal mortality observed in the actively immunized progenitors. To ensure that this result was not due to a low or non-existing GBS bacterial colonization in any of the groups, we quantified the vaginal colonization, one day after birth, by performing a vaginal lavage. Both vaginal mucosa presented high bacterial colonization by GBS with no significant differences between them (Fig. 13B). Moreover, these results also confirmed that their progeny was exposed to GBS during birth.

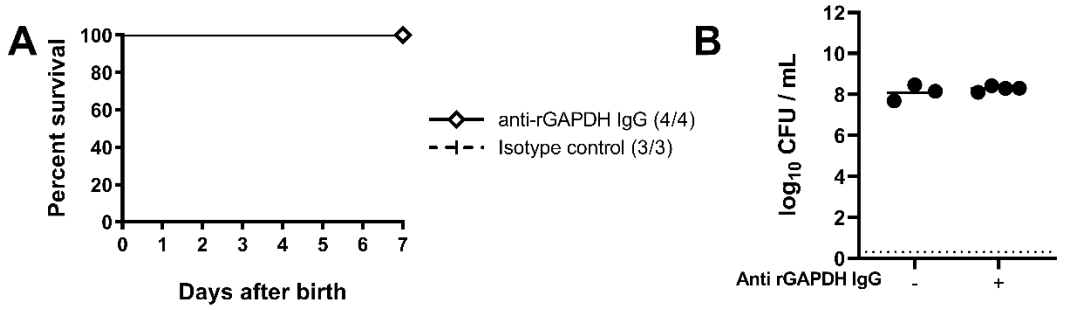

Figure 13. Passive immunization does not affect maternal health. Pregnant female BALB/c were passively immunized with IgG antibodies anti-rGAPDH (anti-rGAPDH IgG), or with non-specific IgGs (Isotype control), at gestational day 16. Pregnant BALB/c mice were intra-vaginally inoculated with $8x10^4$ CFU of GBS BM110 at gestational days 17 and 18. (A) Kaplan-Meier survival curve monitored for 7 days. Numbers in parenthesis represent surviving versus the total animals. (B) GBS vaginal colonization levels were determined using vaginal lavages and posterior culture in selective CHROMagar

media to count GBS colony-forming units (CFU). Each symbol indicates data from a single progenitor. Horizontal line represents the mean, n= 3 (Isotype control) and n=4 (anti-rGAPDH IgG).

Altogether, these data suggest that rGAPDH antibodies do not seem to be contributing for the immunopathology observed in the rGAPDH-vaccinated females upon delivery but are able to confer protection to neonates born from passively immunized mothers.

Deviation from a Th2 response to a Th1/Th17 response

Considering that the mortality observed was not mediated by rGAPDH antibodies, we next sought to investigate the cellular immune response of the progenitors. It has been shown that in vaccines against certain bacteria or virus, the $CD4^+$ T cells contributed to the development of immunopathology [139, 141, 142].

Therefore, we investigated the $CD4^+$ T cells response in Sham- and rGAPDH-vaccinated, in the context of GBS colonisation during pregnancy. To this end, splenocytes from Sham-, healthy and sick rGAPDH-vaccinated mothers were collected 5 days after birth (consistent with the prevalence of onset of disease) and *ex vivo* stimulated with medium alone (RPMI), fixed bacteria (GBS_{fix}), rGAPDH (from GBS and mouse), and Concanavalin A (ConA) as a positive control of T cell proliferation. As rGAPDH (both from mouse and GBS) is a recombinant protein purified from *Escherichia coli* (*E.coli*), splenocytes were also stimulated with low levels of LPS, to ensure that the potential differences were not due to LPS contamination. After 72 hours, the supernatants were collected and posteriorly the cytokines IL-4, IL-6, IL-17, IFN-γ, and IL-10 were quantified by ELISA.

Contrary to what was expected, the levels of IL-4 obtained from the splenocytes supernatants were extremely low with only values obtained from stimulation with ConA and stimulation with GBS_{fix} in the sick rGAPDH-vaccinated group surpassing the detection level for this cytokine (Fig. 14A). These results are the opposite from previous unpublished data from our group, when the cellular response was analysed in uninfected females. In this experiment splenocytes from rGAPDH-vaccinated females produced IL-4 when stimulated with rGAPDH protein (Unpublished data). The adjuvant Alhydrogel®, an adjuvant with a chemical basis of aluminium hydroxide (alum), was used in the rGAPDH vaccine formulation and is an inducer of T helper (Th) 2 cellular responses [143], thus IL-4 levels in the supernatant were expected. This lack of IL-4 production is suggestive that the cellular immune response could shift to another subset of Th cells.

One pro-inflammatory cytokine analysed that belongs to the Th17 subset was IL-17A. This pro-inflammatory cytokine is an important inducer of neutrophil recruitment to infection sites [144]. Results show an increased, however non-significant, production of IL-17A in splenocytes from sick rGAPDH-vaccinated progenitors stimulated with GBS_{fix}, when compared with splenocytes from Sham-vaccinated progenitors (P = 0.2317) (Fig.14C). Splenocytes from healthy rGAPDH-vaccinated females also produced IL-17A, but not as high as the levels found on sick females (Fig. 14C). In response to the remaining stimuli except for RPMI, supernatants from healthy progenitors responded with higher levels of IL-17A than Sham- and sick rGAPDH-vaccinated animals, but no statistically significant differences were found (Fig. 14C). Regarding the positive stimulus, all groups responded equally. Levels of IL-6 were also assessed, as this cytokine is a marker of inflammation and is involved in the differentiation of Th17 cells, and although splenocytes from healthy rGAPDH-vaccinated females produced this cytokine when stimulated with either mouse or GBS rGAPDH, no significant differences between groups were found (Fig. 14B).

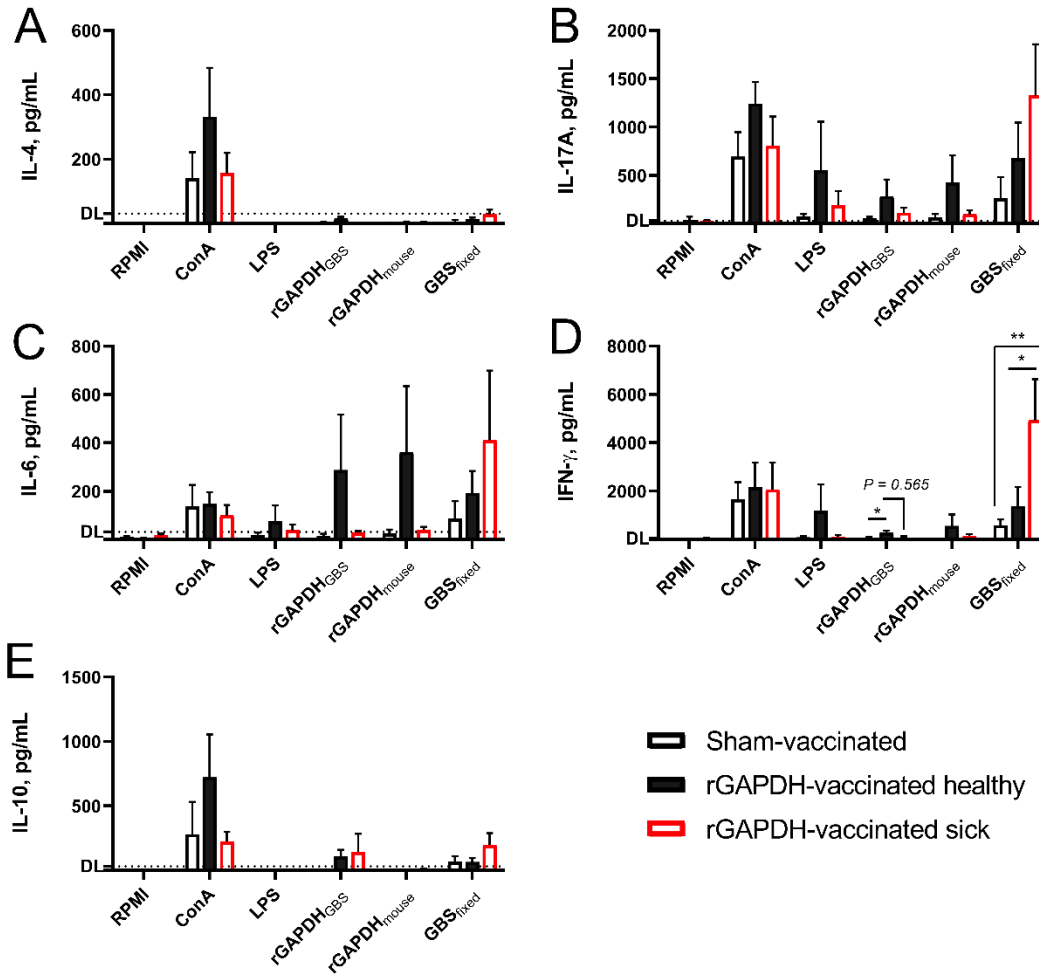

Figure 14. Splenic cells from rGAPDH-vaccinated mice exhibit a shift towards a Th1 and Th17 response. Female BALB/c mice were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). Pregnant BALB/c mice were intra-vaginally inoculated with $8x10^4$ CFU of GBS BM110 at gestational days 17 and 18. Spleens were collected 5 days after delivery and *ex vivo* stimulated for 72 hours with media (RPMI), ConA and LPS, as controls, with rGAPDH from GBS ($rGAPDH_{GBS}$) and mouse ($rGAPDH_{mouse}$), and fixed GBS (GBS_{fix}). The concentration of each cytokine was quantified by ELISA. Data are presented as the mean ± SEM [n = 3-7 (Sham-vaccinated), n=3-5 (rGAPDH-vaccinated healthy) and n=3-6 (rGAPDH-vaccinated sick)]. DL, detection limit. *P < 0.,05, **P < 0.01.

Interestingly, the production of IFN-γ, a pro-inflammatory cytokine belonging to the Th1 subset, was significantly increased in splenocytes from sick rGAPDH-vaccinated mothers stimulated with the GBS_{fix}, when compared with both Sham-vaccinated and healthy rGAPDH-vaccinated animals (Fig. 14D). When splenocytes were stimulated with rGAPDH from GBS, a significantly increase in the production of IFN-γ was observed in the healthy group, when compared with both Sham- or sick rGAPDH-vaccinated progenitors, however in substantially lower quantities than the ones observed in the supernatants from GBS_{fix} stimulation (Fig. 14D).

The anti-inflammatory cytokine IL-10 was also quantified in the supernatants and no major differences between groups and stimulus were found (Fig. 14E).

Given the differences observed in the spleen, and knowing that changes in the levels of cytokines are associated with systemic inflammation and contribute to a number of pathological processes, we next assessed the concentration of these cytokines in the sera of the progenitors from the three groups, 5 days after birth.

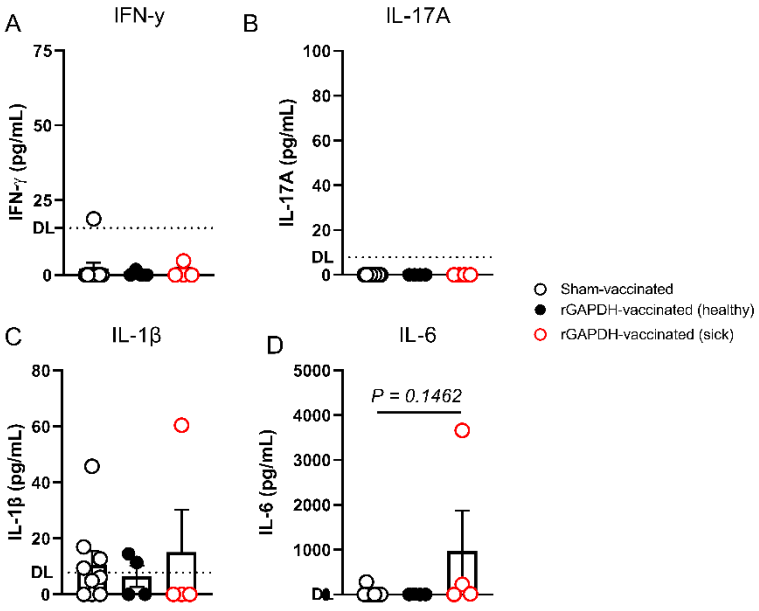

Figure 15. Sera from sick rGAPDH-vaccinated progenitors do not present significantly different cytokine concentrations. Female BALB/c mice were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). Pregnant BALB/c mice were intra-vaginally inoculated with $8x10^4$ CFU of GBS BM110 at gestational days 17 and 18. Sera was collected 5 days after delivery and the indicated cytokines were quantified by ELISA. Each symbol indicates data from a single progenitor and columns the mean ± SEM [n =9 (Sham-vaccinated), n=4 (rGAPDH-vaccinated healthy) and n=4 (rGAPDH-vaccinated sick)]. DL, detection limit.

Surprisingly, the serum levels of the evaluated cytokines were barely above detection level and showed no significant differences between groups (Fig. 15). Although these results do not suggest that systemic inflammation is being responsible for the mortality observed in sick rGAPDH-vaccinated females, we cannot exclude the fact this analysis was performed in the serum and this could be influencing the results. It has been shown that cytokine levels in plasma are more stable compared with serum, being a better matrix than serum for the evaluation of cytokines in clinical or research analyses [145].

Of note, 1 out of 4 sick rGAPDH-vaccinated progenitors presented high levels of IL-6. Elevated levels of this cytokine in the serum in pregnant women have been reported to be a predictor of histological chorioamnionitis alongside the occurrence of preterm-

prelabor rupture of membranes (PPROM) [146]. Interestingly, the progenitor with high IL-6 values was severely colonized in the placenta (Fig. 11) which can indicate that the sick progenitor developed chorioamnionitis even though PPROM did not occur as all three fetuses were deceased.

Altogether, these results show that the immune profile in sick rGAPDH-vaccinated group was shifted towards a more inflammatory response, likely in an attempt to control GBS dissemination, and that the serum levels of cytokines are poor predictors of maternal pathology.

Vaginal immune response to GBS infection

Given the obtained differences in the spleen and the lack of increased inflammation systemically, we next sought to determine whether sick rGAPDH-vaccinated progenitors presented a distinct cytokine production within the female reproductive tract compared with the other groups. To this end, vaginal lavages from Sham-, healthy and sick rGAPDH-vaccinated progenitors were collected 5 days after birth and the cytokines IFN-γ, IL-17A, IL-1β and IL-6 were quantified by ELISA. As shown in Figure 16, IFN-γ and IL-17A were barely above the detection limit (Fig. 16A-B). However, IFN-γ was detected in 3 out of 8 Sham-vaccinated females, and in 1 out of 4 healthy rGAPDH-vaccinated progenitors, but in none of the sick rGAPDH-vaccinated females (Fig. 16A). Sick rGAPDH-vaccinated progenitors produced more IL-1β, an important mediator of the inflammatory response, than Sham- and healthy rGAPDH-vaccinated animals, although it did not reach significant, probably due to the low number of animals used in this group (Fig. 16C). A tendency towards increased production of IL-1β was observed in sick rGAPDH-vaccinated progenitors when compared with the Sham-vaccinated group (*P = 0.0747*) (Fig. 16C). Regarding IL-6, only one female from the sick rGAPDH-vaccinated group presented high levels of this cytokine while the remaining had very low levels (Fig. 16). Increased levels of IL-1β and IL-6 have already been shown in a murine model of vaginal GBS colonization, in the vaginal homogenates two days after inoculation [126]. These cytokines are involved in the differentiation of Th17 cells, which contribute to neutrophil recruitment and sustained inflammation [144].

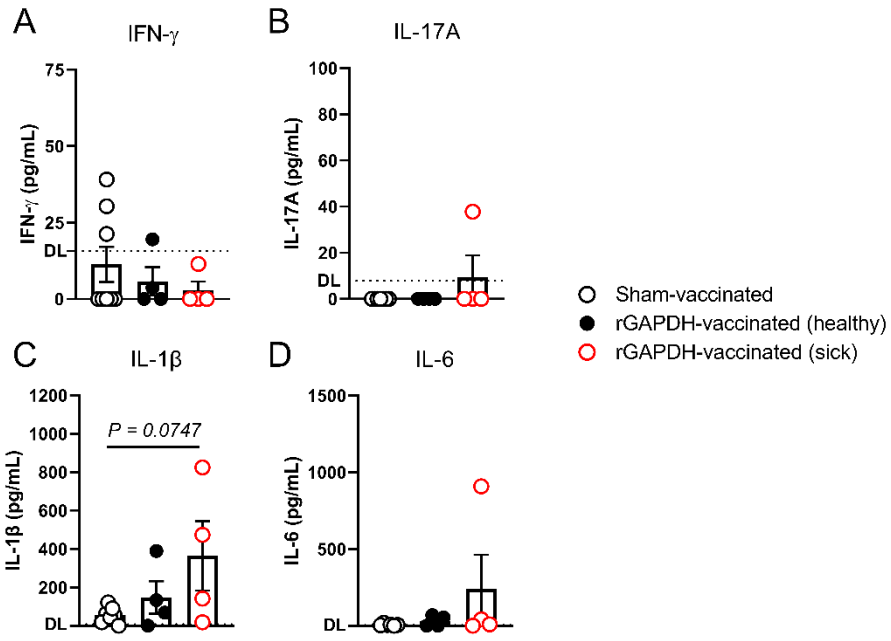

Figure 16. Vaginal lavages of sick rGAPDH-vaccinated progenitors exhibit a tendency for higher levels of IL-1β. Female BALB/c mice were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). Pregnant BALB/c mice were intra-vaginally inoculated with $8x10^4$ CFU of GBS BM110 at gestational days 17 and 18. Vaginal lavage was collected 5 days after delivery and the indicated cytokines were quantified by ELISA. Each symbol indicates data from a single progenitor and columns represents the mean ± SEM [n =6-8 (Sham-vaccinated), n=4 (rGAPDH-vaccinated healthy) and n=4 (rGAPDH-vaccinated sick)]. DL, detection limit

Based on the low levels observed, we reasoned that this sampling method is not the best to study cytokine production in response to GBS in the vaginal mucosa as it only allows the quantification of cytokines released to the vaginal space and does not consider cytokines inside the mucosa and also inside the correspondent producing cells. Moreover, in a vaginal lavage fluid, cytokines can be highly diluted and also highly susceptible to degradation. Therefore, we next quantified these cytokines in lysate of uterus from all groups. Cytokine concentrations obtained from uterus lysates were generally very low for all cytokines studied (Fig. 17). Except for IL-6, in which we verified higher levels of this cytokine in one sick rGAPDH-vaccinated female, the cytokine levels were very similar between all groups studied (Fig. 17D). Thus, we hypothesize that the immune response might be occurring in a more localized manner and not throughout the entire organ. Our analysis using uterus lysates contemplates the entire organ and makes it difficult to detect cytokines produced more locally since those cytokines are probably very diluted in our sample. Additionally, these lysates undergo a lot of processing which can lead to the degradation of the cytokines.

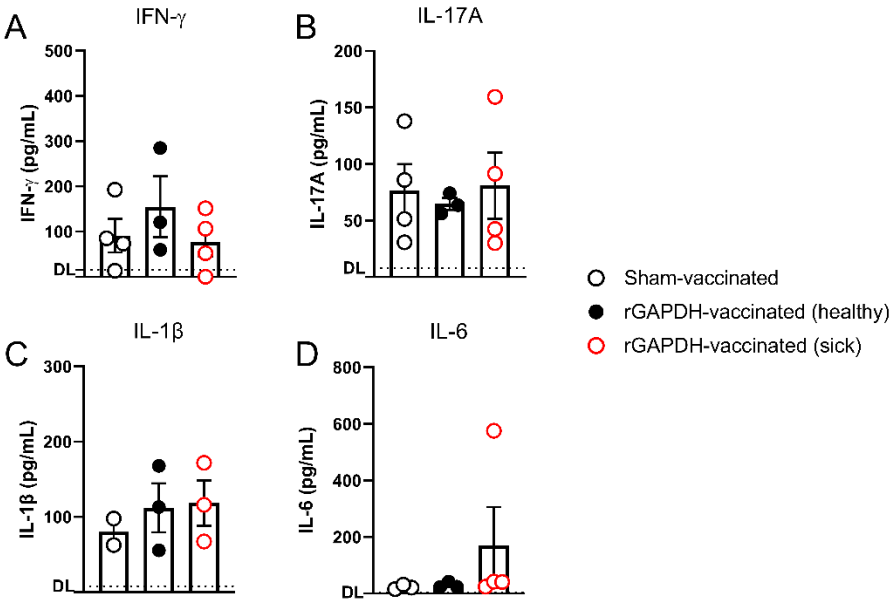

Figure 17. Lysates of the uterus do not present significantly different cytokine concentrations. Female BALB/c mice were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). Pregnant BALB/c mice were intra-vaginally inoculated with $8x10^4$ CFU of GBS BM110 at gestational days 17 and 18. Uterus were collected 5 days after delivery and the indicated cytokines were quantified by ELISA. Each symbol indicates data from a single progenitor and columns represents the mean ± SEM [n =2-4 (Sham-vaccinated), n=3 (rGAPDH-vaccinated healthy) and n=3-4 (rGAPDH-vaccinated sick)]. DL, detection limit.

Due to the differences observed in the cellular response in the spleen, and in an attempt to unravel the role of the local response to the observed pathology, we next sought to analyse the expression of IFN-γ and IL-17A in the cervix/uterus portion by real-time PCR (RT-PCR). The results obtained for IL-17 mRNA expression levels in the portion of the reproductive tract analysed showed no significant differences between Sham-vaccinated and healthy rGAPDH-vaccinated progenitors (Fig. 18A). However, a tendency for increased upregulation of IL-17A expression was found in the sick rGAPDH-vaccinated mothers, compared with the Sham-vaccinated group ($P = 0.0855$) (Fig. 18A). This result suggests that the production of IL-17A is increased in sick rGAPDH-vaccinated, which could lead to the recruitment of neutrophils to the infection site as an attempt to control the increased GBS colonization observed in these progenitors (Fig. 5), and thus perpetuating the inflammatory state.

Surprisingly, the opposite was observed regarding IFN-γ expression, as a significant downregulation was found in both rGAPDH-vaccinated groups, when compared with Sham-vaccinated controls (Fig. 18B). We had hypothesized that IFN-γ was being produced in exacerbated quantities and thus leading to a deleterious effect

on the rGAPDH-vaccinated progenitors. However, vaccinated progenitors presented decreased local expression of IFN-γ.

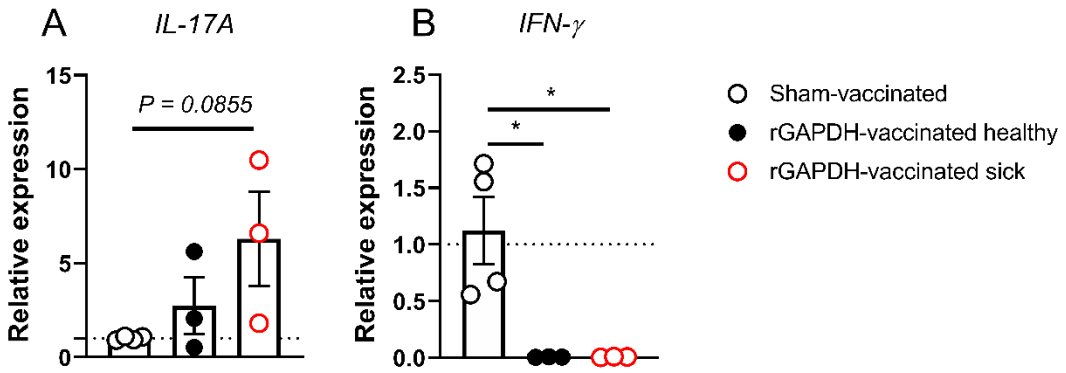

Figure 18. IL-17A is upregulated in sick progenitors and IFN-γ is downregulated in the uterus. Female BALB/c mice were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). Pregnant BALB/c mice were intra-vaginally inoculated with 8x104 CFU of GBS BM110 at gestational days 17 and 18. Uterus were collected on day 5 after delivery and a portion of cervix/uterus was snap-frozen, and later analysed by qRT-PCR. Each symbol indicates data from a single progenitor and columns represents the mean ± SEM [n = 4 (Sham-vaccinated), n=3 (rGAPDH-vaccinated healthy) and n=3 (rGAPDH-vaccinated sick)].

Several studies have demonstrated that IFN-γ can be produced early during streptococcal infections [147]. Indeed, IFN-γ has been shown to be protective against GBS infection in newborns [148] and contribute to the microbicidal activity of human endothelial cells against GBS [149]. Thus, it is likely that at this time point, the increase local GBS colonization observed in the rGAPDH-vaccinate groups is due to reduced IFN-γ production in the uterus. An inability to control GBS growth, might lead to its dissemination and disease. Absence of IFN-γ has been shown to lead to exacerbated T cells responses in a uterine infection caused by *Chlamydia trachomatis* [150]. Yet, we only quantified mRNA levels in the cervix and adjacent areas of the vagina and uterus, and thus this cannot be representative of the entire reproductive tract. Moreover, analysis of the vaginal lavage, showed that the Sham-vaccinated group had already cleared GBS, consistent with the higher levels of IFN-γ mRNA observed, whereas 2 out 5 females from both rGAPDH-vaccinated groups were still colonized in the vaginal lavage (Fig. 19). To better understand the role of this cytokines for GBS clearance, evaluation of their mRNA expression levels in different areas of the reproductive tract will be performed, one and three days after birth, before the onset of disease.

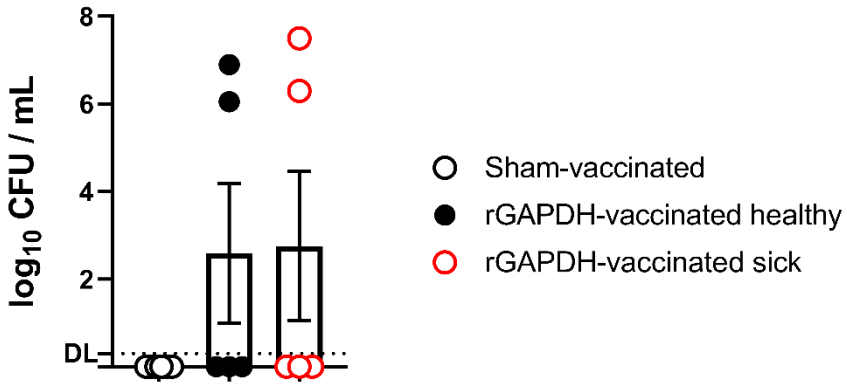

Figure 19. Vaginal colonization is higher o rGAPDH-vaccinated females on the 5th day after delivery. Female BALB/c mice were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). Pregnant BALB/c mice were intra-vaginally inoculated with $8x10^4$ CFU of GBS BM110 at gestational days 17 and 18. GBS vaginal colonization levels was determined in vaginal lavage by enumerating onto CHROMagar StrepB selective media GBS colony-forming units (CFU), 5 days after delivery. Each symbol indicates data from a single progenitor and columns represents the mean ± SEM [n =4 (Sham-vaccinated), n=5 (rGAPDH-vaccinated healthy) and n=5 (rGAPDH-vaccinated sick)].

Alongside the cytokine concentration analysis made, histopathology of the uterus of Sham- and both healthy and sick rGAPDH-vaccinated progenitors was performed to determine if the vaccine was influencing the recruitment of immune cells, such as neutrophils and lymphocytes.

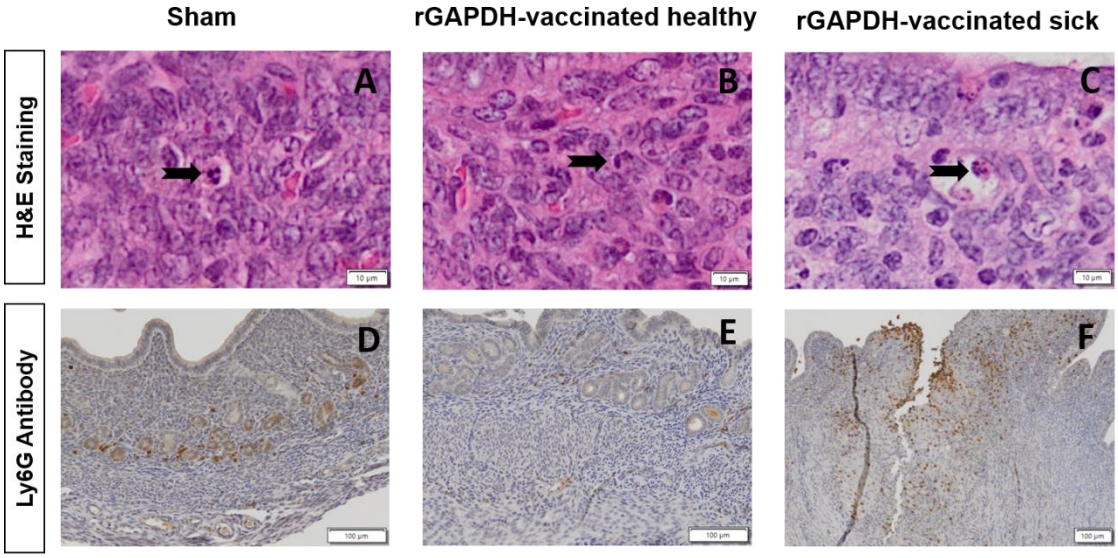

Figure 20. Histopathology analysis of uterus upon delivery. Female BALB/c were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH plus Alum (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). Progenitors who presented external signs of disease are included in the sick rGAPDH-vaccinated group. Organs were collected on day 5 after delivery and the corresponding sections were subjected to H&E staining (A-C) and immunohistochemistry (D-F) for neutrophil detection (Ly6G Antibody). Representative sections are shown. Scale bar, 10 and 100 µm. Black arrows indicate neutrophils.

In a primary analysis of each uterus, it was observed that all uterus, regardless of the treatment group, presented neutrophils dispersed on the endometrium (Fig. 20A-

C). However, as sometimes it might be difficult to identify all neutrophils through H&E staining, an immunohistochemistry specific for neutrophil detection was performed. Differences were observed regarding the number and location of neutrophils on sick rGAPDH-vaccinated progenitors compared to both sham and healthy rGAPDH-vaccinated progenitors (Fig. 20D-F). In slides from sham and healthy rGAPDH-vaccinated progenitors, neutrophils were only located in the stromal layer of the endometrium and were found mostly around the glands (Fig. 20D-E). However, the sick rGAPDH-vaccinated progenitor also presented additional neutrophils on the uterus epithelium (Fig. 20F). Although this could be a result of the recent birth the female underwent, in this group GBS could be contacting with subsequent layers of the vaginal tissue, leading to neutrophil recruitment and further perpetuating local inflammation. Additionally, a few neutrophils could also be found on the myometrium of this progenitor (data not shown). Histological analysis of the vaginal portion of the reproductive tract, as well as quantification of the cytokine's levels in this area, as this is the first barrier to interact with GBS, will be performed in the future.

The observed type of neutrophil distribution is not surprising since their recruitment, alongside with other leukocytes, to the mouse uterus is regulated by steroid hormones like estrogen and progesterone [151]. The influx of these cells when regulated by estrogen, have pro-inflammatory effects on the uterus, while progesterone can both induce or inhibit the recruitment of these cells, depending on the phase of the cycle [151]. Moreover, it has been increasing recognized the impact of sex hormones in both arms of immunity, innate and adaptive, with particular impact in females [152-155]. Therefore, the levels of sex-hormones in these females will be quantified in the future.

The vaginal microbiota suffers extensive remodelling during pregnancy [156] and an association with reduced levels of *Lactobacillus* and poor outcomes during pregnancy has been described [99]. Since GAPDH is a highly conserved enzyme, anti-rGAPDH antibodies could interfere with the vaginal microbiota composition. Thus, 16S ribosomal RNA (rRNA) gene sequencing of vaginal samples from both groups will also be performed.

Non-pregnant rGAPDH-vaccinated mice produce more IFN-γ upon GBS infection

Since the gestational model has proven to be challenging, to shed new light in the role of IFN- γ and IL-17 in the context of vaginal colonization by GBS in vaccinated

animals, we sought to perform an experimental set up in non-pregnant females. To this end, Sham- and rGAPDH-vaccinated females were treated with 17 β-estradiol 48 hours prior to GBS inoculation, to synchronize the mice in the estrus phase of the reproductive cycle and promote the persistent of GBS colonization in the vaginal tract [157].

One week after inoculation, the animals were sacrificed and bacterial levels in the vaginal mucosa determined by vaginal lavage. Although it did not reach significance, a tendency to higher bacterial levels were found in rGAPDH-vaccinated females, when compared to Sham-vaccinated controls (*P* = 0.0835) (Fig. 21). Moreover, similarly to what has been observed in the gestational model, the rGAPDH vaccine does not lead to a reduction of GBS counts in the vaginal mucosa, with some rGAPDH-vaccinated animals presenting even higher bacterial levels than the Sham-vaccinated group (Fig. 21). Of note, none of these females presented any external sign of disease.

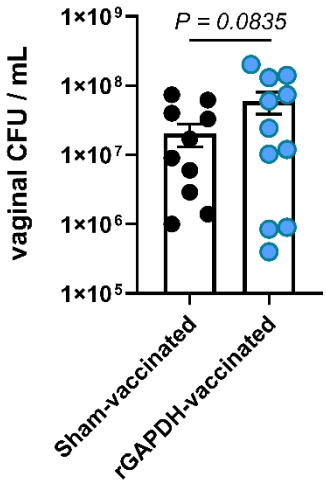

Figure 21. Both treatment groups present persistent GBS colonization. Female BALB/c mice were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). Mice were injected subcutaneously with β-estradiol in sesame oil and after two days females were intra-vaginally inoculated with $2x10^8$ CFU of GBS BM110. GBS vaginal colonization levels was determined in vaginal lavage by enumerating onto CHROMagar StrepB selective media GBS colony-forming units (CFU), 7 days after inoculation. Data are presented as the mean ± SEM [n = 12 (Sham-vaccinated) and n=12 (rGAPDH-vaccinated).

To determine which lymphoid populations could respond by producing IFN-γ or IL-17, the spleen and reproductive tracts from mid-uterine horn to just proximal of the vulva were excised. Single cell suspensions were incubated for 4 hours with ionomycin, PMA and Brefeldin A, and analysed by flow cytometry. Figure 22 shows a representation of the gating strategy used throughout this experiment.

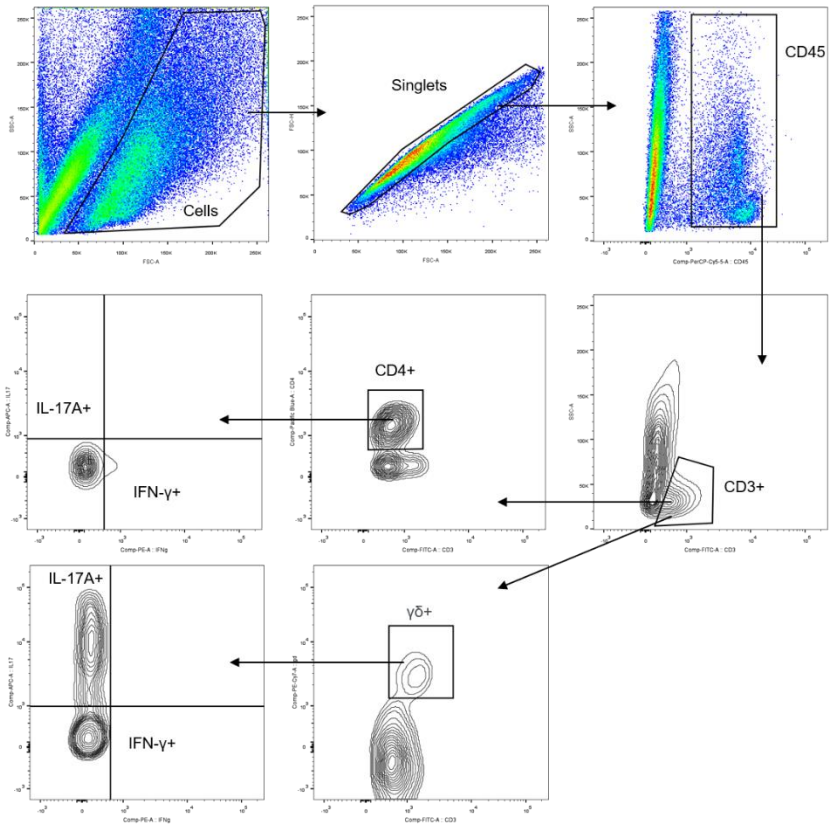

Figure 22. Representation of the gating strategy used for the flow cytometry analysis of vaginal samples. Representative gating strategy used to define the different IFN-γ and IL-17 producing cells in the vaginal mucosa. All cells were first gated on singlets (FSC-A vs FSC-H dot plot) and a gate for total cells was made from FSC-A vs SSC-A dot plot. The remaining cells were gated on $CD45^+$. Populations were defined based on the expression of indicated surface markers as represented. All T cell subsets were analysed within the CD^3 cells.

No differences were found neither in the IFN-γ production nor IL-17 production by splenic $CD3^+CD4^+$ T cells, or $CD3^+\gamma\delta^+$ T cells, between both groups (Fig. 23A-D). However, a significant increase was found in the production of IFN-γ by $CD3^+CD4^+$ T cells present in the reproductive tissue of rGAPDH-vaccinated females, compared to Sham-vaccinated animals, which could correspond to the Th1 subset (Fig. 23E). Moreover, tendency for a higher production of IFN-γ by $CD3^+\gamma\delta^+$ T cells was also found in the rGAPDH-vaccinated progenitors. when compared to Sham-vaccinated females (*P = 0.0918*) (Fig. 23G). No differences were found between groups regarding IL-17 producing $CD3^+CD4^+$ or $CD3^+\gamma\delta^+$ T cells (Fig. 23F,H).

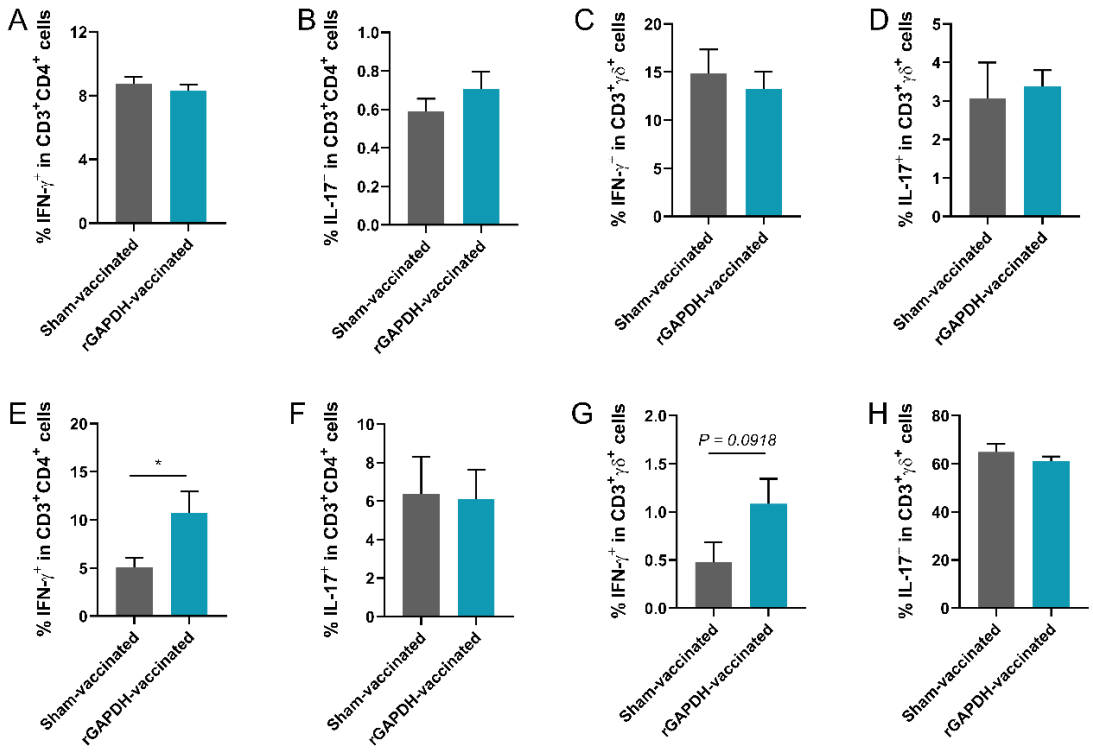

Figure 23. IFN-γ is more produced by CD3+γδ+ cells and CD3⁺ CD4⁺ cells of rGAPDH-vaccinated progenitors. Female BALB/c mice were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). Mice were injected subcutaneously with β-estradiol in sesame oil and after two days females were intra-vaginally inoculated with $2x10^8$ CFU of GBS BM110. Mice reproductive tissue was excised 7 days after inoculation, digested with collagenase-D and analysed by flow cytometry. Data are presented as the mean ± SEM [n = 6-10 (Sham-vaccinated) and n = 9-12 (rGAPDH-vaccinated)]. *P < 0.05.

Altogether these results show that the vaginal mucosa of rGAPDH-vaccinated females responds to GBS challenge with the production of IFN-γ, which is mediated mainly by the CD4 T cell population. As this experiment was performed outside the context of pregnancy, it can be difficult to relate with the results obtained with the gestational model. Moreover, no systemic dissemination of bacteria was found in this model (data not shown), with clear contrast with data from the gestational model. This data is also suggestive that the rGAPDH vaccine can promote a heighten IFN-γ production, that in a systemic environment, as the spleen, could lead to an uncontrolled immune response during and after the pregnancy. Also, the differences observed could be associated with the level of bacterial colonization found in these animals. This experiment leads to higher levels of GBS in the vaginal mucosa, than the ones found in the gestational model, being even higher in the rGAPDH-vaccinated group. It is, therefore, reasonable to speculate that perpetuated presence of GBS could lead to IFN-γ.

In order to understand how the rGAPDH vaccine would influence GBS infection throughout the female reproductive of mice, ovaries, uterine horns, cervix and vagina were collected 7 days after GBS inoculation and were submitted to histological analysis.

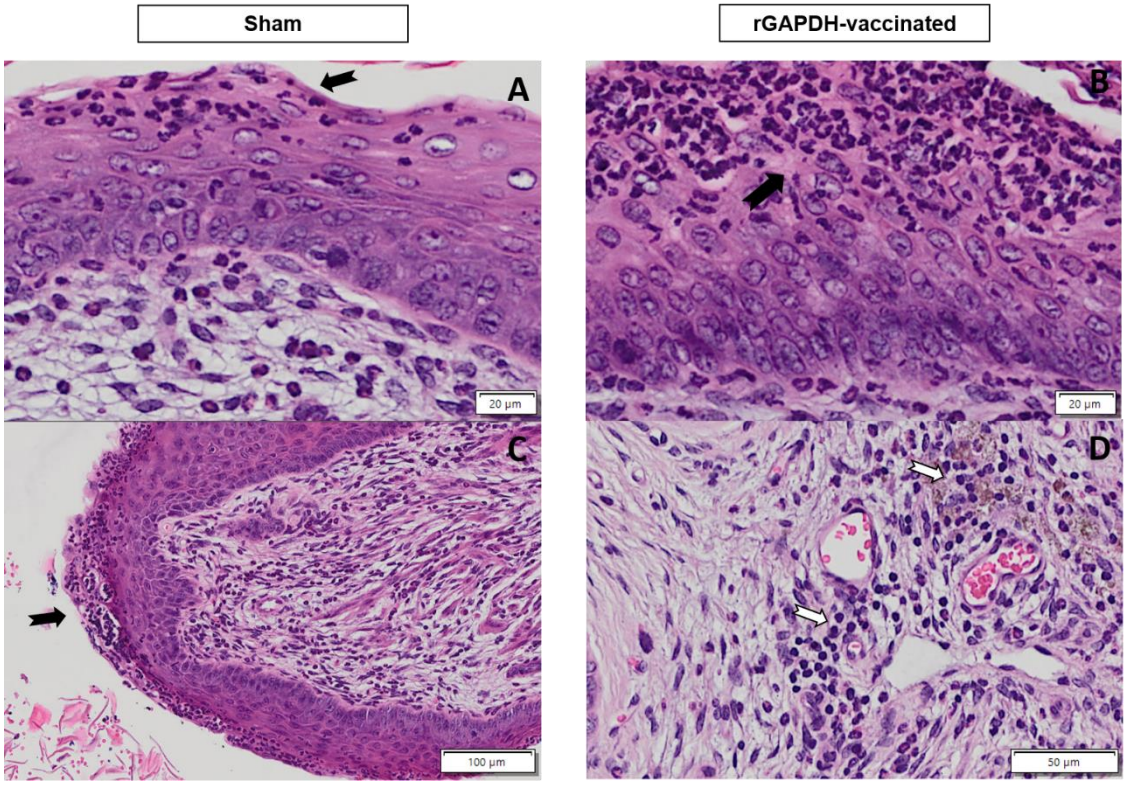

Figure 24. rGAPDH-vaccinated progenitors present more inflammation in the vaginal tract with lymphocytic and neutrophilic infiltration. Female BALB/c mice were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). Mice were injected subcutaneously with β-estradiol in sesame oil and after two days females were intra-vaginally inoculated with $2x10^8$ CFU of GBS BM110. Mice reproductive tissue was collected 7 days after inoculation and subjected to H&E staining. Representative sections are shown. Scale bar, 20, 50 and 100 µm. Black arrows indicate the presence of neutrophils and white arrows indicate lymphocytes.

Images of the vagina show an inflammatory response with many neutrophils on the vaginal epithelia, sometimes extending to its surface forming a layer, in both groups (Fig. 24 A-C). This inflammation is not continuous across the entire vaginal epithelia as only some regions are affected. Additionally, rGAPDH-vaccinated progenitors presented these patches of epithelial inflammation more often and were also more exacerbated than the ones found on Sham-vaccinated progenitors (Fig. 24 A,B). Both groups presented neutrophil recruitment to these areas and oedema on the stroma associated with inflammation (Fig. 24 C,D). Aggregates of lymphocytes were only found on the stroma of rGAPDH-vaccinated animals (Fig. 23D). These aggregates could constitute by T cells responsible for the high IFN-γ production observed in the flow cytometry results. Nonetheless, this immune response does not seem to be contributing to a diminish vaginal GBS colonisation as confirmed in Figure 21.

The cervix and ovaries of these females were also analysed but no pathology was found on those areas (data not shown).

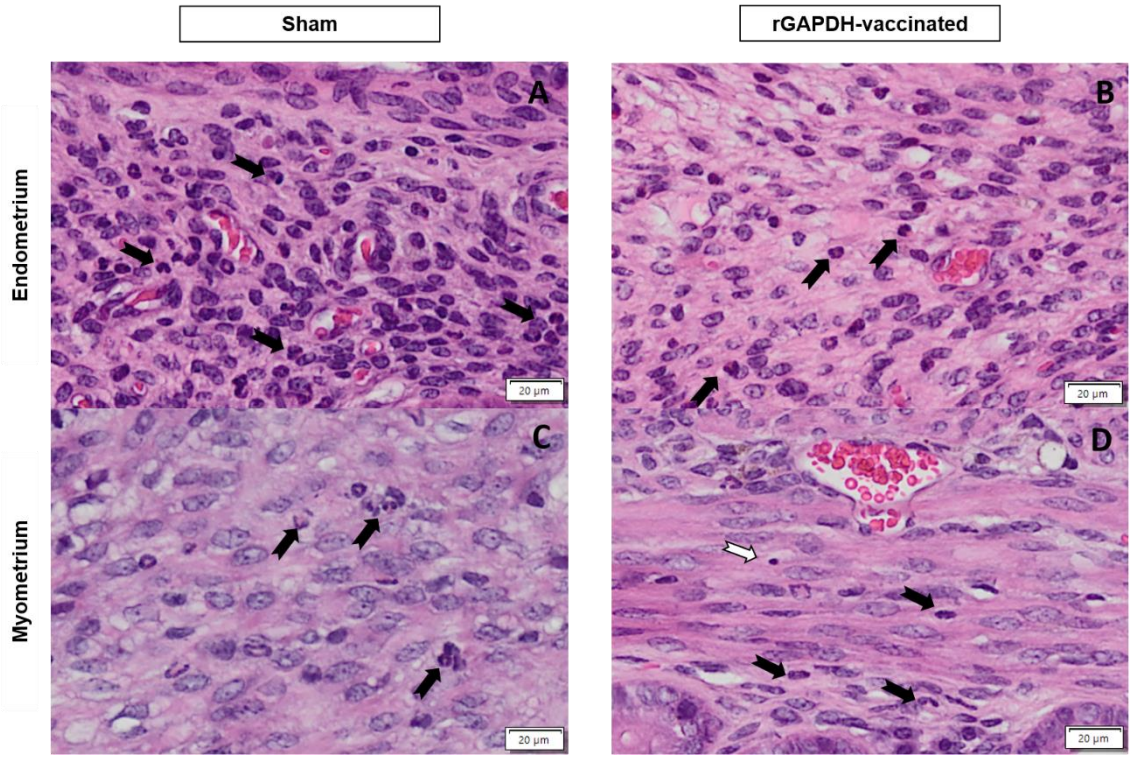

Figure 25. Neutrophils and lymphocytes are dispersed both across the endometrium and myometrium. Female BALB/c mice were immunized three times, spaced three weeks apart, with 25 µg of rGAPDH (rGAPDH-vaccinated) or Alum alone (Sham-vaccinated). Mice were injected subcutaneously with β-estradiol in sesame oil and after two days females were intra-vaginally inoculated with $2x10^8$ CFU of GBS BM110. Mice reproductive tissue was collected 5 days after inoculation and subjected to H&E staining. Representative sections are shown. Scale bar, 20 µm. Black arrows indicate the presence of neutrophils and white arrows indicate lymphocytes.

Another area of the reproductive tract analysed was the uterus. No differences were found between the two groups as all uterus presented dispersed neutrophils both on the endometrium and myometrium (Fig. 25), and they can be found on all tissues of the female reproductive tract under healthy conditions [158]. Similarly, some lymphocytes were also found dispersed on the endometrium and myometrium (Fig. 25).

Altogether, this data indicates that in the context of infection, both during pregnancy and outside of pregnancy, the rGAPDH vaccine seems to induce an augmented inflammatory state when compared to the Sham-vaccinated group, with increased local production of IL-17A, and increased systemic production of IFN-γ during the postpartum period. Moreover, data from non-pregnant females are suggestive that persistent GBS infection leads to IFN-γ production mainly by $CD4^+$ T cells. It is thus tempting to speculate that IFN-γ presents a dual function in the context of GBS infection: 1. being essential for GBS clearance; 2. low production could lead to GBS local

persistence in the uterus, increased neutrophil recruitment, and later dissemination. Its persistence in the host leads to induced heighten IFN-γ production systemically, and uncompensated immune response, which could culminate in disease.

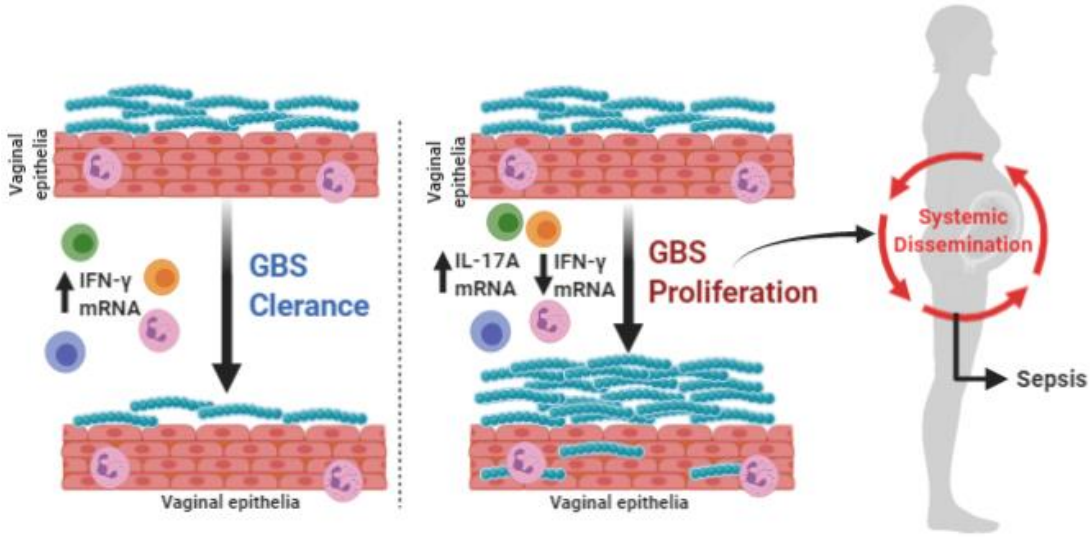

Figure 26. Schematic representation of the dual role of IFN-γ in the context of GBS infection. Small arrows symbolise augment (pointing upwards) or diminished (pointing downwards) production.

Taken into account the results obtained on non-pregnant females that presented a higher percentage of IFN-γ producing cells, it is necessary to understand if an exacerbated production of this cytokine would be detrimental for maternal health and studies on mice who receive a recombinant form of IFN-γ will be performed in the future. Moreover, the potential contribution of IL-17A to the local immunity against GBS will be analysed as heightened IL-17A mediated responses may also lead to pathology [159].

Our results highlight the need for the development of proper experimental design and development of accurate animal models in pre-clinical experiments. Changes in the infection route used to access the efficacy of the same vaccine, generated opposite results, with an effect on the immune response developed by rGAPDH-vaccinated animals and appearance of unexpected pathology in the same group.

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