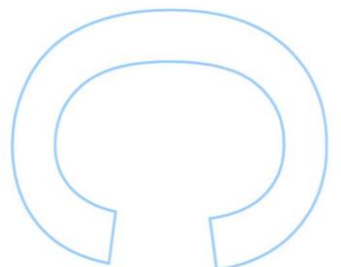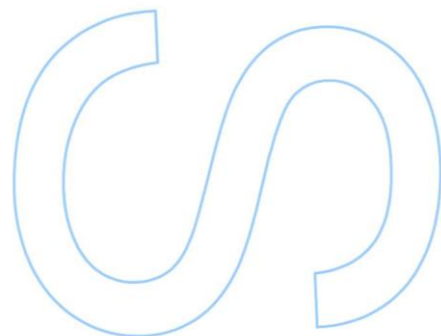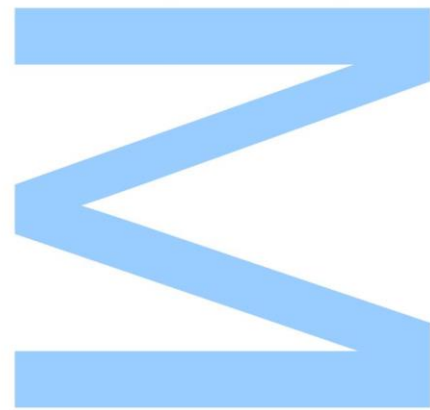# Maternal GAPDH vaccination: evaluation of haematopoietic changes in their offspring

Inês Sampaio e Lorga
Master's degree in Cellular and Molecular Biology
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Supervisors
Elva Bonifácio Andrade, Researcher, ICBAS-UP
Paula Ferreira Silva, Associate Professor, ICBAS-UP

Author
Inês Sampaio e Lorga
up201403198@fc.up.pt

Supervisors
Elva Bonifácio Andrade, Researcher
ebandrade@icbas.up.pt
Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto (ICBAS-UP)
Rua de Jorge Viterbo Ferreira, 228,
4050-313 Porto
+351 220 428 000
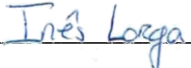
Paula Ferreira Silva, Associate Professor
pauferr@icbas.up.pt
Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto (ICBAS-UP)
Rua de Jorge Viterbo Ferreira, 228,
4050-313 Porto
+351 220 428 000
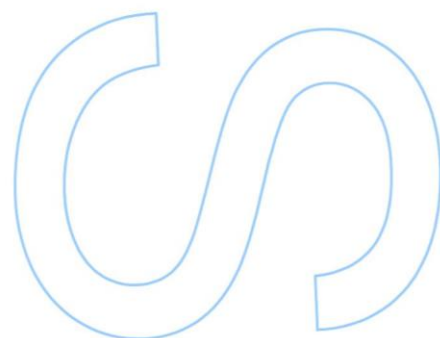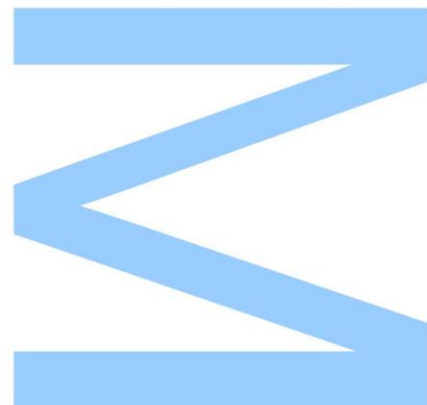
Declaração de Compromisso Anti-Plágio

Eu, Inês Sampaio e Lorga, aluna de Mestrado em Biologia Celular e Molecular da edição de 2017/2018, com o nº 201403198, declaro por minha honra que sou a autora da totalidade do texto apresentado, intitulado "Maternal GAPDH vaccination: evaluation of haematopoietic changes in their offspring", bem como do trabalho prático desenvolvido. Declaro ainda que reconheço as consequências disciplinares e legais de uma situação de plágio.

Porto, 22 de novembro de 2019

_____ Inês Lorga _____

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Resumo

A bactéria Estreptococos do grupo B (EGB), comensal dos tratos intestinal e urinário em 50% dos adultos saudáveis, é um agente patogénico para os recém-nascidos. A prevenção da infeção neonatal por EGB baseia-se na administração de antibióticos *intrapartum* a mulheres colonizadas com esta bactéria. No entanto, o crescente aumento da resistência desta bactéria a antibióticos, bem como o atraso na colonização da microbiota intestinal causado por este tratamento, tornam urgente o desenvolvimento de novas estratégias preventivas/terapêuticas. O nosso grupo de investigação identificou a forma extracelular da gliceraldeído-3-fosfato desidrogenase (GAPDH) como um potencial candidato a ser usado como uma vacina contra o EGB. No entanto, este enzima é altamente conservado, pelo que os anticorpos anti-GAPDH têm o potencial de reconhecer a GAPDH de bactérias filogeneticamente relacionadas. A colonização intestinal pela microbiota durante o período pós-natal tem um enorme impacto na saúde. Resultados não publicados pelo nosso grupo mostraram que os anticorpos produzidos contra a GAPDH do EGB reconhecem duas bactérias comensais isoladas do intestino neonatal do ratinho. Mais ainda, a vacinação materna com GAPDH parece interferir com o sistema imune da sua descendência. Recentemente, tem sido demonstrada a importância do microbioma intestinal em regular a hematopoiese. Assim, neste estudo, avaliamos o efeito da vacinação materna com GAPDH no desenvolvimento do sistema imune e na hematopoiese da sua descendência.

Com esse propósito, as fêmeas de murganho foram imunizadas com GAPDH recombinante (rGAPDH) juntamente com o adjuvante (grupo rGAPDH vacinado), ou apenas com o adjuvante (grupo Sham vacinado). A frequência e o número dos progenitores hematopoiéticos, bem como as populações mieloides e linfoides foram estudadas por citometria de fluxo na medula óssea, fígado e baço da descendência de ambos os grupos, ao longo do desenvolvimento. Os resultados mostram alterações significativas na hematopoiese das crias nascidas de progenitoras vacinadas com GAPDH, quando comparadas com o grupo nascido de progenitoras Sham vacinadas. Em particular, verifica-se uma diminuição significativa na frequência das células indiferenciadas pluripotentes na medula óssea e no baço, bem como uma tendência para uma diminuição da frequência das mesmas no fígado dos ratinhos nascidos de progenitoras vacinadas com GAPDH, no dia pós-natal (P) 5, quando comparadas com as crias nascidas do grupo controlo. Na medula óssea, os promielócitos (GMP, do Inglês *granulocyte-monocyte progenitors*) estão igualmente diminuídos nas crias nascidas de progenitoras vacinadas com GAPDH. No baço, as populações diminuídas são os

linfoblastos (CLP, do Inglês *common lymphoid progenitors*) e as células precursoras de megacariócitos e eritrócitos (MEP, do Inglês *megakaryocyte-erythroid progenitors*). No entanto, no fígado, o número total de mieloblastos (CMP, do Inglês *common myeloid progenitors*) e de GMP está aumentado nas crias nascidas de progenitoras vacinadas com GAPDH relativamente aos controlos, sugerindo um mecanismo compensatório. Mais ainda, o fígado e o baço das crias nascidas de progenitoras vacinadas com GAPDH apresentam um aumento significativo de células fagocíticas e monócitos Ly6C^{high} inflamatórios, quando comparados com crias provenientes de progenitoras Sham vacinadas. Embora menos afetadas, as células linfoides apresentam um aumento significativo na frequência e no número das células B e das células CD5$^+$ B-1 no fígado de ratinhos nascidos de progenitoras vacinadas com GAPDH comparativamente com os mesmos nascidos de progenitoras Sham vacinadas, em P5. O número de células CD5$^+$ B-1 no baço está igualmente aumentado no primeiro grupo, na mesma idade. Em P8, a frequência e número das células T e das células T $\gamma\delta$ estão diminuídos no fígado de crias provenientes de progenitoras vacinadas com GAPDH, quando comparados com os controlos.

Estes resultados levaram-nos a estudar a resposta imune neonatal à *Escherichia coli*. Desta forma, ratinhos recém-nascidos de progenitoras provenientes de ambos os grupos, foram infetados oralmente com *E. coli* IHE3034 (uma estirpe K1), em P4. Não foram encontradas diferenças na percentagem de sobrevivência. Três dias após infeção os ratinhos foram eutanasiados para determinar a colonização bacteriana no sangue, fígado, pulmões e cérebro. Não foram verificas diferenças entre os grupos.

Adicionalmente, estudamos o impacto da vacinação materna no metabolismo da sua descendência. A análise histopatológica do fígado, através da coloração de H&E, demonstra que o metabolismo dos ratinhos de progenitoras vacinadas com GAPDH está atrasado/alterado, relativamente ao verificado nos ratinhos controlo. É verificada uma maior vacuolização nos ratinhos nascidos de progenitoras vacinadas com GAPDH, aos dias P8 e P14, comparativamente com os respetivos controlos. Mais ainda, fêmeas provenientes de progenitoras vacinadas com GAPDH parecem ter uma maior predisposição para um aumento de peso quando submetidas a uma dieta rica em lípidos durante 30 dias.

Concluindo, nesta dissertação é descrito o efeito negativo da vacinação materna com GAPDH quer nos precursores hematopoiéticos, quer nas populações imunes maduras, na sua descendência. É ainda mostrado um efeito para além do período neonatal, com uma provável incidência de doenças metabólicas.

Abstract

Group B *Streptococcus* (GBS), a commensal of the human intestinal and genitourinary tract in up to 50% of healthy adults, is a life-threatening pathogen for neonates. The prevention of GBS neonatal infection is based on *intrapartum* antibiotic administration to colonised women. However, the increasing bacterial resistance to antibiotics, along with the delay of gut microbiota colonisation caused by this treatment, makes the development of new strategies mandatory. Our research group identified the extracellular form of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a valuable GBS vaccine candidate. Nevertheless, GAPDH is a highly conserved protein and anti-GAPDH antibodies have the potential to recognize phylogenetically related bacteria. Gut microbial colonisation during the post-natal and early infant periods have a profound influence on both health and disease. Unpublished work by our group showed that antibodies produced against GBS GADPH recognize two commensal bacteria of pup's intestines. Moreover, maternal GAPDH vaccination seems to interfere with their offspring's immune system, at post-natal day (P) 8. Emerging data show the importance of the intestinal microbiome in regulating and shaping haematopoiesis. Therefore, in this study, we evaluated the effects of maternal GAPDH vaccination on the neonatal haematopoiesis and immune system development of their offspring.

For that purpose, female mice were immunized with recombinant GAPDH (rGAPDH) and the adjuvant Alhydrogel (rGAPDH-vaccinated group) or only with the adjuvant (Sham-vaccinated group). The frequency and number of haematopoietic progenitors, as well as the myeloid and lymphoid mature populations were studied by flow cytometry in the bone marrow (BM), liver and spleen of pups born from both groups, throughout development. Results show significant alterations in the haematopoiesis of pups born from rGAPDH-vaccinated dams when compared to those born from Sham-vaccinated mothers. Specifically, significantly decreased frequency of haematopoietic stem cells and multipotent progenitors (LSK) is observed in the BM and spleen, and a tendency to decrease frequency in the liver, in pups born from rGAPDH-vaccinated mothers, at P5, compared to the ones born from Sham-vaccinated mothers. In the BM, granulocyte-monocyte progenitors (GMP) are also significantly decreased in the rGAPDH-vaccinated progeny, whereas in the spleen the diminished populations are common lymphoid progenitors (CLP) and megakaryocyte-erythroid progenitors (MEP). However, in the liver, the total number of common myeloid progenitors (CMP) and GMP are increased in pups born from rGAPDH-vaccinated mothers relative to controls, suggesting a compensatory mechanism. Moreover, both the liver and spleen of pups

from the rGAPDH-vaccinated mothers have a significant increase in phagocytic cells and inflammatory Ly6C^{high} monocytes, compared to those born from Sham-vaccinated controls. The lymphoid populations are less but significantly affected, with an increase in both frequency and number of B cells and CD5$^+$ B-1 cells, in the liver of pups born from rGAPDH-vaccinated progenitors comparing to those born from Sham-vaccinated females, at P5. The splenic number of CD5$^+$ B-1 cells is also increased in the former group, at P5. At P8, both the frequency and number of T cells and $\gamma\delta$ T cells are decreased in the liver of pups from the rGAPDH-vaccinated mothers, comparing to the Sham-vaccinated progeny.

These results prompted us to study neonatal immune response to *Escherichia coli*. For that, newborn mice born from Sham- and rGAPDH-vaccinated mothers, were orally infected with *E. coli* IHE3034 (a K1 strain), at P4. No differences were found in the percentage of survival of pups born from Sham- or rGAPDH-vaccinated mothers. Three days post-infection mice were sacrificed to assess their blood, liver, lungs and brain for bacterial colonisation. No differences were found between groups.

In addition, we studied the impact of maternal vaccination on the metabolism of their offspring. Histopathological liver analysis, through H&E staining, demonstrates an altered/delayed metabolism in pups born from rGAPDH-vaccinated mothers, when compared to those born from Sham-vaccinated females. Higher vacuolation in the pups born from rGAPDH-vaccinated mothers is observed at P8 and P14 than in those born from Sham-vaccinated progenitors. Additionally, the female progeny of rGAPDH-vaccinated progenitors seems to be more predisposed to weight gain, when submitted to a high-fat diet for 30 days.

In conclusion, in this thesis, we describe a negative effect of maternal immunization with rGAPDH in both haematopoietic progenitors and mature immune populations of their progeny, and uncover an impact beyond the neonatal period, with a likely probability towards metabolic disorder.

Keywords

GBS, GAPDH maternal vaccination, neonatal haematopoiesis, gut microbiota, infection, host metabolism

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Abbreviation List

A

AGM- Aorta-gonad-mesonephros
ALT- Alanine aminotransferase
AP- Alkaline Phospatase
APC- Antigen-presenting cell
ATP- Adenosine Triphosphate

B

BBB- Blood-brain barrier
BM- Bone Marrow
BSA- Bovine Serum Albumin

C

CD- Cluster of Differentiation
CFU- Colony-forming units
CLP- Common lymphoid progenitors
CMP- Common myeloid progenitors
CNS- Central Nervous System
CPS- Capsular Polysaccharides

D

DAP- Diaminopimelic acid
DC- Dendritic cell
DGAV- Direção Geral de Alimentação e Veterinária
DNA- Deoxyribonucleic acid
DPBS- Dulbecco's Phosphate-Buffered Saline
dpi- days post-infection

E

EDTA- Ethylenediaminetetraacetic acid
ELISA- Enzyme-Linked Immunosorbent Assay
EOD- Early-onset diseases
ExPEC- Extraintestinal pathogenic *Escherichia coli*

F

FcRn- Neonatal Fc receptor
FVD- Fixable viability dye

G

G- Gestational day
GAPDH- Glyceraldehyde-3-phosphate dehydrogenase
GBS- Group B *Streptococcus*
G-CSF- Growth colony-stimulating factor
GF- Germ-free

GMP- Granulocyte-monocyte progenitors

H

H&E- Haematoxylin and eosin
HFD- High-fat diet
HSC- Haematopoietic stem cell

I

IAP- *Intrapartum* antibiotic prophylaxis
Ig- Immunoglobulin
IL- Interleukin
ILC- Innate lymphoid cell
ILF- Intestinal lavage fluid
IRF3- Interferon Regulatory Factor 3

J

K

L

LOD- Late-onset diseases
LPS- Lipopolysaccharide
LSK- Lin$^-$Sca-1$^+$c-Kit$^+$

M

MDSC- Myeloid-derived suppressor cells
MEP- Megakaryocyte-erythroid progenitors
M-MDSC- Monocyte myeloid-derived suppressor cells
MyD88- Myeloid differentiation factor 88
MZ- Marginal zone

N

NAD- Nicotinamide adenine dinucleotide
NAFLD- Non-alcoholic fatty liver disease
NASH- Non-alcoholic steatohepatitis
NBH- Neutrophil B helper
NK- Natural Killer cell
NOD- Nucleotide-binding oligomerization domain

O

OCT- Optimal cutting temperature
ORO- Oil Red O

P

P- Post-natal day
PBS- Phosphate-buffered saline
PIg- Plasminogen

Q

R

RA- Retinoic acid
rGAPDH- Recombinant GAPDH
RT- Room temperature

S

s.c.- subcutaneously
SCFA- Short-chain fatty acid
ST- Sequence type

T

T2D- Type 2 diabetes
Th- T Helper cell
TH- Todd-Hewitt
TLR- Toll-Like Receptor
TNF- Tumour Necrosis Factor
TRAF3- TNF Receptor Associated
Factor
Treg- Regulatory T cell

U

UNICEF- United Nations Children's
Fund

V

VIP- Virulence-associated
immunomodulatory protein

W

WHO- World Health Organization

X

Y

YS- Yolk sac

Introduction

Group B Streptococcus and neonatal diseases

According to the World Health Organization (WHO) and United Nations Children's Fund (UNICEF) joint statement, approximately one-fifth of the world's annual 2.7 million neonatal deaths are due to infections (1). *Streptococcus agalactiae*, more commonly known as Group B *Streptococcus* (GBS), is the leading cause of life-threatening neonatal bacterial infections (2, 3).

GBS, a Gram-positive, diplococcus, facultative anaerobic, β-haemolytic human commensal bacterium (4, 5), is present in the urogenital and gastrointestinal tracts of up to 50% of healthy adults (6). Regarding pregnant women, it is estimated that nearly 20% worldwide are colonised with this bacterium in the vaginal mucosa (7, 8), representing a risk factor for transmitting the infectious agent to the newborns during the perinatal period. Neonatal bacterial infections can be caused by vertical transmission, *i.e.*, from mother to child, during labour or *in utero* (5, 9), or by horizontal transmission, through breast milk (10), via health care workers and other infants (11-13).

Neonatal GBS infections may lead to two types of diseases, depending on the time in which pathologic manifestations begin: early-onset diseases (EOD) if it is developed in the first week of life (0-6 days), and late-onset diseases (LOD) if it occurs between the first week and three months of life (7-90 days) (5, 15). EOD is typically related to vertical transmission of GBS during birth and it is characterized by pneumonia, respiratory failure, and septicaemia (15, 16). LOD is associated with both vertical and horizontal transmission to the newborns and it is characterized by bacteraemia and meningitis (5,
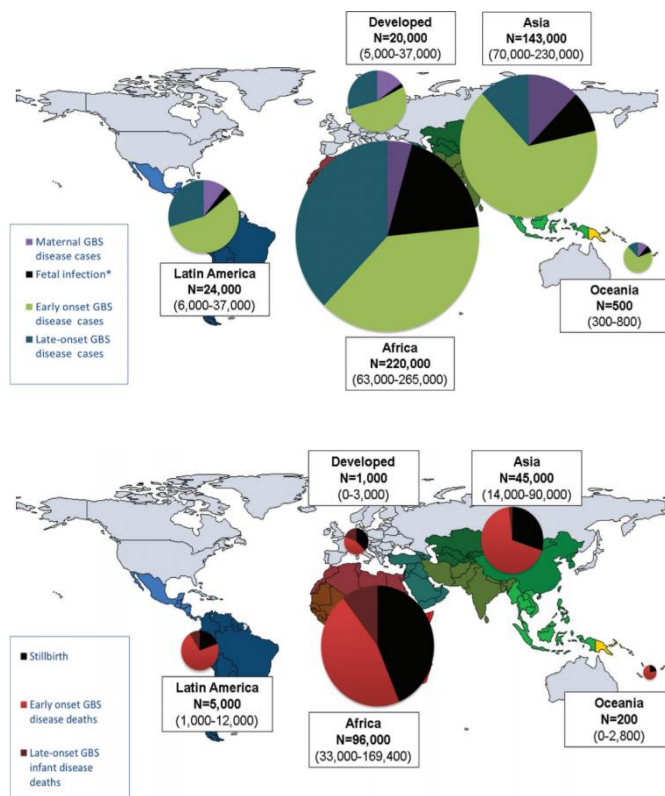
Figure 1 | Estimated cases for *Group B Streptococcus* diseases (upper image) and infants' deaths (lower image) worldwide. *Stillbirths represent a minimum estimate of foetal infection cases. Adapted from (14).

15). The mortality rates for EOD and LOD are 10% and 2-6%, respectively, and, among the survivors, approximately 50% suffer from permanent neurological sequelae, such as

deafness, uncontrolled seizures, hydrocephalus, hearing loss, cortical blindness, and speech and language delay (6).

GBS is currently divided in ten different serotypes (Ia, Ib, II-IX), based on the immunogenic capsular polysaccharides (CPS) that it possesses, a feature that facilitates immune system evasion by the bacterium, promoting colonisation and invasive disease (5, 17). Serotypes Ia, Ib, II, III and V are the most frequently found in neonatal diseases, with serotype III being responsible for a significant proportion of EOD and the most prevalent in LOD. The serotype III strains belonging to the sequence type 17 (ST-17) are referred to as hypervirulent strains, being associated with the majority of GBS meningitis cases (80%) (5, 18, 19).

GBS GAPDH: a virulence factor

GBS is a commensal in adults and a pathogen in neonates. Its pathogenicity is due to several virulence factors such as adhesion factors (like cell-surface pili proteins), pore-forming toxins and immune evasion factors (as CPS). These factors contribute to either GBS adhesion and/or modulation of the host immune response, increasing its survival, or to GBS mobility, facilitating the migration across the blood-brain barrier (BBB) and the intestinal epithelium (20, 21).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a well-known glycolytic enzyme, responsible for the production of adenosine triphosphate (ATP) through catalysation of oxidative phosphorylation of D-glyceraldehyde 3-phosphate into 1,3-bisphosphoglycerate, with the consumption of nicotinamide adenine dinucleotide (NAD) (22). Despite its original catalytic function, GAPDH has several different functions being, therefore, called a "moonlight protein" (23). This enzyme was identified by our group as an important virulence factor for GBS host immune evasion (24). It can be found in the cell cytoplasm, the main location, on the cell surface and in the extracellular space (25). Our group showed that GAPDH present on GBS surface is a host plasminogen receptor, which converts plasminogen into plasmin, a protease that, upon activation by host-derived activators, leads to fibronectin degradation, a major extracellular matrix protein (26). This promotes bacterial invasion of the central nervous system (CNS) via BBB migration, contributing to meningitis (27). In the extracellular space, our group identified GAPDH as a virulence-associated immunomodulatory protein (VIP) since it prevents the development of an immune response against GBS, by inducing the early production of anti-inflammatory cytokine Interleukin 10 (IL-10), favouring the host-

microbial colonisation (28, 29). Furthermore, we also showed that GBS infection induces high levels of this immunosuppressive cytokine through Toll-Like Receptor 2 (TLR2), impairing neutrophils recruitment, cells of the innate immune system responsible for the first line of defence against bacterial invasion (28). Moreover, maternal GAPDH vaccination, or passive immunization of neonates with anti-GAPDH immunoglobulin G (IgG) antibodies, confer protection to pups due to neutralization of IL-10 production, increasing neutrophil recruitment to infected organs, leading to bacterial elimination (29).

Preventing neonatal GBS infections- Current Treatment

The current guidelines for the prevention of GBS infections rely on prenatal screenings using vaginal and rectal cultures in pregnant women between 35 and 37 weeks of gestation and *intrapartum* antibiotic prophylaxis (IAP) to women colonised with GBS (5, 30, 31). This treatment reduced GBS colonisation, helping to decrease deaths due to EOD (32, 33), but it had no impact on LOD prevention since IAP did not diminish LOD incidence nor LOD-associated deaths (3, 34). Additionally, the widespread administration of antibiotics has led to the emergence of new GBS and *Escherichia coli* resistant isolates, having become a major issue for the scientific community (33, 35). Moreover, several studies have already reported the negative impact of the use of antibiotics in the gut microbiota of newborns, by delaying the appearance of beneficial bacteria and/or by leading to an incomplete microbiota recovery after antibiotic administration (36-38). Gut microbiota modifications can have serious implications for the neonates' health, since they play an important role in the correct post-natal development of systems, including the brain, gut, metabolic and immune systems. Therefore, gut microbiota alterations can lead to the development of numerous diseases, such as obesity and inflammatory bowel disease (39).

Taking all these concerns into account, a new and effective approach is necessary to protect neonates from GBS diseases, such as maternal vaccination (6, 31, 34, 40).

Maternal vaccination against GBS

Maternal immunization has the potential to decrease the risk of infection, reducing the morbidity and mortality after birth. This vaccination strategy confers protection through vertical transmission of specific IgG across the placenta (41). Several vaccines

against GBS have been developed using two different approaches: the conventional ones, based on the cultivation of pathogens and identification of possible antigens through standard biochemical and microbiological techniques, and new approaches based on genomics, proteomics, gene expression and *in situ* technologies for identification of possible candidates (6). The first evidence that maternal GBS vaccination could prevent invasive GBS disease in their offspring dates back to the 1970s, where studies have demonstrated an inverse correlation between the levels of maternal serotype-specific capsular antibodies and newborns' susceptibility to GBS disease (6, 42).

The first developed vaccines used CPS as an antigen in the unconjugated form and later coupled with tetanus toxoid, to improve efficacy. Despite decreasing the risk of infection, the existence of various GBS serotypes with diverse CPS composition and different geographic distribution increases the challenge of producing a globally effective vaccine against this bacterium (6, 41). Alternative vaccines have been developed targeting conserved surface proteins of GBS, such as pili. These proteins are also GBS virulence factors but, unlike CPS, are common in all invasive strains. However, pili proteins are not essential for GBS survival, being able to suffer mutations for a survival strain (43, 44). Therefore, the ideal target to be used in a human GBS vaccine is an immunogenic, surface-exposed virulence factor, essential for GBS growth/survival, being present in all GBS strains (*i.e.*, a structurally conserved target) (40).

Our group developed a vaccine against GBS GAPDH and since it is a conserved glycolytic enzyme, indispensable for GBS energy production, is less susceptible to selective pressure. Moreover, we showed that GAPDH is present in the supernatants of several GBS isolates, belonging to different serotypes (29). Therefore, GAPDH has great potential as a target for a human vaccine development against GBS, which may overcome the problems presented by the vaccines mentioned above.

Our group demonstrated that the antibodies raised against GBS GAPDH did not recognize the native nor the denatured form of human GAPDH (29). We showed that GAPDH vaccine protects neonates and susceptible adult mice from GBS infections (29, 40). Lastly, all parameters required to establish the safety and stability of this vaccine candidate were tested in pre-clinical trials (40). However, given the high conservation of GAPDH in all species, the antibodies produced through maternal vaccination may be able to recognize not only the GBS GAPDH but also GAPDH from other species present in the human microbiota, possibly interfering with the normal neonatal colonisation, that, in turn, could influence early-life immune system development (22).

The neonatal immune system development

The immune system starts to develop *in utero* by 3-4 weeks of gestation in human and on embryonic day 8 in mice (45). The first haematopoietic stem cells (HSCs) derive from the yolk sac (YS) and the ventral wall of the aorta in the aorta-gonad-mesonephros (AGM). Around week 5 of human gestation and embryonic day 10 in mice, HSCs migrate to the foetal liver, where they suffer expansion, maturation and give origin to erythroid, myeloid and lymphoid cells. Thus, the foetal liver is the main haematopoietic site during human and mice gestation and this organ is responsible for the HSCs colonisation of foetal spleen and thymus. In these organs occur B and T cells differentiation, respectively, and HSCs expansion but not the *de novo* generation. In the second trimester of human gestation and just before birth in mice, the bone marrow (BM) is colonised and is responsible for HSCs maintenance and haematopoiesis throughout life (45-48). This transitional phase implies that, during the neonatal period, the liver and spleen are still haematopoietic organs (49).

Newborns lack immunological memory because environmental exposure to antigens before birth is limited, relying only on the innate immunity for protection (50). However, this system is not fully developed, being the innate cells less polyfunctional, with each one producing fewer cytokines when compared to adult innate cells (48). Therefore, the unique characteristics of the neonatal immune system make it more susceptible to infectious diseases. The neonatal immune system is essentially controlled by immunosuppressive mechanisms in order to avoid massive inflammatory responses that are harmful to the neonate because, during this period, it is highly exposed to bacterial colonisation (51). Antigen-presenting cells (APCs) produce less pro-inflammatory cytokines, such as Tumour Necrosis Factor (TNF-α) and IL-1β, and T helper 1 (Th1) promoting cytokines (52). However, neonatal APCs are able to produce equal levels of Th17 promoting cytokines, like IL-6, when compared to adult APCs, enhancing resistance to infection and healing of injured tissues (53, 54). Moreover, neonatal monocytes (appearing right after HSCs seed the foetal liver) and dendritic cells (DCs) produce more IL-10 than the ones from adults, highlighting the importance of anti-inflammatory responses in early life (51).

Neutrophils are the dominant leukocytes at birth, being crucial for the innate immune system since they are the first circulating immune cells to defend against infectious microorganisms. The number of circulating neutrophils in the first week of life can exceed the number of adult neutrophils (48). Besides circulating neutrophils, these cells have storage pools in the BM, liver and spleen. However, neonatal neutrophils have

reduced ability to adhere and extravasate from the bloodstream and lower chemotaxis activity (48, 55). Also, neutrophils are less able to release bactericidal substances, to form extracellular traps and to phagocyte (48, 55).

Regarding Natural Killer (NK) cells, they reach maximum levels at birth, meaning that neonates present a considerable quantity of these cells compared to adults (48). Nevertheless, NK cells' cytotoxicity is much lower in neonates, probably due to poor levels of activating cytokines (48). The complement system is also vital as a component of the innate immune system since it contributes to cell recruitment to the infection site, to opsonization and pathogen lysis. Nevertheless, proteins from this system are found in lower quantities in neonates (48, 56).

Adaptive immunity starts to develop before birth, since T and B cell progenitors can be detected in the foetal liver from the 8th week of gestation and are abundant at birth, albeit the poor functionality (57). Both cell types massively increase during the first weeks of life, exceeding adult's cell numbers, and can develop into memory cells (48, 57). T cells are divided into cytotoxic CD8$^+$ T lymphocytes, responsible for pathogen killing, and CD4$^+$ T helper cells, responsible for providing cytokine stimulus to other cells, enhancing their functions. Neonatal CD8$^+$ cells are less responsive and require more stimulus than adult ones (48). CD4$^+$ cells are further subdivided into different subclasses of effector cells (Th1, Th2 and Th17) and regulatory T cells (Tregs) (48, 57). Neonatal effector cells present different implications on pathogen's elimination capacity, depending on the dominant type (48). While Th1 cells induce the inflammatory responses to infectious agents (51), a Th2 polarization leads to the opposite function, increasing the production of anti-inflammatory cytokines (58). Th17 cells increase neonatal resistance to infections, as described above (51). Regarding Tregs, these cells contribute to the immunosuppressive responses of neonates (48, 57).

Neonatal B cells are mainly naïve and immature, consisting predominantly of transitional 1 and 2 B cells (48, 59-62). IgM is the first and most commonly found antibody secreted by B cells, after antigen encounter, followed by IgG and IgA, which are formed after class-switch events (48). The neonatal antibody responses are delayed and present decreased ability to class-switch (62, 63). Naïve B cells also express low levels of cell-surface receptors such as CD40, CD80, and CD86, required for its activation and proliferation (64).

Given the immaturity of neonatal humoral immunity, the protection of neonates largely depends on maternal transfer of IgG across the placenta, mainly during the third

trimester, and IgA through breast milk (65, 66). These antibodies confer immunity to the newborn and protect against enteric infections.

In sum, the neonatal immune system is still in development in early life, and its unique features appear to promote tolerance to initial bacterial colonisation. Due to this, newborns rely on mother's passive immunization for protection against bacterial infections.

The crosstalk between gut microbiota and the immune system

More than 100 trillion microbes are present in the human body, including approximately 1000 bacterial species carrying 150 times more genes than the entire human genome (39). The commensal colonisation of neonates is crucial for the correct development of the immune system (67-69). Recently, experimental and clinical studies demonstrated the presence of microbial communities harboured in the placenta and amniotic fluid, as well as of intrauterine bacterial DNA, indicating that microbial colonisation may start even before birth, *in utero* (70, 71). This is important to help the neonate adapting to extrauterine life at immune and brain levels (69, 72, 73). Notwithstanding, the major exposure to microbes is after birth and microbiome development is dependent on several factors, such as maternal microbiota, method of delivery, diet and administration of antibiotics (74).

The normal intestinal bacterial colonisation is composed of five phases: phase one comprises the intrauterine colonisation and phase two the extrauterine colonisation from mother's vaginal microbiota; phase three corresponds to colonisation from oral feeding, either from breast milk or formula, and phase four from solid food, after weaning.

Bacterial colonisation is complete in phase five, at two years of age (69). The human microbiome differs in composition and function depending on several factors, like region, age, sex, ethnicity and host diet, being as unique to the individual as a fingerprint (75).

Gut microbiota, rather than causing inflammation, is beneficial for the intestinal immune system and general health. Colonizing bacteria stimulate enterocytes

Figure 2 | Gut microbiota has active participation in the modulation of the immune system.

and mucosal macrophages, lymphocytes and DCs, as well as prime Th cells subsets, which work towards immune homeostasis and defence against pathogens at sites of colonisation (67, 69, 76). Dysbiosis (*i.e.*, abnormal colonisation of the intestine) can alter the intestinal immune system, leading to several pathologies, such as inflammatory bowel disease and autoimmune diseases (77). However, the impact of commensal microbiome is not only limited to the immune system at mucosal sites, being able to interfere at systemic level. Changes in the microbial composition modulate the microbial molecules, which can be sensed by the immune system through TLRs, dictating the tone of the immune response (78). Furthermore, lipopolysaccharide (LPS)-induced production of IL-6 was found to be inversely correlated to the relative amount of commensal bacterium *Bacteroides fragilis* (79). Thus, microbiota shapes TLR and LPS responsiveness, influencing systemic immunity. Moreover, in mouse models of multiple sclerosis and autoimmune arthritis, the presence of specific bacteria and consequent Th17 induction lead to systemic inflammation and autoimmunity, reinforcing the role of gut microbiota in the systemic immune response (80).

Recently, several studies have been focused on haematopoietic tissues in order to understand the impact of the gut microbiome on haematopoiesis (68, 81-84). Experiments with antibiotic-treated or germ-free (GF) adult mice demonstrated to negatively affect haematopoiesis in both primary (BM, thymus and foetal liver) and secondary (spleen) lymphoid tissues due to decreased or altered microbiota/microbiota signals (68, 81-84). Myelopoiesis in BM, foetal liver and spleen, as well as lymphopoiesis in BM and thymus, were reduced due to decreased numbers of HSCs, common lymphoid and myeloid progenitors and differentiated cells, such as neutrophils, monocytes and B cells (68, 81-84). Besides, there was a reduction in spleen and thymus' weight, reinforcing the lower B and T cell generation, respectively (83). However, regarding neonatal mice, few studies have been performed so far, although one already reported an altered myelopoiesis in the BM of 3-14 days old mice born from antibiotic-exposed progenitors, with reduced number of cells' populations, at different stages of differentiation (82). Some of these studies presenting impaired myelopoiesis in mice lacking normal microbiota also showed that it leads to increased susceptibility to bacterial pathogens, such as *Listeria monocytogenes*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* (81, 82, 84).

In addition to neonatal commensal bacteria, maternal microbiota-derived compounds were also proven to be important for the offspring's haematopoiesis. Retinoic acid (RA), a metabolite of vitamin A (retinol), was shown to accelerate B cell differentiation in the BM, as the implantation of *trans*-retinoic acid to mice significantly

decreased lymphoid progenitors with concomitant increase of B cells, and *in vitro* experiments shortened the interval between lymphoid primitive progenitors and differentiated B cells (85). Moreover, an *ex vivo* treatment of the different cell populations of B lineage of the foetal liver with RA decreased B lymphopoiesis (86). Additionally, in the spleen, short-chain fatty acids (SCFAs) appear to regulate lymphopoiesis, influencing Treg differentiation in the offspring (87).

The mechanism by which microbiota can influence haematopoiesis is still unclear, but it is thought to be through the interaction between microbial compounds, such as LPS, and TLR4 and myeloid differentiation factor 88 (MyD88). LPS is a ligand for TLR4, which is upstream of MyD88. This interaction, although through an unclear pathway, leads to induction of IL-17 by group 3 innate lymphoid cells (ILCs), increasing plasma levels of granulocyte colony-stimulating factor (G-CSF) and, consequently, the number of BM and circulating neutrophils (81, 82, 88). Other proposed mechanism is through nucleotide-binding oligomerization domain-containing protein 1 (NOD1) activation by bacterial amino acid meso-diaminopimelic acid (DAP), inducing several haematopoietic cytokines (89, 90). Both pathways share the same downstream signalling cascade, with the intervention of the same molecules, such as TNF receptor-associated factor (TRAF3), which signals to interferon regulatory factor 3 (IRF3) to induce interferon production, interfering with haematopoiesis in a similar way (90).

Thus, gut microbiota plays a crucial role in the development and functioning of the neonate's immune system and, consequently, is determinant for the infant's health.

The host-gut microbiota metabolic symbiosis

Metabolic disorders, such as obesity and type 2 diabetes (T2D), are a major concern nowadays since their prevalence has been exponentially increasing in the last decades, reaching an overwhelming stage worldwide (91-97). About one-third of the adult world population is classified as overweight or obese (91, 98), and 1 in 11 adults has diabetes mellitus, 90% of whom have T2D (92). Regarding children and adolescents, overweight and obesity have also been alarmingly increasing worldwide (91, 95), being estimated that in the next few years this health problem will only aggravate, as well as its consequences, namely T2D (99-101). Among several factors, such as the current sedentary lifestyle, increased food consumption/calorie intake and genetic factors (102-105), there is growing evidence that the gut microbiota plays an important role in the metabolic homeostasis and, consequently, on metabolic diseases (106-108).

The gut microbiota is a key factor in the normal host metabolism, contributing to important physiological processes such as digestion, bile acid and vitamin synthesis, production of SCFAs, amongst others (109). When gut microbiota is altered, it can lead to metabolic disturbances, predisposing the host to obesity and associated comorbidities (108, 110). The pathways linking the gut bacterial composition to metabolic diseases are not fully understood, but there are already several proposed mechanisms (summarized in Table 1): specific gut microbiota can ferment polysaccharides that are not metabolized by humans, increasing SCFAs production which, in one hand, leads to lipid and triglyceride storage (111-113) and, on the other hand, activates nuclear factors like

Table 1 | Current proposed mechanisms for gut microbiota metabolic modulation that may predispose the host to obesity and diabetes. From (110).

Mechanism	Molecular mediators	Ultimate effects
Complex polysaccharide (starch, glycans) degradation to monosaccharides	Microbial transport proteins, glycoside hydrolases, and fermentation enzymes	Increased CHO uptake from the diet
Increased glucose absorption	Increased Glut1 expression in small intestine enterocytes	Increased CHO enterocyte absorption
Increased monosaccharide transfer to portal circulation	Increased microbiota-driven density of capillaries underlying the small intestinal villus epithelium	Increased CHO portal flow to the liver
Enhanced de novo lipogenesis	ChREBP- and SREBP-1-mediated expression of lipogenic enzymes	Increased liver and adipose tissue Tg accumulation
Increased hydrolisis of circulating Tg-rich lipoproteins	Reduced intestinal Fiaf secretion, leading to increased adipose tissue LPL activity	Increased storage of circulating Tg in adipose tissue
Reduced FFA oxidation	Reduced Fiaf-induced (PGC)-1α expression of mitochondrial FFA oxidative enzymes	Reduced FFA oxidation in liver and muscle
Reduced FFA oxidation	Reduced AMPK-induced expression of mitochondrial FFA oxidative enzymes	Reduced FFA oxidation in liver and muscle
Reduced intestinal transit time and increased L-FABP enterocyte expression	Increased Gpr41-mediated PYY secretion induced by microbial production of SCFA from dietary polysaccharides	Increased energy harvest from the diet
Modulation of host liver and adipose tissue fatty acid composition	Increased linoleic acid conversion to c9,t11 CLA by gut microbiota, increased liver and adipose tissue abundance of DHA and EPA through unknown mechanisms	Altered tissue composition of biologically active fatty acids
Modulation of bile acid enterohepatic cycle through bacterial deconjugation and dehydroxylation of primary bile acids	Modulation of intestinal lipid absorption, and hepatic and adipose tissue gluconeogenesis, de novo lipogenesis, FFA oxidation, and triglyceride-rich lipoprotein metabolism through FXR activation and cAMP-dependent thyroid hormone activation	Modulation of bile acid–regulated energy homeostasis, glucose and lipid metabolism in the liver and adipose tissue
Reduction of choline bioavailability for synthesis of phosphatidylcholine in the liver	Microbial conversion of dietary choline to hepatotoxic methylamines	Hepatic accumulation of toxic methylamines; impaired hepatic VLDL secretion, resulting in NAFLD
Production of LPS by gut microbiota	LPS-TLR4-mediated induction of proinflammatory cytokines SOCS-1, SOCS-3, IL-6, TNF-α, MCP-1 in adipose tissue, liver and macrophages	Systemic, hepatic, and adipose tissue inflammation and insulin resistance
Regulation of GLP-2 secretion by intestinal enteroendocrine L cells	Unknown signaling pathways linking gut microbes to L cells	Modulation of intestinal barrier function

Abbreviations: AMPK, enzyme adenosine monophosphate–activated protein kinase; CHO, carbohydrates; ChREBP, carbohydrate response element binding protein; cAMP, cyclic adenosine monophosphate; CLA, conjugated linoleic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FFA, free fatty acid; FXR, farnesoid X receptor; GLP, glucagon-like peptide; IL, interleukin; L-FABP, liver fatty acid binding protein; LPL, lipoprotein lipase; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; NAFLD, nonalcoholic fatty liver disease; PGC, peroxisomal proliferator-activated receptor coactivator; PYY, peptide YY; SCFA, short-chain fatty acid; TLR4, toll-like receptor 4; SOCS, suppressor of cytokine signaling; SREBP, sterol response element binding protein 1; TNF, tumor necrosis factor; VLDL, very low-density lipoprotein.

peroxisome proliferator-activated receptors (PPARs), important for the correct host metabolic regulation (114, 115); another mechanism is the increased systemic LPS due to higher levels of Gram-negative commensals, leading to low-grade inflammation which, in turn, increases insulin resistance (116-118).

It has been shown an association of obesity with enrichment in *Firmicutes* to *Bacteroidetes* phylum [the two main phyla present in the gut of both mice and humans (119)], contributing to a higher harvest and storage of energy from the host diet (120-122). Several studies report the increased relative abundance of *Firmicutes* in obese mice (122, 123). Moreover, diabetes has also been linked to gut dysbiosis, with increased levels of *Lactobacillus* and *Clostridium* and decreased butyrate-producing bacteria (124, 125). Therefore, modulation of the gut microbiota has been proposed as a potential therapy for the current incurable metabolic diseases, being performed either through adjustments in the host diet, treatment with probiotics or by faecal transplantations (126-128). Experiments reported the reversibility of microbial composition in obese mice when these animals received a normal diet (122, 123). Faecal transplantation of gut microbiota from normal diet mice has also been proven to ameliorate the metabolic alterations in obese mice (121, 129, 130) and, regarding probiotics administration, it leads to lower glucose fasting, inflammation and insulin levels (131-134).

During early life, as aforementioned, neonates are exposed to a tremendous quantity and variety of microorganisms and depending on several factors, they are uniquely colonised (69, 74, 75). Therefore, it is expectable this phase to be determinant for the predisposition of the subject for metabolic disturbances. In fact, antibiotics treatment in weaned mice leads to metabolic changes due to altered gut microbiota, increasing the SCFAs levels (135). Human studies also demonstrated that administration of antibiotics during the first months of life is associated with increased body mass and risk for obesity at childhood (136, 137), highlighting the importance of early colonisation for the infants' health. Although there are no studies reporting the risk for diabetes due to neonatal dysbiosis, the same tendency was observed in adults, where microbial changes by antibiotic exposure were associated with a higher risk for the development of this disease (138, 139).

In sum, given the importance of commensals for the correct metabolism of the host and the association between dysbiosis and metabolic disorders, the early life colonisation might be crucial and dictate the predisposition to metabolic complications later in life.

Objectives

Unpublished data from our group showed that antibodies produced against GBS GAPDH during maternal vaccination recognize two commensal bacteria present in the gut of neonatal mice (Pinho B, BSc thesis). Furthermore, the vaccine seems to interfere with neonatal immune system development at mucosal (intestine), systemic and central nervous system levels (Mesquita P, BSc thesis; Pinho B, BSc thesis; Geraldo R, BSc thesis) when analyzed one week after birth. Moreover, there is now mounting evidence, for the role of gut microbiota in driving immune cell development *via* promoting haematopoiesis.

This project aims to assess whether maternal rGAPDH vaccination changes their offspring's haematopoiesis. More specifically, the objectives are:

1. To evaluate the impact of maternal rGAPDH vaccination on the haematopoietic progenitors of their offspring in the bone marrow, spleen and liver, during the first week of life;

2. To verify the impact of rGAPDH maternal vaccination on the development of neonatal immune system;

3. To evaluate the susceptibility of neonates born from vaccinated mothers to the enteric *Escherichia coli* infection;

4. To uncover if the progeny of rGAPDH-vaccinated mothers is more prone to metabolic disorders.

This will be done through a comparative study among the offspring born from rGAPDH-vaccinated and Sham-vaccinated mothers.

This study will provide a better understanding on the extension and severity of a maternal vaccine based on GBS rGAPDH on the neonatal immune system, and how it affects resistance to pathogens and susceptibility to metabolic diseases.

Materials and Methods

Animal handling and ethics statement

Male and female BALB/c mice were purchased from Charles River Laboratory and housed at the Instituto de Ciências Biomédicas Abel Salazar's animal facility under a 12 h alternating cycle of light/dark. All experimentations were conducted in conformity with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and Directive 2010/63/EU and Portuguese rules (DL 113/2013). All protocols concerning animals were approved by the Direção Geral de Alimentação e Veterinária (DGAV) and the institute's Animal Ethical Committee. All efforts were made to minimise animal suffering and to reduce the number of animals used. Investigators were not blinded during experimental mice allocation and outcome assessment.

Maternal GBS rGAPDH vaccination

Female BALB/c mice were divided into two experimental groups, being subcutaneously (s.c.) injected thrice, in a three-week interval period, with one of the following suspensions: 100 μL of 25 μg of recombinant GBS GAPDH (rGAPDH) in a 1:40 phosphate-buffered saline (PBS)/Alhydrogel suspension (Aluminium hydroxide Gel) (rGAPDH-vaccinated group) or 100 μL of 1:40 PBS/Alhydrogel suspension (Sham-vaccinated). Seven days after last injection, peripheral blood was collected, and serum levels of rGAPDH-specific IgG antibodies were determined by Enzyme-Linked Immunosorbent Assay (ELISA). Upon confirmation of high levels of anti-rGAPDH IgG antibodies in vaccinated animals, female mice were mated. The day of delivery was designated as post-natal day (P) 0. Pregnant females delivered by spontaneous partum and pups were kept with their mothers throughout the experiment.

Determination of rGAPDH-specific IgG antibodies (ELISA)

For anti-rGAPDH IgG antibodies determination, blood from vaccinated mice was collected, allowed to clot and centrifuged for 15 minutes (min) at 13000 g. The sera were stored at -80 ºC.

For the assay, high-binding 96 wells flat-bottom plates (Nunc) were coated with 50 μL/well of rGAPDH (5 μg/mL) in PBS and incubated overnight at 4 ºC. The coating solution was then discarded, plates were washed with TBST Washing Solution [TBST: Tris-Buffed Solution with 0,05% Tween-20 (Sigma Aldrich)] and blocked with 180 μL of blocking solution [TBST-BSA (Bovine Serum Albumin, Sigma Aldrich) 2% (m/v)]. After 1 hour (h) of incubation at room temperature (RT), plates were washed with TBST Washing

Solution and incubated with 50 μL/well of diluted sera. Samples were initially diluted 1:30 in TBST-BSA (1%), followed by threefold serial dilutions. Plates were left to incubate at RT for 2 h and washed again. Alkaline Phosphate (AP) conjugated goat anti-mouse IgG (Southern Biotech) was diluted 1:1000 in TBST-BSA (1%) and 50 μL were added to each well. After 1 h incubation at RT and further washing, 50 μL/well of p-nitrophenyl disodium phosphate (Sigma Aldrich) diluted in AP buffer was added. Plates were incubated at RT in the dark for 15 minutes. The reaction was stopped by adding the same volume 0.1 M Ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich) and the absorbance was read at 405 nm in a Thermo Multiskan Ex Spectrometer.

Sera antibody titres correspond to the minimal serum dilution to detect absorbance signal. Using Microsoft Excel Software, the line tendency was drawn considering the absorbance values in the exponential phase. The obtained equation was used to calculate the value when y=0, which corresponds to the titre.

Collection of intestinal lavage fluid (ILF)

At indicated time points, ILF was collected. Animals were killed by decapitation, the entire mouse small intestine removed, and clamped at one end of the intestine. Carefully, 500 μL of ice-cold Dulbecco's Phosphate-Buffered Saline (DPBS, Sigma Aldrich) was introduced in the intestine avoiding its puncture. The intestinal content was removed to a microtube and was suspended by extensive vortexing followed by centrifugation at 4000 g for 15 min. The supernatant was removed to a new tube and centrifuged for 20 min, at 10000 g, at 4 ºC. The clear supernatants were stored at – 80ºC until analysis. Specific IgG antibodies to rGAPDH were determined as described above.

Immune cells isolation

Haematopoietic progenitors from bone marrow, spleen and liver, as well as mature myeloid and lymphoid populations from spleen, liver and thymus were isolated for flow cytometry analysis.

Mice at P3, P5, P8, P14 and P30 were anaesthetized with isoflurane (IsoFlo® Esteve) and transcardially perfused with saline. The liver, thymus and spleen were excised and weighed. The tibias, femurs, and blood were also collected at indicated time points. Spleens and thymuses were gently pressed through 100 μm nylon mesh cell strainer with a sterile plastic plunger to yield a single cell suspension, in 5 mL (for neonates) or 10 mL (for older animals) of Roswell Park Memorial Institute (RPMI, Sigma Aldrich) 1640 Medium. Cells were then washed with DPBS solution, without Ca^{2+} nor Mg^{2+}, by centrifuging at 500 g for 7 min, and red blood cells in the spleen were lysed with

lysis solution [0.01 M Tris, 0.15 M NH4Cl in deionized water (pH=7.2)] (lysis was not performed in the thymic suspension). Cells were then washed twice with ice-cold DPBS, resuspended in ice-cold RPMI 1640 containing 2% Foetal Bovine Serum (FBS, Biowest) and counted.

Livers were cut into small fragments and incubated in 10 mL of digestion solution [DPBS containing 1 mg/mL of collagenase D (Sigma Aldrich)], for 15 min, at 100 rpm. Cells were passed through a 70 µm nylon mesh cell strainer and centrifuged for 3 min at 25 g to remove hepatocytes. The resulting supernatants were centrifuged for 7 min at 500 g and red blood cells were lysed. Cells were then washed, resuspended and counted.

Femurs and tibias were removed from euthanized mice and cleaned from the remaining muscle tissue. Bones were gently homogenized in ice-cold DPBS, passed through a 100 µm cell strainer and cells were washed twice with ice-cold DPBS, resuspended and counted.

Blood was collected in heparinized tubes (BRAND) and analysed either through flow cytometry, for P3 mice, or directly through ProCyte Dx Haematology Analyser (IDEXX), for the remaining time points. In the case of flow cytometry analysis, blood was lysed for 10 min with lysis buffer, washed twice with ice-cold DPBS, resuspended in RPMI with 2% FBS and counted.

Flow cytometry analysis

After cell isolation, 1×10^6 cells were added to each U-shaped well of a 96-well plate, washed once with DPBS, for 2 min, at 1200 rpm. For dead cell exclusion, cells were stained with a fixable viability dye (FVD, eBioscience) diluted 1:1000 in DPBS, for 30 min, in the dark and on ice. Cells were then washed with FACS buffer (1% BSA, 0.01% sodium azide in DPBS) for 2 min, at 1200 rpm and resuspended in Fc Block (1:100 dilution; Biolegend), for 10 min, on ice, for the elimination of nonspecific binding. Cells were then surface stained with prediluted antibodies.

All antibodies were from Biolegend except otherwise indicated. For haematopoietic progenitors' analysis, splenic, hepatic and bone marrow cells were stained with an anti-mouse lineage cocktail of antibodies to exclude lineage populations: anti-CD4 (Clone RM4-5, BD Biosciences), anti-CD8 (Clone 53-6.7 BD Biosciences), anti-CD11c (Clone N418), anti-Ly6G (Clone 1A8, BD Biosciences), anti-B220 (Clone RA3-62B BD Biosciences) and anti-Ter-119 (Clone TER-119), all conjugated to FITC and 1:200 diluted. Cells were also marked with anti-IL-7Rα PE (CD127; Clone SB/199), anti-

c-Kit PerCP-Cy5.5 (CD117; Clone 2B8), anti-Sca-1 Pe-Cy7 (Clone D7), anti-FcγR APC (CD16/32; Clone93) and anti-CD34 BV421 (Clone RAM34, BD Biosciences), all 1:100 diluted, with the exception of anti-CD34, 1:50 diluted.

For myeloid lineage, splenic and hepatic cells were stained with anti-CD45 FITC (Clone 30-F11; 1:200 diluted), anti-Siglec-F PE (Clone 50-2440, BD Biosciences; 1:100), anti-Ly6C PerCP-Cy5.5 (Clone HK1.4; diluted 1:200), anti-CD11b Pe-Cy7 (Clone M1/70; 1:300), anti MHC-II IA/IE APC (Clone M5/114.15.2; 1:100), anti-CD11c APC-Cy7 (Clone N418; 1:100) and anti-Ly6G Pacific Blue (Clone 1A8; 1:100).

Regarding lymphoid lineages, the same tissues were stained with a mix containing anti-CD45 FITC (Clone 30-F11), anti-CD5 PE (Clone 53-7.3, BD Biosciences), anti-CD3 Pe-Cy7 (Clone 17A2), anti-TCR γδ APC (Clone GL3) and anti-CD19 APC-Cy7 (Clone 6D5), all 1:100 diluted except for CD45 (1:200).

Thymuses were stained with anti-CD45 FITC (Clone 30-F11; 1:200 diluted), anti-CD8 PerCP-Cy5.5 (Clone 53-6.7; 1:200), anti-CD3 Pe-Cy7 (Clone 17A2, 1:100) and anti-CD4 Pacific Blue (Clone RM4-5; 1:100).

Blood was stained with anti-CD45 FITC (Clone 30-F11; 1:200), anti-F4/80 PE (Clone BM8; 1:100), anti-Ly6C PerCP-Cy5.5 (Clone HK1.4; 1:200), anti-CD3 Pe-Cy7 (Clone 17A2; 1:100), anti-CD19 APC (Clone 6D5; 1:100), anti-CD11b AF700 (Clone M1/70; 1:100) and anti-Ly6G Pacific Blue (Clone 1A8; 1:100).

After 20 min of incubation on ice, in the dark, cells were washed twice with ice-cold DPBS and fixed with 1% formaldehyde (Biotium) in DPBS for 10 min at RT. Cells were then washed twice and resuspended in FACS buffer.

Single stains were performed using compensation beads (Invitrogen) or cells (FVD) to obtain clearly defined positive and negative populations for compensation. The samples were acquired within 24 h after fixation on a FACSCanto II flow cytometer (BD Biosciences), using the FACS Diva Software (Becton Dickinson, Franklin Lakes, NJ, USA). Data were analysed as described in the results section using the FlowJo Software version 10 (Tree Star).

E. coli IHE3034 infection

The extraintestinal pathogenic *E. coli* (ExPEC) IHE3034, belonging to the serotype O18:K1:H7, ST95 is a neonatal meningitis associated K1 strain isolated in Finland in 1976. Overnight cultures of *E. coli* IHE3034 in Todd-Hewitt (TH, BD Biosciences) medium were subcultured 1:100, grown until mid-log phase for ~3 h at 37

⁰C, washed twice with sterile phosphate-buffered saline (PBS) and resuspended in the same solution. The absorbance of the bacteria was adjusted to 0.450 at 600 nm (Jenway 6300 Spectrophotometer), corresponding to ~2×10^8 colony-forming units (CFU)/mL. Neonatal mice at P4 were orally inoculated with 20 µL containing 1×10^7 CFU of *E. coli,* using a micropipette. Infected pups were kept with their mothers during the course of the experiment and were assessed daily. Neonatal monitoring was maintained until any of the humane points (lethargic, darker red colour and with no observable milk spot) was observed. Survival curves were determined in a 21-day period.

To access bacterial colonisation, three days post-infection pups were euthanized by decapitation, and the brain, lungs and liver were aseptically removed. Tissues were homogenized in PBS using a pellet mixer (VWR). Serial dilutions were prepared in sterile PBS and plated on solid TH medium. Plates were incubated for 24 h at RT, for CFU counts. Blood was collected by decapitation to avoid extreme hypovolaemia associated with stress and pain, serially diluted and plated.

Histological Analysis

Left lateral liver lobes from pups born from the two experimental groups were removed at indicated time points, fixed in 10% buffered formalin, routinely processed, and embedded in paraffin. Histologic 2 µm-thick sections were cut for haematoxylin and eosin (H&E) staining. Slides were deparaffinized in xylene, with 2 changes of 10 min each, and then hydrated by passing the slides slowly (~2 min) through a series of decreasing concentrations of alcohols: 100%, 96% and 70%. Then, slides were briefly washed in deionized water and stained for 3 min with haematoxylin (Merck). After washing in running water, they were rapidly passed through acid alcohol for differentiation and washed again. Slides were then coloured with eosin Y (Merck) for 1 min and washed in running water to remove the excess of dye. Finally, they were dehydrated through rapid changings in an increased gradient of alcohols, diaphonized in xylene and mounted with Enttelan mounting medium (Merck). H&E-stained sections were analysed by a pathologist (A.F.). Slides were assigned random numbers that had no relevance to the experiment for blind analysis.

Fat diet administration

Female and male BALB/c mice born from Sham- and rGAPDH-vaccinated mothers were weaned at P27. Mice were separated by gender, with a maximum of 2 animals per cage. Mice were fed with food and water *ad libitum* and a mixture of 3.07 g of pork lard (Porminho) plus 1.93 g of powdered food per animal was given. The mixture

was replaced every day, and the animals weighted daily. The first day of the experience was defined as day 1. After 30 days of fat diet administration, mice were sacrificed and the spleen was analysed by flow cytometry as aforementioned. The livers were excised for bacterial colonisation analysis.

Analysis of serum ALT

Serum alanine aminotransferase (ALT) activity was measured using a commercially available kit (Idexx laboratories), according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, California USA). Means and standard errors of the means (SEM) were calculated, corresponding to the indicated independent experiments. The log-rank (Mantel-Cox) test was used to analyse the survival curve. CFU and IgG titters data were log10 transformed. The differences between the 2 groups were analysed by Students' t-test, when appropriate for $\alpha=0.05$. The normality of the data was verified by Shapiro-Wilk normality test. When it was not verified, Mann-Whitney tests were performed. In the case of ratios, the values were normalized using the formula: $Arcsin(\sqrt{(Value/100)}) \times 180/\pi$, previous to statistical analysis. Significance was represented by the following symbols: $^{*,\#}P < 0.05$, $^{**,\#\#}P < 0.01$, $^{***,\#\#\#}P < 0.001$, $^{****,\#\#\#\#}P < 0.0001$.

Results

This work is part of the study presented as:

Poster on the XLV Annual Meeting of the Portuguese Society for Immunology, at Coimbra (15 to 17 May, 2019) - Maternal GAPDH vaccination against Group B Streptococcus affects postnatal haematopoiesis (**Inês Lorga** *et. al*).

Maternal rGAPDH vaccination interferes with the development of their offspring Immune System

The initial bacterial communities in the neonatal gastrointestinal tract are crucial for their correct immune system development (67, 69, 76). Unpublished work from our group showed similarities between the GAPDH of GBS with the ones from other relevant commensal microorganisms by *in silico* evaluation (Lopes I, MSc thesis) and that the antibodies produced against GBS recombinant GAPDH (rGAPDH) cross-react with the enzyme present in phylogenetically related commensal bacteria (Pinho B, BSc thesis). Moreover, it was found that, at post-natal day (P) 8, maternal vaccination with GBS rGAPDH interferes with the immune system, at systemic (Pinho B, BSc thesis) and intestinal level (Mesquita P, BSc thesis), and of the central nervous system (Geraldo R, BSc thesis) of their offspring. Thus, we performed a kinetic study in order to understand the impact of maternal vaccination on the immune system maturation of their offspring, during development, in the bone marrow (BM), liver, spleen, thymus and blood.

1. Maternal vaccination with rGAPDH leads to rGAPDH-specific IgG antibodies in the intestinal tract of their offspring

Female BALB/c mice were subcutaneously immunized with rGAPDH plus the adjuvant Alhydrogel (rGAPDH-vaccinated group) or with PBS and Alhydrogel (Sham-vaccinated group). One week after the last vaccination dose, the serum levels of anti-rGAPDH IgG antibodies were quantified by ELISA. As expected, only the rGAPDH-vaccinated females exhibited high levels of anti-rGAPDH IgG antibodies (Figure 3). Thereafter, females were allowed to mate (to be guaranteed that the vaccination did not occur during pregnancy).
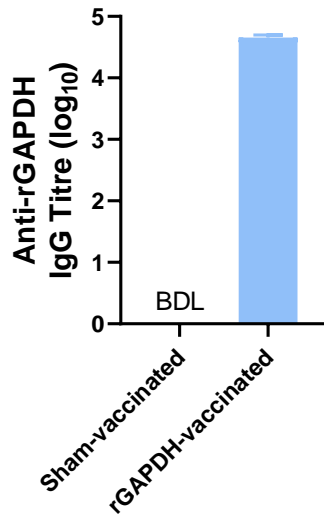
Figure 3 | Serum titre of anti-rGAPDH IgG antibodies. Female BALB/c mice were subcutaneously immunized thrice, in a three-week interval, with 25 µg of rGAPDH in a PBS-Alum solution (rGAPDH-vaccinated) or only with PBS-Alum (Sham-vaccinated). Seven days after the last injection, blood samples were collected and serum rGAPDH-specific IgG antibodies were quantified by ELISA. The titre corresponds to the minimal serum dilution required to detect signal. Bars correspond to the mean value ± SEM of 30 mice per group. BDL, below detection limit.

It has been described that IgG is at least partially resistant to digestion in the neonatal intestinal tract (140) and can be retro transported from plasma into intestinal lumen via neonatal Fc receptor (FcRn)-mediated pathway, protecting neonates from enteric infections (141). Therefore, we analysed for the presence of anti-rGAPDH IgG antibodies in the intestinal lavage fluid (ILF) of pups born from Sham- or rGAPDH-vaccinated mothers, at P5 and P8. Anti-rGAPDH IgG antibodies were detected in ILF from pups born from rGAPDH-vaccinated progenitors at both time points tested, while they were below detection limit in pups born from the control group (Figure 4).
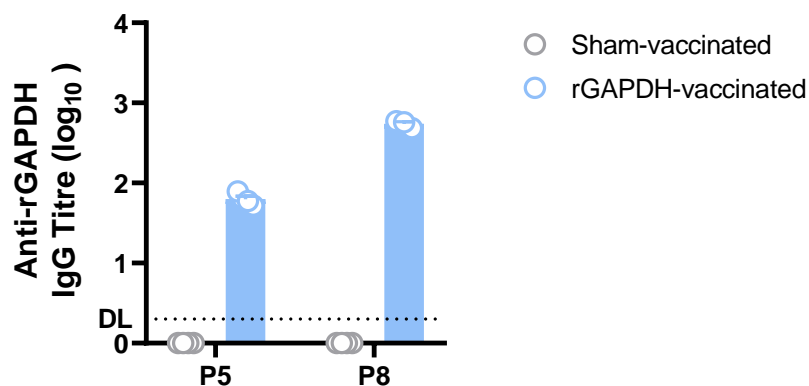
Figure 4 | Titre of anti-rGAPDH IgG antibodies in the neonatal intestinal lavage fluid. Female BALB/c mice were immunized with GBS rGAPDH plus the Alum (rGAPDH-vaccinated) or only with the Alum (Sham-vaccinated). Animals born from the two groups were sacrificed at the indicated time points and the intestines removed for anti-rGAPDH IgG quantification by ELISA. Bars indicate the mean ± SEM. Each symbol indicates data from single pups [mean, n=4 (Sham-vaccinated and P8 rGAPDH-vaccinated); n=3 (P5 rGAPDH-vaccinated)]. DL, detection limit.

2. Maternal vaccination with rGAPDH induces alterations in medullary and extra-medullary myelopoiesis of their offspring

Measurement of weight gain, a sensitive marker of mice well-being, was recorded at different time points throughout the first 30 days of life. As shown in figure 5A and 5B, there was a significant decrease in the body weight of pups born from rGAPDH-vaccinated mothers at P8, when compared with ones born from Sham-vaccinated mothers. This result indicates that rGAPDH maternal vaccination causes a slight decrease in the weight gain of their progeny at the age of 8 days. Comparisons of the weight of young animals, at P14 and P30, showed no differences between groups (Figure 5A).
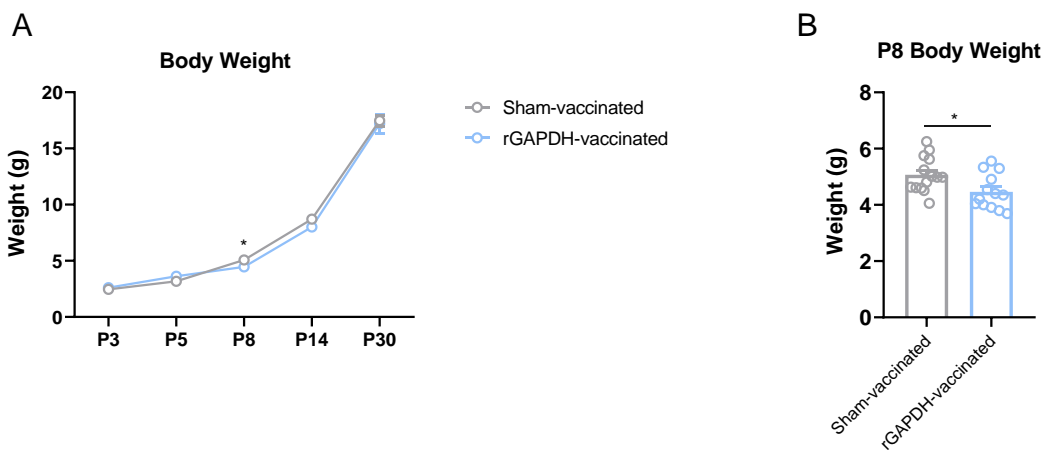
Figure 5 | Body weight during development. Female BALB/c mice were vaccinated with rGAPDH plus the Alum (rGAPDH-vaccinated) or only with the Alum (Sham-vaccinated). (A-B) Body weight of mice born from both groups, at indicated post-natal days (P). Data are presented as the mean ± SEM of pups pooled from 2 to 4 independent litters [n=10 (P3 Sham-vaccinated); n=13 (P3 rGAPDH-vaccinated); n=6 (P5 Sham-vaccinated); n=9 (P5 rGAPDH-vaccinated); n=14 (P8 Sham-vaccinated); n=13 (P8 rGAPDH-vaccinated); n=10 (P14 Sham-vaccinated); n=8 (P14 rGAPDH-vaccinated); n=5 (P30 Sham-vaccinated); n=7 (P30 rGAPDH-vaccinated)]. Comparisons by Student's t-test. *$P < 0.05$.

During embryonic mouse development, the primary source of haematopoietic progenitors is the foetal liver (49). Near or at birth, cells then migrate from the liver to the BM, where they remain throughout the animal's adult life. However, upon delivery, the neonatal liver still contributes to the haematopoietic homoeostasis, until eventually the bone marrow is fully established and takes over (49). The spleen is also a haematopoietic site. Thus, to investigate if maternal vaccination alters their offspring haematopoiesis, we analysed whether haematopoietic progenitor populations were affected in the BM, liver and spleen of neonates born from Sham- or rGAPDH-vaccinated mothers during their first week of life. Specifically, these populations were analysed at P3, P5 and P8 by flow cytometry.

The gating strategy used to define the different progenitor populations is shown in Figure 6.
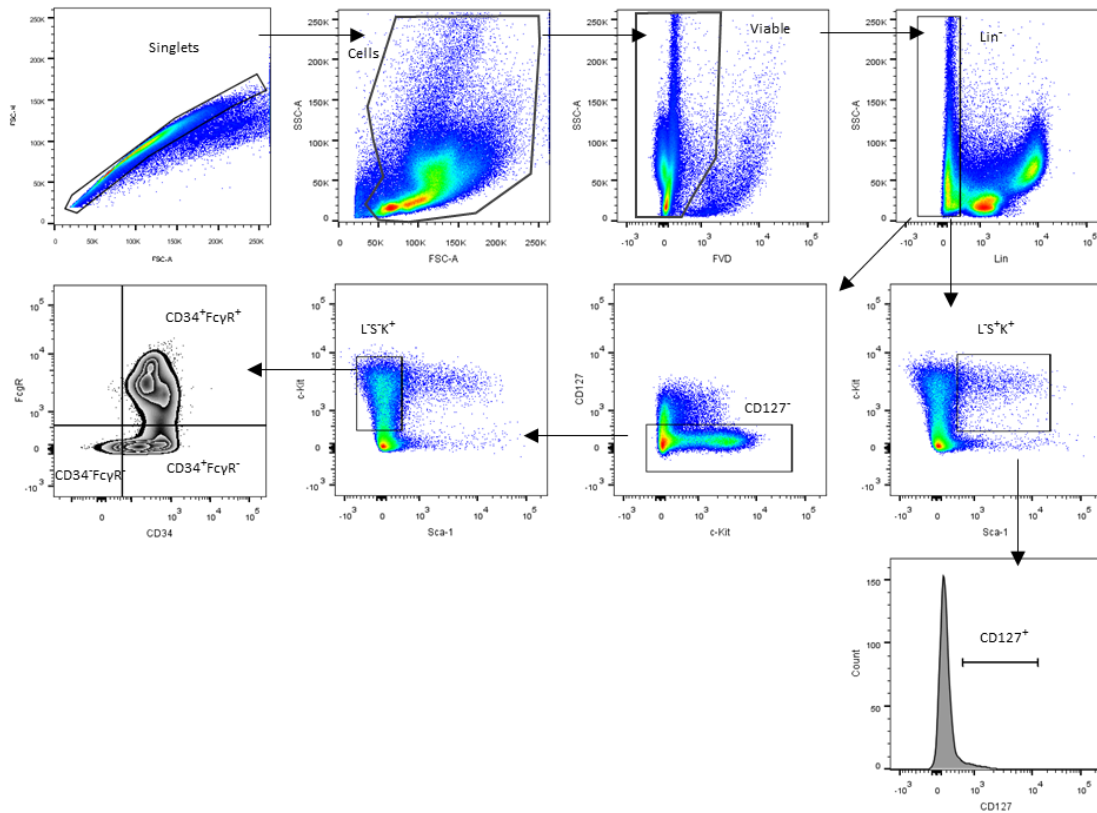
Figure 6 | Flow cytometry gating strategy of haematopoietic progenitors. Representative gating strategy used to define the haematopoietic progenitors in the spleen. All cells were first gated on singlets (FSC-A vs FSC-H dot plot) and a gate for total cells was made from FSC-A vs SSC-A dot plot. Dead cells were further removed by staining with a fixable viability dye and the remaining cells were gated on Lin⁻. Haematopoietic stem cells and multipotent progenitors were further defined as Sca-1⁺c-Kit⁺ (LSK). Common lymphoid progenitors (CLP) were further defined as CD127⁺. For the myeloid and erythroid lineages, cells were gated on CD127⁻ and further on Sca-1⁻c-Kit⁺. Based on the expression of CD34 and FcγR, cells were then characterized as common myeloid progenitors (CMP, CD34⁺FcγR⁻), granulocyte-monocyte progenitors (GMP, CD34⁺FcγR⁺) or megakaryocyte-erythroid progenitors (MEP, CD34⁻FcγR⁻).

No differences were found between pups born from Sham- or rGAPDH-vaccinated mothers at P3, in the BM (Figure 7), liver (Figure 8) or spleen (Figure 9).

However, at P5, the offspring of rGAPDH-vaccinated females showed significantly decreased frequency of either BM Lin⁻Sca-1⁺c-Kit⁺ (LSK) population (composed by haematopoietic stem cells and multipotent progenitors) and granulocyte-monocyte progenitors (GMP, Lin⁻CD127⁻Sca-1⁻c-Kit⁺CD34⁺FcγR⁺), when compared to pups born from Sham-vaccinated mothers (Figure 7A and D). Although not significant, a tendency to decreased levels of megakaryocyte-erythroid progenitors (MEP, Lin⁻ CD127⁻ Sca-1⁻c-Kit⁺CD34⁻FcγR⁻) was also observed in the BM of pups born from rGAPDH-vaccinated progenitors, at this time point, in pups born from rGAPDH-vaccinated mothers compared to the pups born from Sham-vaccinated mothers (Figure 7E). Analysis of

animals at P8 showed that the frequency of common myeloid progenitors (CMP, Lin$^-$CD127$^-$Sca-1$^-$c-Kit$^+$CD34$^+$FcγR$^-$), GMP and MEP were significantly increased in the pups born from rGAPDH-vaccinated females when compared to neonates born from Sham-vaccinated mothers (Figure 7C-E). However, a more careful analysis of these populations in the control animals, *i.e.*, pups born from Sham-vaccinated progenitors, revealed that the frequency increases from P3 to P5, where it peaks, and then decreases at P8 (Figure 7C-E). This tendency was not observed in the offspring of rGAPDH-vaccinated mothers, as the values continuously increased from P3 to P8. Specifically, the frequencies of CMP, GMP, and MEP in the BM of pups born from rGAPDH-vaccinated mothers increased from P5 to P8, reaching at P8 similar values to those quantified in the Sham-vaccinated group at P5 (Figure 7 C-E).

No differences were found in the common lymphoid progenitors (CLP, Lin$^-$Sca-1$^+$c-Kit$^+$CD127$^+$), between groups (Figure 7B).
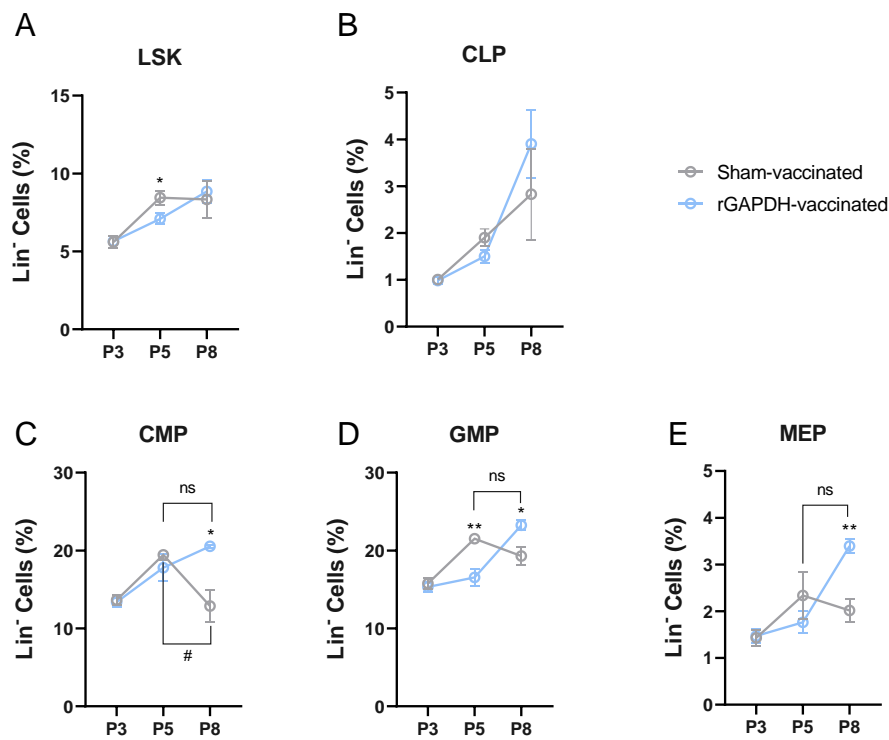
Figure 7 | Maternal rGAPDH-vaccination affects the bone marrow haematopoiesis of their offspring. Female BALB/c mice were immunized with GBS rGAPDH plus the Alum (rGAPDH-vaccinated) or only with the Alum (Sham-vaccinated). Animals born from the two groups were sacrificed at indicated time points and the bone marrow collected for flow cytometry analysis of haematopoietic progenitors. (A-E) Frequency of indicated haematopoietic progenitors within Lin$^-$ cells. Each symbol indicates the mean ± SEM and represents data pooled from 1 (P8 rGAPDH-vaccinated) to 4 independent litters [n= 6 (Sham-vaccinated); n=8 (P3 rGAPDH-vaccinated); n=9 (P5 rGAPDH-vaccinated); n=4 (P8 rGAPDH-vaccinated)]. Comparisons by Student's t-test or Mann-Whitney test. ns, not significant *,#$P < 0.05$; **$P < 0.01$. LSK, Lin$^-$Sca-1$^+$c-Kit$^+$; CLP, common lymphoid progenitors; CMP, common myeloid progenitors; GMP, granulocyte-monocyte progenitors, MEP, megakaryocyte-erythroid progenitors.

Analysis of the liver haematopoiesis showed that the frequency of CMP and GMP populations followed a similar pattern to that observed in the BM of pups born from Sham-vaccinated mothers, increasing from P3 to P5, and decreasing thereafter (Figure 7C and D, and Figure 8C and D, left). This was not observed in pups born from rGAPDH-vaccinated mothers, as the percentages of these populations increased from P5 to P8,
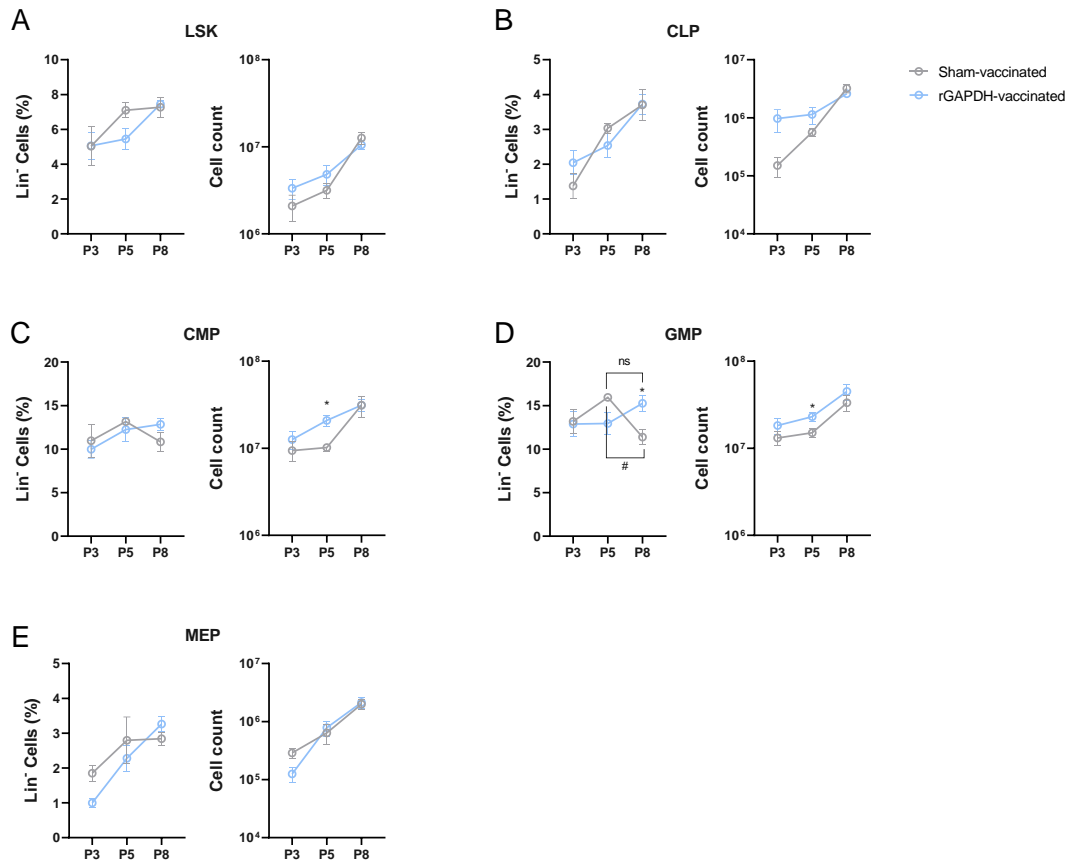
Figure 8 | Maternal rGAPDH-vaccination affects the liver myelopoiesis of their offspring. Female BALB/c mice were immunized with GBS rGAPDH plus the Alum (rGAPDH-vaccinated) or only with the Alum (Sham-vaccinated). Animals born from the two groups were sacrificed at indicated time points and the liver removed for flow cytometry analysis of haematopoietic progenitors. (A-E) Frequency and number of indicated haematopoietic progenitors. Each symbol indicates the mean ± SEM and represents data pooled from 1 (P8 rGAPDH-vaccinated) to 4 independent litters [n=7 (P3 Sham-vaccinated); n=8 (P3 rGAPDH-vaccinated); n=6 (P5 Sham-vaccinated); n=9 (P5 rGAPDH-vaccinated); n=5 (P8 Sham-vaccinated); n=4 (P8 rGAPDH-vaccinated)]. Comparisons by Student's t-test or Mann-Whitney test. ns, not significant; *,#$P < 0.05$ **$P < 0.01$. LSK, Lin⁻Sca-1⁺c-Kit⁺; CLP, common lymphoid progenitors; CMP, common myeloid progenitors; GMP, granulocyte-monocyte progenitors, MEP, megakaryocyte-erythroid progenitors.

reaching at P8 the values obtained at P5 in the control group (Figure 8C and D). Moreover, despite the frequency of GMP cells being significantly increased at P8 in the progeny of rGAPDH-vaccinated dams when compared to pups born from Sham-vaccinated mothers, this was not reflected in differences in total cell numbers of this population, between both groups (Figure 8D). A slight increase in the total cell numbers of CMP and GMP was also observed in pups born from rGAPDH-vaccinated mothers at P5 when compared to those observed in Sham-vaccinated animals, that is no longer found by day 8 (Figure 8C and D, right). Moreover, at P5, the frequency of GMP is

decreased in the neonates born from rGAPDH-vaccinated females, despite not reaching significance (P=0.0789) when compared to the ones born from Sham-vaccinated mothers (Figure 8D, left). No statistical differences were observed in the LSK, but a slight decrease was detected in the frequency at P5 (P=0.0727) in the pups born from rGAPDH-vaccinated females (Figure 8A). No differences were observed between groups in both frequency and number of CLP and MEP (Figure 8B and E).

Considering splenic haematopoiesis, the frequency of LSK, CLP and MEP populations were significantly decreased at P5 in pups born from rGAPDH-vaccinated mothers, comparing to those born from Sham-vaccinated progenitors (Figure 9A, B and E, left). However, their total number did not differ between groups, at this time point
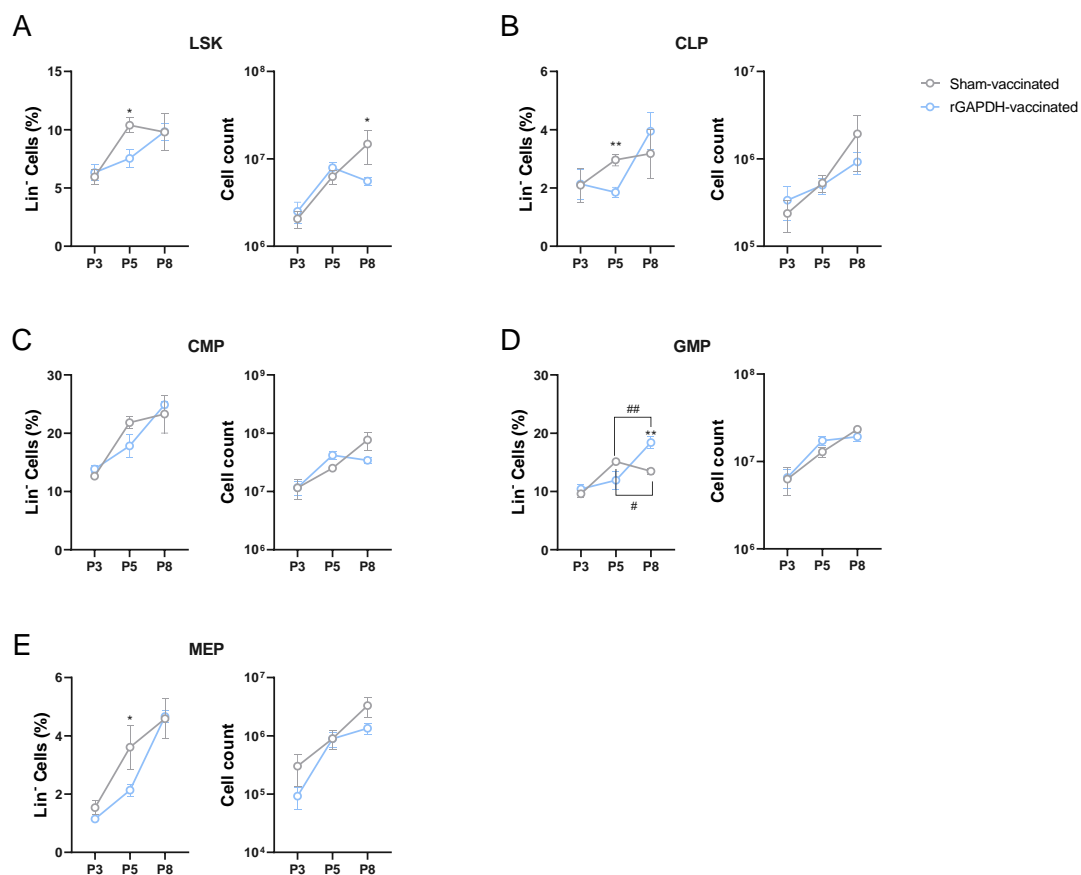
Figure 9 | Maternal rGAPDH-vaccination alters their offspring spleen haematopoiesis. Female BALB/c mice were immunized with GBS rGAPDH plus the Alum (rGAPDH-vaccinated) or only with the Alum (Sham-vaccinated). Animals born from the two groups were sacrificed at indicated time points and the spleen removed for flow cytometry analysis of haematopoietic progenitors. (A-E) Frequency and number of indicated haematopoietic progenitors. Each symbol indicates the mean ± SEM and represents data pooled from 1 (P8 rGAPDH-vaccinated) to 4 independent litters [n=7 (P3); n=6 (P5 Sham-vaccinated); n=9 (P5 rGAPDH-vaccinated); n=5 (P8 Sham-vaccinated); n=4 (P8 rGAPDH-vaccinated)]. Comparisons by Student's t-test or Mann-Whitney test. ns, not significant; $*,\#P < 0.05$ $**,\#\#P < 0.01$. LSK, Lin⁻Sca-1⁺c-Kit⁺; CLP, common lymphoid progenitors; CMP, common myeloid progenitors; GMP, granulocyte-monocyte progenitors, MEP, megakaryocyte-erythroid progenitors.

(Figure 9A, B and E, right). By P8, we did not find differences in the frequency and total numbers of CLP and MEP between both groups (Figure 9A and E). Regarding LSK,

although its relative percentage was similar between groups, the total cell counts were significantly decreased in the rGAPDH-vaccinated offspring comparing to the pups born from Sham-vaccinated mothers (Figure 9A). No differences were observed between groups in both frequency and number of CMP (Figure 9C). Analysis of the frequency of GMP revealed that it continuously increased overtime in the pups born from rGAPDH-vaccinated animals, reaching significant higher values at P8, when compared to age-matched controls (Figure 9D, left). However, this did not correlate with absolute cell number of this population, as no differences were observed between groups (Figure 9D, right).

Overall, these results demonstrate that maternal vaccination with rGAPDH affects the haematopoiesis of their progeny, particularly the myeloid compartment, that appears to have a delay in maturation.

3. Maternal rGAPDH vaccination interferes with mature myeloid cells of their progeny

In view of the described alterations, we next hypothesized that maternal vaccination with rGAPDH-induced alterations at the haematopoietic compartment may result in downstream effects at the mature populations level in pups born from rGAPDH-vaccinated mothers. To investigate this hypothesis, we performed a detailed analysis of the offspring hepatic and splenic mature populations by flow cytometry. Pups were analysed at P3, P5 and P8 in both the liver and spleen. Older animals (P14 and P30) were only analysed in the spleen.

The gating strategy to characterize the different myeloid populations is shown in Figure 10.
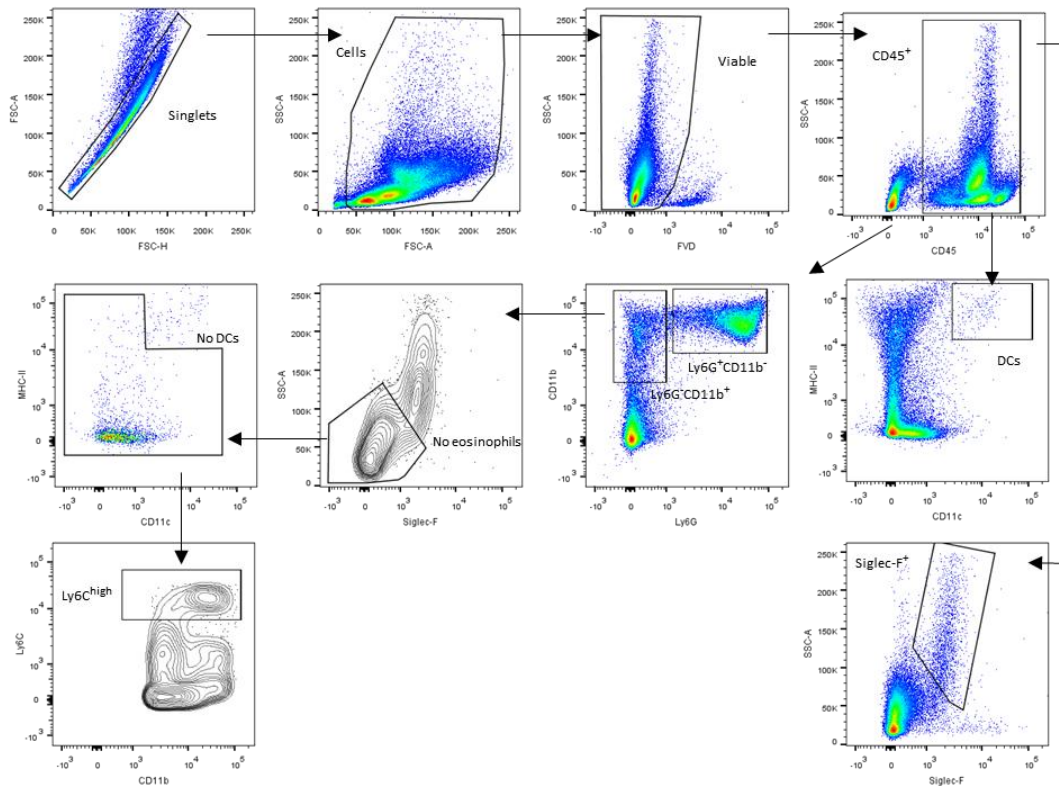
Figure 10 | Flow cytometry gating strategy of the myeloid populations. Representative gating strategy used to define the different myeloid cells in the spleen. All cells were first gated on singlets (FSC-A vs FSC-H dot plot) and a gate for total cells was made from FSC-A vs SSC-A dot plot. Dead cells were further removed by staining with a fixable viability dye and the remaining cells were gated on CD45+. Eosinophils were further defined as Siglec-F+SSC-A$^{int/high}$, neutrophils as CD11+Ly6G+ and dendritic cells as CD11c+MHC-II+. Macrophages were identified as CD11b+Ly6G-Siglec-F-DC- [CD11c+MHC-II+] and, within this population, inflammatory monocytes were defined as Ly6C^{high}.

Analysis of total hepatic cells revealed that its numbers increased over time, in both studied groups (Figure 11A). However, at P5, pups born from rGAPDH-vaccinated mothers presented a significant increase in total liver cells when compared to the Sham-vaccinated offspring (Figure 11A).
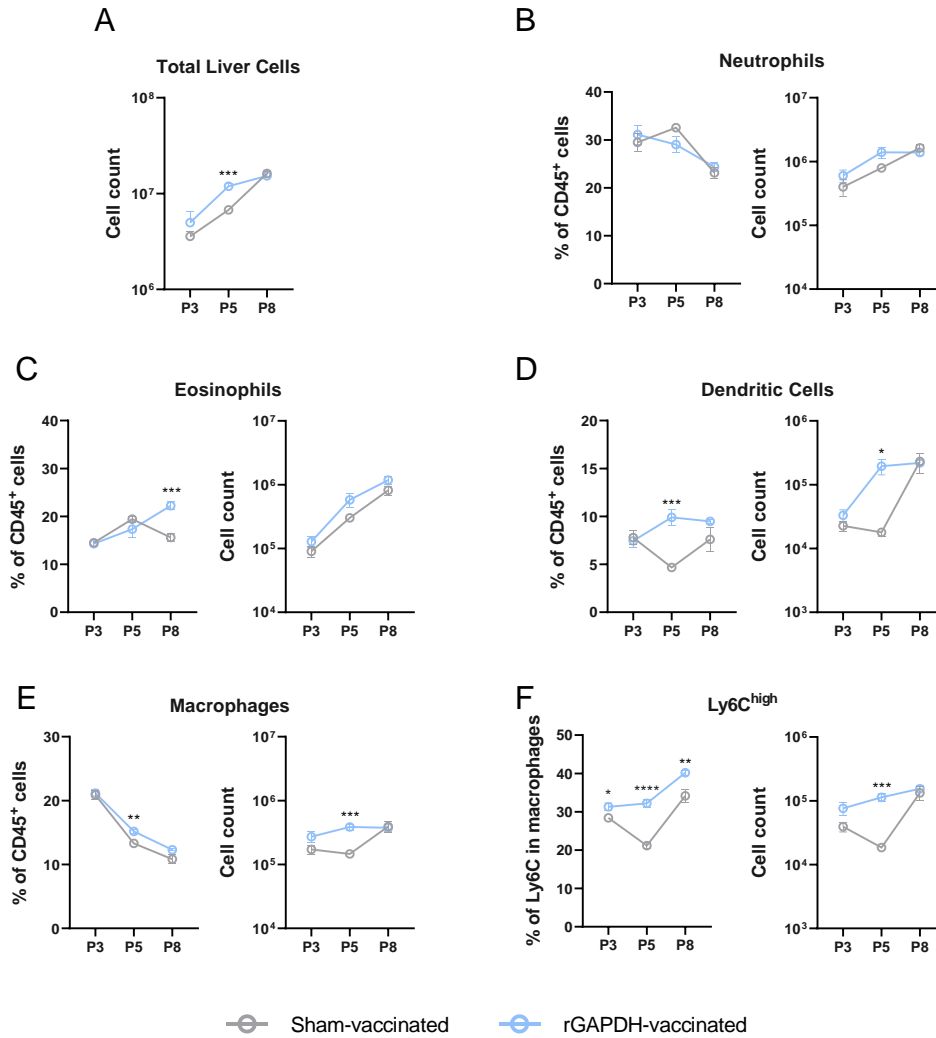
Figure 11 | Maternal rGAPDH-vaccination increases liver myeloid populations of their offspring in both frequency and number. Female BALB/c mice were immunized with GBS rGAPDH plus the Alum (rGAPDH-vaccinated) or only with the Alum (Sham-vaccinated). Animals born from the two groups were sacrificed at indicated time points and the liver excised for flow cytometry analysis of myeloid populations. (A) Absolute number of total cells per liver. (B-F) Frequency and number of indicated myeloid populations. Each symbol indicates the mean ± SEM and represents data from 1 (P8 rGAPDH-vaccinated) to 4 independent litters [n=10 (P3 Sham-vaccinated); n=11 (P3 rGAPDH-vaccinated); n=6 (P5 and P8 Sham-vaccinated); n=9 (P5 rGAPDH-vaccinated); n=4 (P8 rGAPDH-vaccinated)]. Comparisons by Student's t-test or Mann-Whitney test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

No differences were found in the myeloid cells at P3 in pups born from Sham- or rGAPDH-vaccinated mothers, with the exception of CD11b$^+$Ly6C^{high} cells (inflammatory monocytes), which frequency was slightly increased in the rGAPDH-vaccinated group (Figure 11F). This population was increased in the progeny of rGAPDH-vaccinated mothers at all time points studied (Figure 11F). Analysis of neutrophils (CD11b$^+$Ly6G$^+$) showed no differences between groups in both frequency and number, at all time points tested (Figure 11B). Regarding eosinophils (Siglec-F$^+$SSC-A$^{int/high}$), despite the observation of a significant increase in their frequency in pups born from rGAPDH-vaccinated mothers, at P8, when compared to those born from Sham-vaccinated

progenitors, no differences were observed in their absolute numbers (Figure 11C). A significant increase in both frequency and number of dendritic cells (CD11c$^+$MHC-II$^+$) was observed in pups born from rGAPDH-vaccinated mothers, at P5, comparing to the neonates born from Sham-vaccinated females (Figure 11D). By P8, this difference was no longer observed (Figure 11D). A significant increase in the frequency and number of macrophages (CD11b$^+$Ly6G$^-$Siglec-F$^-$DC$^-$[CD11c$^+$MHC-II$^+$]) was observed in the liver of neonatal mice born from rGAPDH-vaccinated mothers when compared to the control pups, at P5 (Figure 11E).

Regarding splenic myeloid cells, no differences were found at P3 (Figure 12). Significant differences appear 5 days after birth, with pups born from rGAPDH-vaccinated mothers presenting increased absolute number of total splenic cells (Figure 12A). Higher relative frequency and number of the phagocytic cells, dendritic cells (Figure 12D) and macrophages (Figure 12E), as well as Ly6C^{high} monocytes (Figure 12F), were observed in the progeny of rGAPDH-vaccinated mothers, at P5, when compared to the offspring of Sham-vaccinated females. Dendritic cells and macrophages' differences disappear by P8. At this time point, the Ly6C^{high} monocytes maintained significantly increased, albeit in less extension, in the rGAPDH-vaccinated progeny when compared to Sham-vaccinated offspring (Figure 12F). Moreover, decreased numbers of neutrophils (Figure 12B, right) and eosinophils (Figure 12C, right) were observed at this age (P8) in the rGAPDH-vaccinated offspring, comparing to the control.

Two other time points were studied in the spleen, P14 and P30. At P14, pups born from rGAPDH-vaccinated mothers presented fewer eosinophils (Figure 12C, right), similarly to the previous time point, and dendritic cells showed increased frequency (Figure 12D), similarly to what was observed 5 days after birth, compared to the Sham-vaccinated progeny. Regarding the Ly6C^{high} population, as shown in figure 12F, the observed tendency at P14 was the opposite of this observed at P5, with lower frequencies when compared to Sham-vaccinated progeny. No further differences were found at this time point.
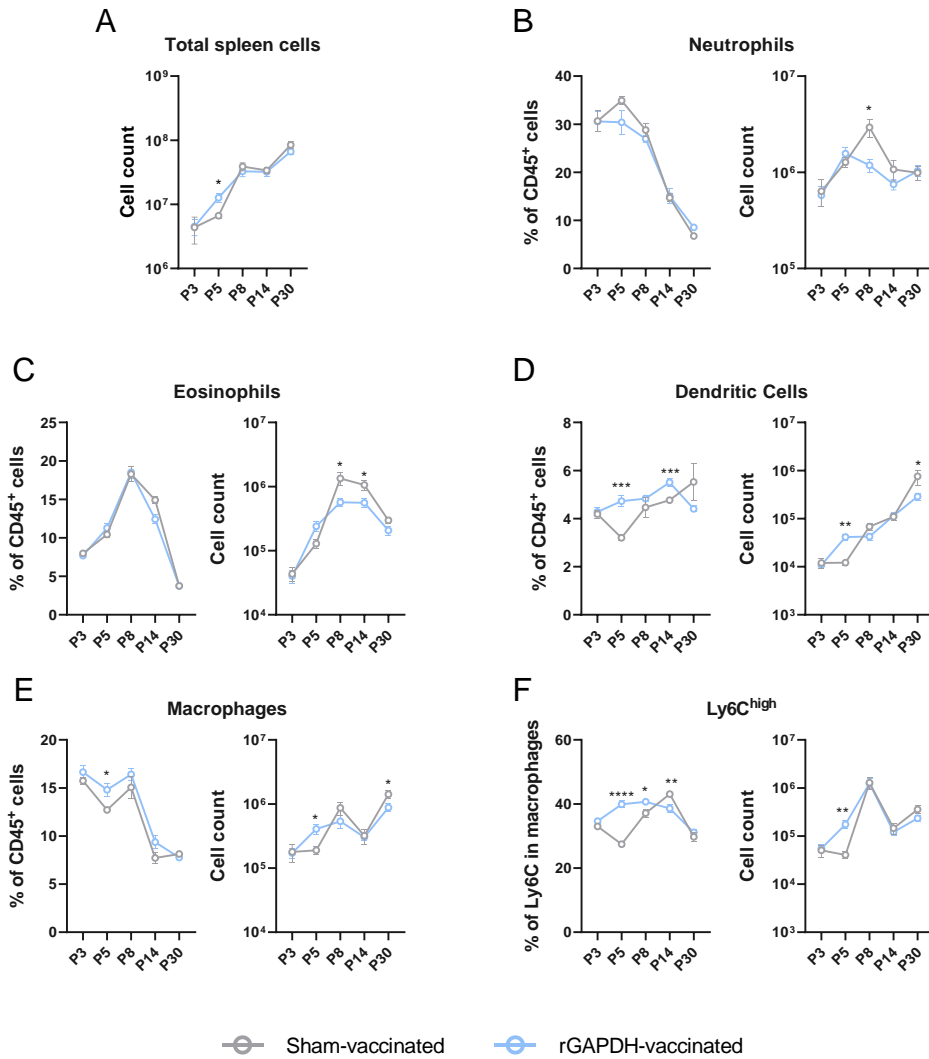
Figure 12 | Maternal rGAPDH-vaccination impact on their offspring splenic myeloid cells. Female BALB/c mice were immunized with GBS rGAPDH plus the Alum (rGAPDH-vaccinated) or only with the Alum (Sham-vaccinated). Animals born from the two groups were sacrificed at indicated time points and the spleen excised for flow cytometry analysis of myeloid populations. (A) Absolute number of total cells per spleen. (B-F) Frequency and number of indicated myeloid populations. Each symbol indicates the mean ± SEM and represents data pooled from 2 to 4 independent litters [n=10 (P3 and P14 Sham-vaccinated); n=6 (P3 rGAPDH-vaccinated and P5 Sham-vaccinated); n=9 (P5 rGAPDH-vaccinated); n=13 (P8 Sham-vaccinated); n=4 (P8 rGAPDH-vaccinated); n=8 (P14 and P30 rGAPDH-vaccinated); n=4 (P30 Sham-vaccinated)]. Comparisons by Student's t-test or Mann-Whitney test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

At the oldest time point studied, P30, lower numbers of dendritic cells and macrophages were observed in the offspring of rGAPDH-vaccinated mothers when compared to the progeny of Sham-vaccinated females (Figure 12D and E). All the remaining populations presented no differences between groups (Figure 12).

Overall, maternal rGAPDH vaccination interferes with myeloid cells in the offspring's liver and spleen during development, with higher impact at P5.

4. Maternal rGAPDH vaccination affects the lymphoid cells of their progeny

Lymphoid populations were also analysed in the liver and the spleen of pups born from Sham- or rGAPDH-vaccinated mothers, at the same time points as the myeloid cell's analysis. The gating strategy used to define the different lymphoid cells is represented in Figure 13.
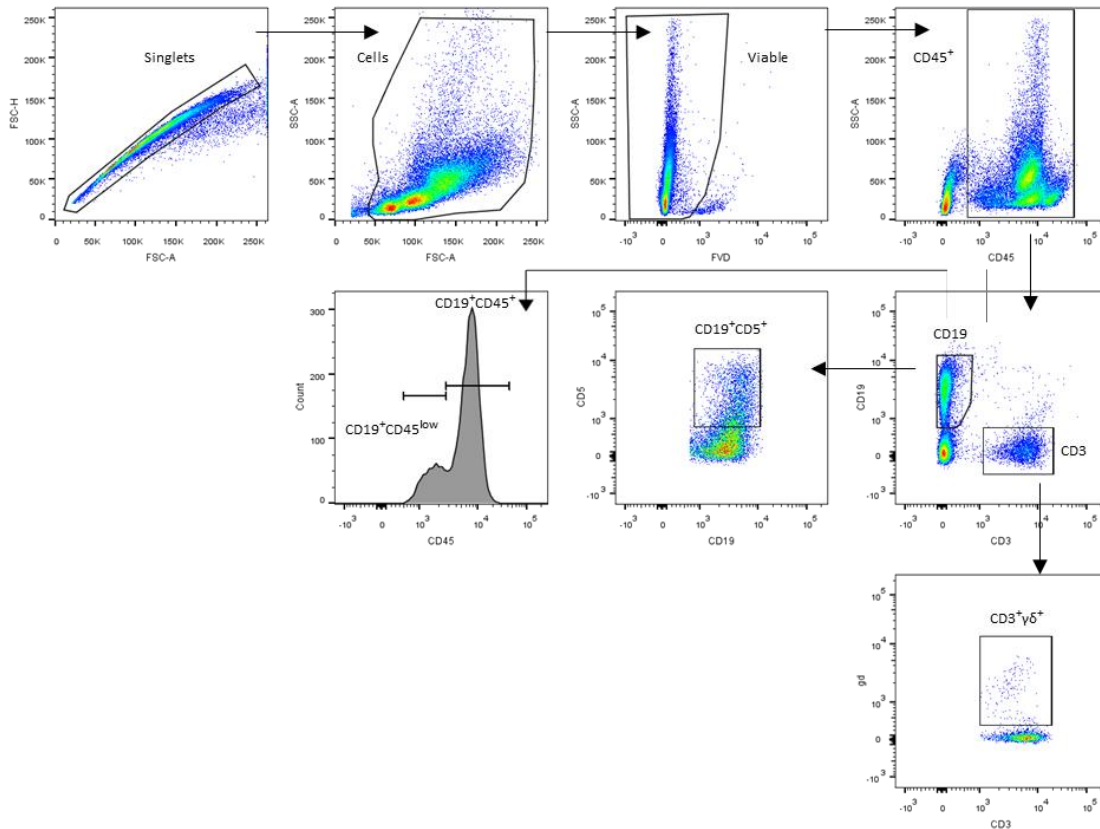
Figure 13 | Flow cytometry gating strategy of lymphoid populations. Representative gating strategy used to define the different myeloid cells in the spleen. All cells were first gated on singlets (FSC-A vs FSC-H dot plot) and a gate for total cells was made from FSC-A vs SSC-A dot plot. Dead cells were further removed by staining with a fixable viability dye and the remaining cells were gated on CD45+. B cells were defined as CD19+CD3-. B-1 were further defined based on CD5 expression and two other populations of B cells were identified based on the expression level of CD45+. T cells were identified as CD19-CD3+ and γδ T cells were defined as CD19-CD3+γδ+.

Similar to what was previously observed, no differences were found between groups in the liver, at P3 (Figure 14). Regarding the B cells compartment, the liver of pups born from rGAPDH-vaccinated mothers showed a significant increase in both the frequency and number of B cells (CD19+CD3-), at P5 (Figure 14A), compared to those born from Sham-vaccinated mothers. When analysing the expression of CD5+ cells (a B-1 cell marker) within the B cell compartment, pups born from rGAPDH-vaccinated mothers presented a tendency to show increased frequency ($P = 0.0587$) and significant higher cell number than those observed in pups born from Sham-vaccinated mothers, at this time point (Figure 14B). However, from P5 to P8, the relative frequency and absolute

numbers of this cell population were barely unchanged in the rGAPDH-vaccinated offspring, in contrast to what was found in pups born from Sham-vaccinated mothers, that showed an increase, reaching significantly higher values (Figure 14B). Still within
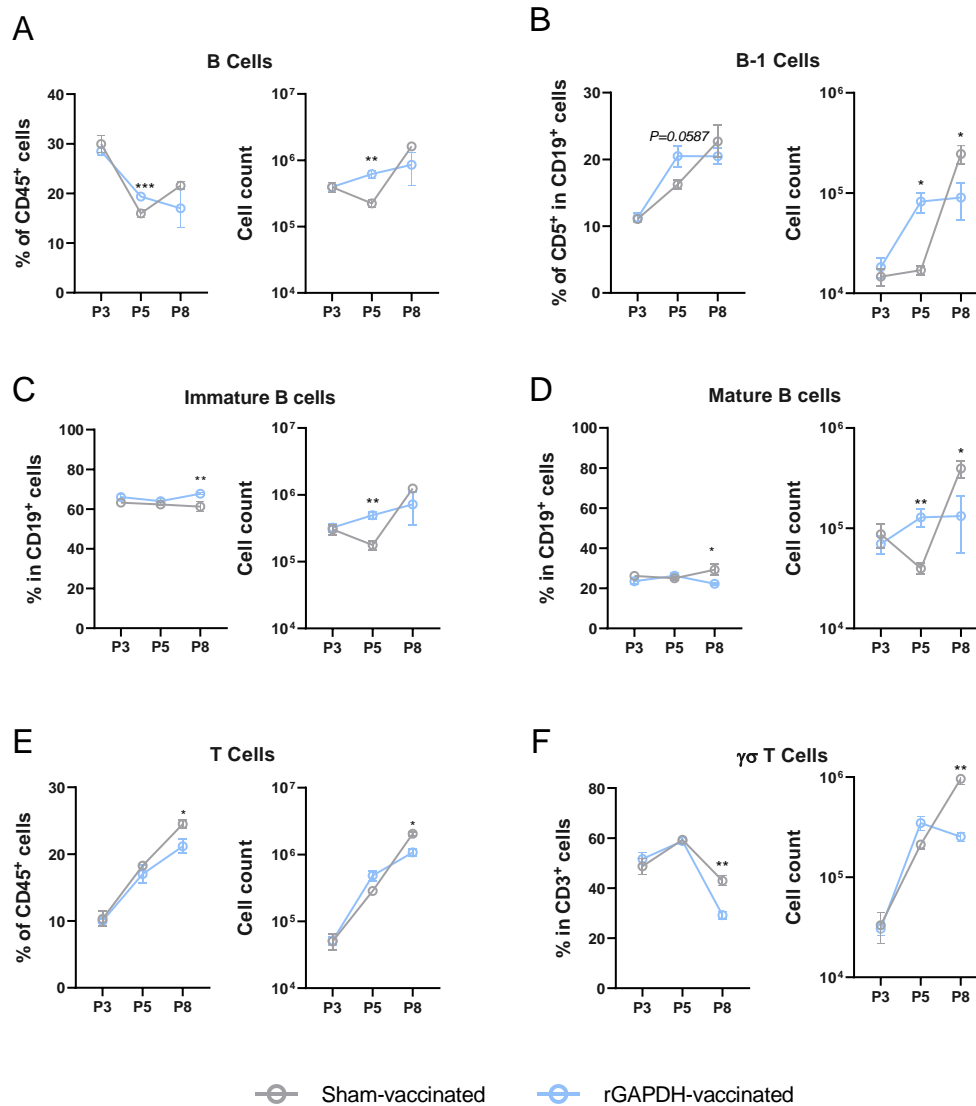

Figure 14 | Maternal rGAPDH-vaccination impact on their offspring hepatic lymphoid cells. Female BALB/c mice were immunized with GBS rGAPDH plus the Alum (rGAPDH-vaccinated) or only with the Alum (Sham-vaccinated). Animals born from the two groups were sacrificed at indicated time points and the liver excised for flow cytometry analysis of lymphoid populations. (A-H) Frequency and number of indicated lymphoid populations. Each symbol indicates the mean ± SEM and represents data pooled from 2 to 4 independent litters [n=10 (P3 Sham-vaccinated); n=13 (P3 rGAPDH-vaccinated); n=6 (P5 and P8 Sham-vaccinated); n=9 (P5 rGAPDH-vaccinated); n=4 (P8 rGAPDH-vaccinated)]. Comparisons by Student's t-test or Mann-Whitney test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

the B cell compartment, both immature (CD45^{low}CD19^{+}) and mature (CD45^{+}CD19^{+}) B cells were also analysed (Figure 14C and D). At P5, their numbers were significantly increased in pups born from rGAPDH-vaccinated mothers when compared to those found in age-matched controls (Figure 14C and D, right). Later, at P8, this tendency was reversed, and higher numbers were found in pups born from Sham-vaccinated mothers,

within B-1 and mature B cells (Figure 14B and D, right). The frequency of immature B cells was significantly increased at this time point, although no differences were observed between groups in their number (Figure 14C).

Regarding T cells, no differences were observed between groups until P8 (Figure 14E and F). Pups born from rGAPDH-vaccinated progenitors presented a slight decrease in frequency and number of both T ($CD19^-CD3^+$) and γδ T cells ($CD19^-CD3^+γδ^+$) (Figure 14E and F), compared to those born from Sham-vaccinated mothers.

The spleen was also affected by maternal rGAPDH-vaccination (Figure 15). Considering B cell populations, we found, at P5, a tendency to increased total B cells count ($P = 0.0554$) (Figure 15A, right), and significantly higher number of $CD5^+$ B-1 cells (Figure 15B, right) in pups born from rGAPDH-vaccinated mothers, compared to those observed in pups born from Sham-vaccinated mothers.

At P8, differences were still observed between groups. The rGAPDH-vaccinated offspring presented decreased relative frequency and number of total B cells (Figure 15A), as well as decreased cell counts regarding both immature and mature B cells (Figure 15C and D, right). The $CD5^+$ B-1 cells in the spleen of pups born from rGAPDH-vaccinated mothers presented increased frequency and number when compared to the Sham-vaccinated progeny (Figure 15B).

Regarding $CD3^+$ T cells, no differences were found (Figure 15E). γδ T cells presented a significant decrease in its relative frequency, at P3, in pups born from rGAPDH-vaccinated mothers, when compared to the progeny of Sham-vaccinated dams (Figure 15F, left). This did not reflect in differences in their total number (Figure 15F, right).
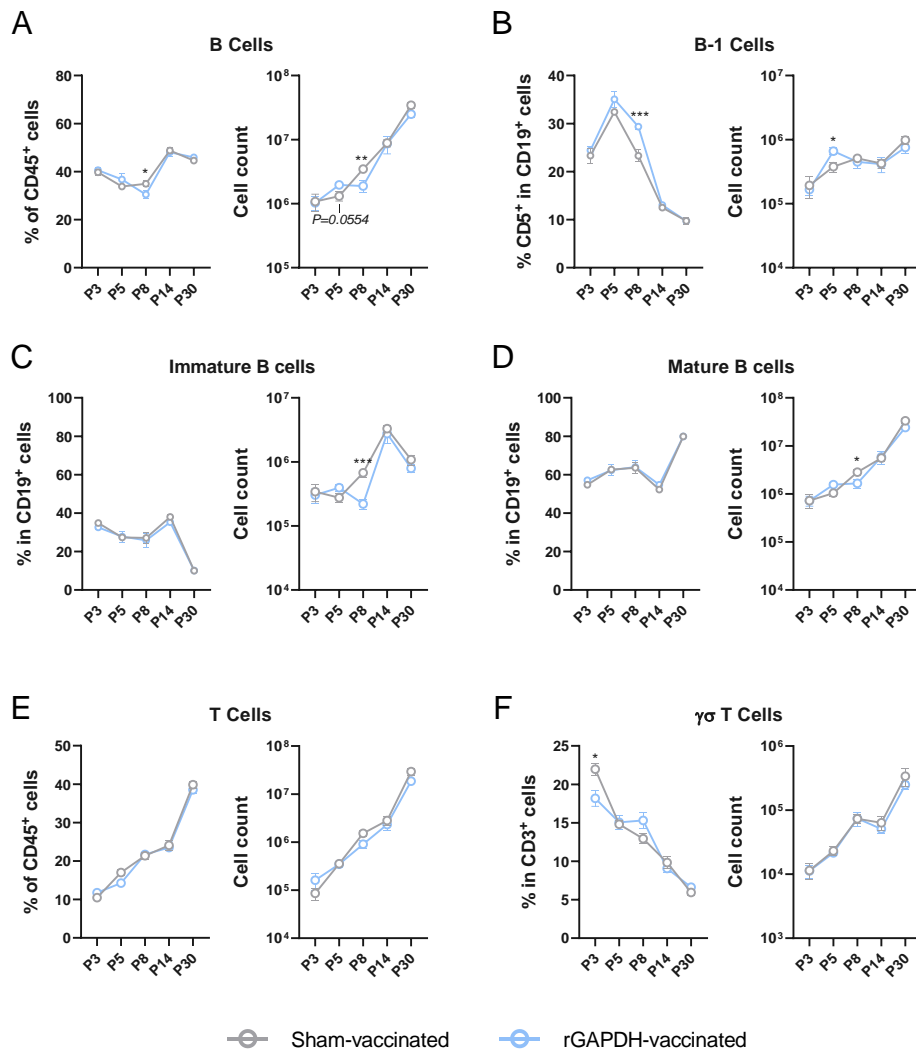
Figure 15 | Maternal rGAPDH-vaccination impact on the splenic lymphoid cells of their offspring. Female BALB/c mice were immunized with GBS rGAPDH plus the Alum (rGAPDH-vaccinated) or only with the Alum (Sham-vaccinated). Animals born from the two groups were sacrificed at indicated time points and the spleen excised for flow cytometry analysis of lymphoid populations. (A-H) Frequency and number of indicated lymphoid populations. Each symbol indicates the mean ± SEM and is represented as pooled from 2 to 4 independent litters [n=10 (P3 and P14 Sham-vaccinated); n=13 (P3 and P8 rGAPDH-vaccinated, P8 Sham-vaccinated); n=6 (P5 Sham-vaccinated); n=9 (P5 rGAPDH-vaccinated); n=8 (P14 and P30 rGAPDH-vaccinated); n=4 (P30 Sham-vaccinated)]. Comparisons by Student's t-test or Mann-Whitney test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Overall, the maternal vaccination with rGAPDH affects the lymphoid compartment within the liver and spleen of their progeny, between P5 and P8.

5. Maternal rGAPDH vaccination does not alter immune blood cells of their progeny

In order to determine if the observed differences in the spleen and liver correlated with alterations in circulating cells, blood was collected from animals of both groups, at P3, P5 and P8. Due to the amount of blood obtained at P3 being very small, these

samples were analysed by conventional flow cytometry, whereas older animals were analysed by hemogram in a Haematology Analyser developed for veterinary use.

At P3, no differences were observed between the two groups (Figure 16).
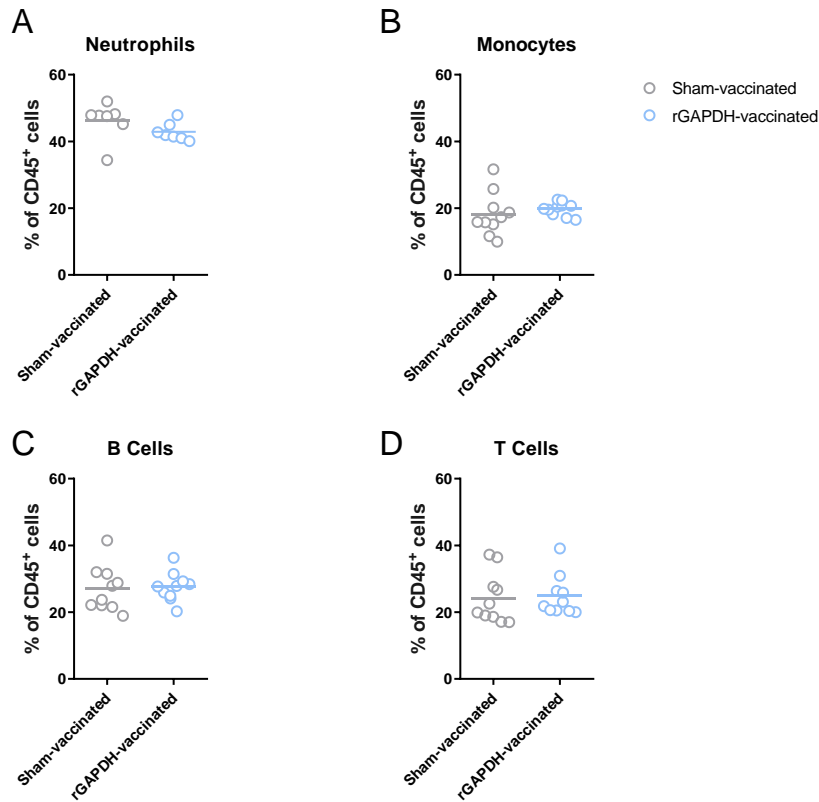
Figure 16 | Maternal rGAPDH-vaccination does not affect circulatory immune cells of their offspring. Female BALB/c mice were immunized with GBS rGAPDH plus the Alum (rGAPDH-vaccinated) or only with the Alum (Sham-vaccinated). Animals born from the two groups were sacrificed at post-natal day 3 and the blood was collected for flow cytometry analysis. (A-D) Frequency of indicated cell populations. Each symbol represents data from an individual mouse. Horizontal lines indicate the mean for each group. The results represent data pooled from 2 to 3 independent litters [n=7 (neutrophils); n=10 for the remaining populations]. Comparisons by Student's t-test or Mann-Whitney test.

Analysis of blood from P5 animals showed that pups born from rGAPDH-vaccinated mothers presented a significant increase on the plateletcrit, *i.e,* the measure of total platelet mass, and on the total number of platelets when compared to the Sham-vaccinated offspring (Figure 17G and H). No more differences were observed between groups (Figure 17).

Figure 17 | Maternal rGAPDH-vaccination does not alter blood populations of their offspring. Female BALB/c mice were immunized with GBS rGAPDH plus the Alum (rGAPDH-vaccinated) or only with the Alum (Sham-vaccinated). Blood from pups born from the two groups was collected and directly analysed through ProCyte Dx Haematology Analyser (IDEXX). (A) Number of red blood cells per litre. (B) Haematocrit. (C) Haemoglobin concentration. (D) Frequency of reticulocytes. (E) Platelet distribution width (PDW). (F) Mean platelet volume (MPV). (G) Percentage of plateletcrit. (H) Number of platelets per litre. (I) Number of white blood cells (WBC) per litre. (J-L) Frequency and number of indicated cell populations. Each symbol indicates the mean ± SEM and represents data pooled from 2 to 3 independent litters [n=5 (P5 Sham-vaccinated and P8 rGAPDH-vaccinated); n=6 (P5 rGAPDH-vaccinated) n=9 (P8 Sham-vaccinated)]. Comparisons by Student's t-test or Mann-Whitney test. *$P < 0.05$.

6. Thymus of pups born from rGAPDH-vaccinated mothers does not appear to be altered

As the thymus is a primary lymphoid tissue, responsible for the maturation of T cells, and we have found that the T cell compartment in the liver of pups born from rGAPDH-vaccinated mothers was slightly altered at P8, this organ was analysed at P5 and P8. The thymocytes and T cell profile were analysed by flow cytometry, by assessing the percentage and number of double-negative (CD4$^-$CD8$^-$) T cells, double-positive (CD4$^+$CD8$^+$) T cells, CD4$^+$ T cells and CD8$^+$ T cells. The gating strategy used to define the different cell populations is represented in Figure 18.
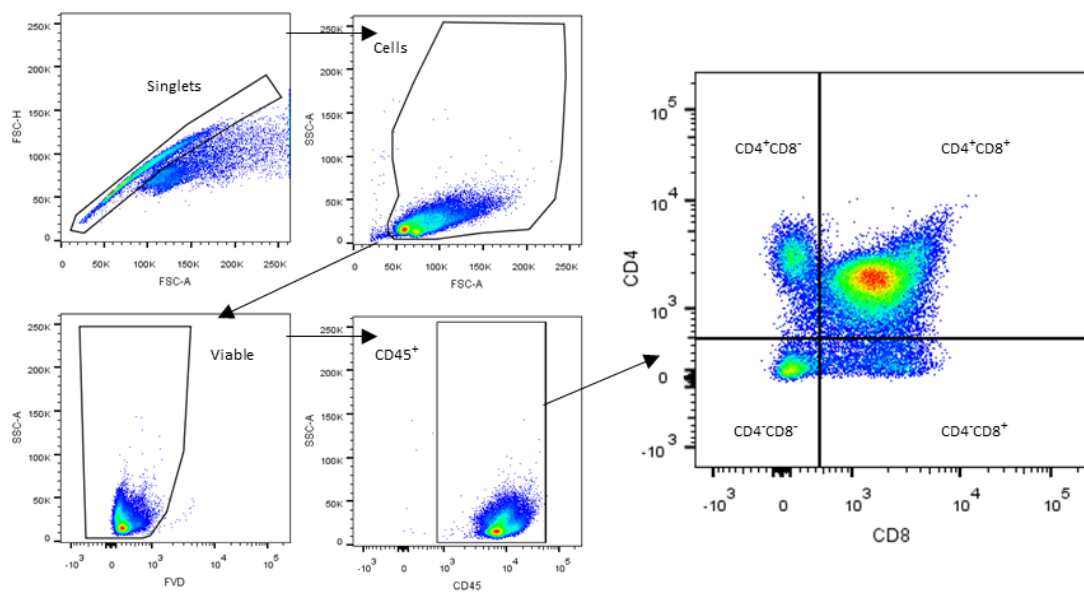
Figure 18 | Flow cytometry gating strategy of thymic populations. Representative gating strategy used to define the different cells in the thymus. All cells were first gated on singlets (FSC-A vs FSC-H dot plot) and a gate for total cells was made from FSC-A vs SSC-A dot plot. Dead cells were further removed by staining with a fixable viability dye and the remaining cells were gated on CD45$^+$ cells. Based on the expression of CD4 and CD8, cells were further defined as double-negative T cells (CD4$^-$CD8$^-$), double-positive T cells (CD4$^+$CD8$^+$), and single-positive T cells (CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$).

No differences were observed in total thymic cells between groups (Figure 19A). Regarding double-positive, double-negative and single-positive CD4$^+$ T cells, no differences were observed between groups, at the studied time points (Figure 19B-D). The frequency of single-positive CD8$^+$ T cells was slightly decreased in pups born from rGAPDH-vaccinated dams at P5, comparing to controls (Figure 19E, left). However, from P5 to P8, the frequency of CD8$^+$ T cells remained fairly stable in pups born from rGAPDH-vaccinated mothers, whereas in the progeny of Sham-vaccinated group it decreased, reaching similar levels (Figure 19E, left). Regarding the absolute number of CD8$^+$ T cells,

no differences were found between both groups, at both time points tested (Figure 19E, right).
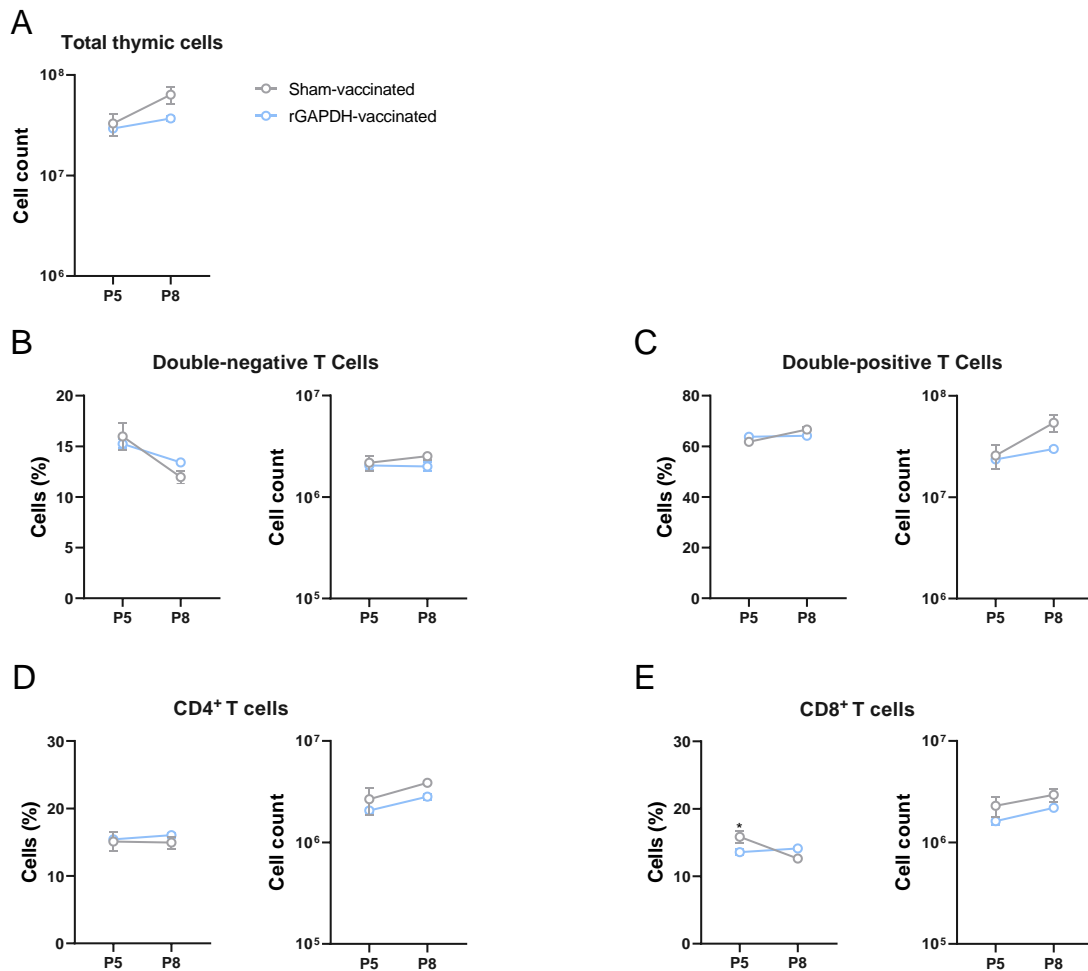
Figure 19 | Maternal rGAPDH-vaccination does not appear to affect their offspring thymic populations. Female BALB/c mice were immunized with GBS rGAPDH plus the Alum (rGAPDH-vaccinated) or only with the Alum (Sham-vaccinated). Animals born from the two groups were sacrificed at indicated time points and the thymus was excised for flow cytometry analysis. (A) Number of total thymic cells. (B-E) Frequency and number of indicated populations. Each symbol indicates the mean ± SEM and represents data pooled from 1 to 3 independent litters [n=6 (P5 Sham- and rGAPDH-vaccinated, and P8 Sham-vaccinated); n=4 (P8 rGAPDH-vaccinated)]. Comparisons by Student's t-test or Mann-Whitney test. *$P < 0.05$.

Maternal vaccination with rGAPDH does not alter the immune response against neonatal *E. coli* infection

Due to the observed differences in the neonatal immune system development of pups born from rGAPDH-vaccinated mothers, we next sought to assess whether it would alter the immune response to a relevant infectious agent. To test this hypothesis, we challenged pups with *E. coli* as it is a major neonatal pathogen, responsible for sepsis and meningitis, particularly the K1 strains. For that purpose, pups were orally infected at

P4 with *E. coli* IHE3034 (a K1 strain) and survival was evaluated. As shown in figure 20, no statistical difference was observed in the percentage of survival of pups born from Sham- or rGAPDH-vaccinated mothers. No death was recorded after 4 or 5 days post-infection (dpi) in rGAPDH- or Sham-vaccinated group, respectively (Figure 20).
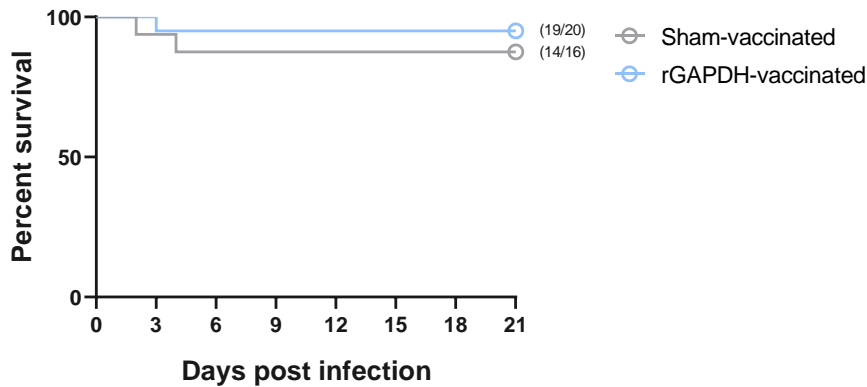
Figure 20 | Neonates survival curve. Female BALB/c mice were immunized with GBS rGAPDH plus the Alum (rGAPDH-vaccinated) or only with the Alum (Sham-vaccinated). Neonates born from the two groups were orally inoculated, at post-natal day 4, with 1×10^7 CFU of *E. coli* IHE3034. Newborns remained with their mothers during the course of the experiment. Kaplan–Meier survival curve of neonatal mice, determined in a 21-day period. Numbers in parenthesis indicate the number of animals that survived versus the total number of infected animals [n=16 (Sham-vaccinated); n=20 (rGAPDH-vaccinated). Results represent data pooled from 3 to 4 independent experiments. Comparisons with log-rank (Mantel-Cox) test.

To evaluate if maternal rGAPDH-vaccination was interfering with the neonatal ability to clear this pathogen, the bacterial load in the blood, brain, lung and liver was determined at 3 dpi. No significant differences were observed in the brain, lung and liver of pups born from Sham- or rGAPDH-vaccinated mothers (Figure 21B-D). Interestingly, neonates born from rGAPDH-vaccinated progenitors presented a higher capacity to clear the bacteria from circulation, as only 1 out of 7 pups were colonised in blood at 3 dpi, whereas in the pups born from Sham-vaccinated mothers bacteria was only not detected in 3 out of 12 pups (Figure 21A). The body weight of the pups was also similar between groups (3.91 ± 0.28 g in mice born from Sham-vaccinated mothers compared to 4.61 ± 0.64 g in pups born from rGAPDH-vaccinated females).
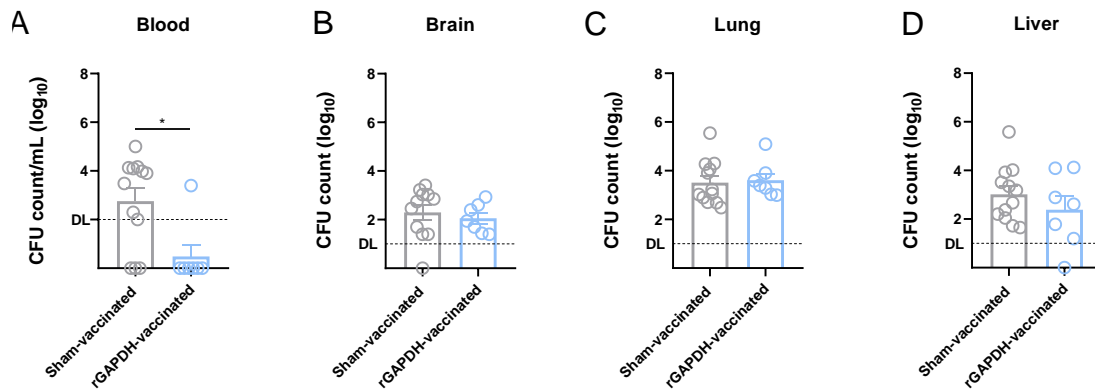
Figure 21 | Organ colonisation in pups after *E. coli* infection. Female BALB/c mice were immunized with GBS rGAPDH plus the Alum (rGAPDH-vaccinated) or only with the Alum (Sham-vaccinated). Neonates born from the two groups were orally inoculated, at post-natal day 4, with 1 × 10^7 CFU of *E. coli* IHE3034. *E. coli* counts in blood (A), brain (B), lung (C) and liver (D). Bars indicate mean + SEM and is represented as pooled from 3 independent experiments [n=12 (Sham-vaccinated); n=7 (rGAPDH-vaccinated)]. Each symbol represents an individual mouse. Comparisons by Student's t-test or Mann-Whitney test. *$P < 0.05$. DL, detection limit.

Altogether, these results show that the alterations observed in the immune cell population induced by maternal vaccination with GBS rGAPDH do not impact host immune response to *E. coli.*

The progeny from rGAPDH-vaccinated dams seems to have a predisposition for metabolic disorder

Despite its role as a haematopoietic site, the neonatal liver is also crucial for the proper organism metabolism, similar to what happens in adults. Moreover, during development, due to the different dietary challenges (pre and post-weaning period), liver experiences not only immune but also morphologic and metabolic alterations (142).

Thus, we next analyzed the overall liver morphology by H&E staining, in animals born from Sham- and rGAPDH-vaccinated mothers, at P5, P8, P14 and P30. We found that the liver of the progeny from rGAPDH-vaccinated dams presented higher organ structure disorganization at P14 and P30 when compared to age-matched controls since fewer sinusoids (black arrows) and hepatocyte cords (blue arrows) are observed (Figure 22E-H). Moreover, we also observed that neonates born from rGAPDH-vaccinated dams seem to be accumulating more lipids in the hepatocytes than the Sham-vaccinated progeny, at P8 (Figure 22C e D). This was evidenced by the higher hepatocyte steatosis observed in pups born from rGAPDH-vaccinated mothers at P8, being more similar to

the liver of P5 controls than to aged-matched ones (Figure 22A, C and D). Although in less extension, the same differences were also observed at P14 (Figure 22E and F). By day 30, this was no longer detected (Figure 22G and H). These data suggest that maternal rGAPDH vaccination could impact the metabolic system of their offspring.
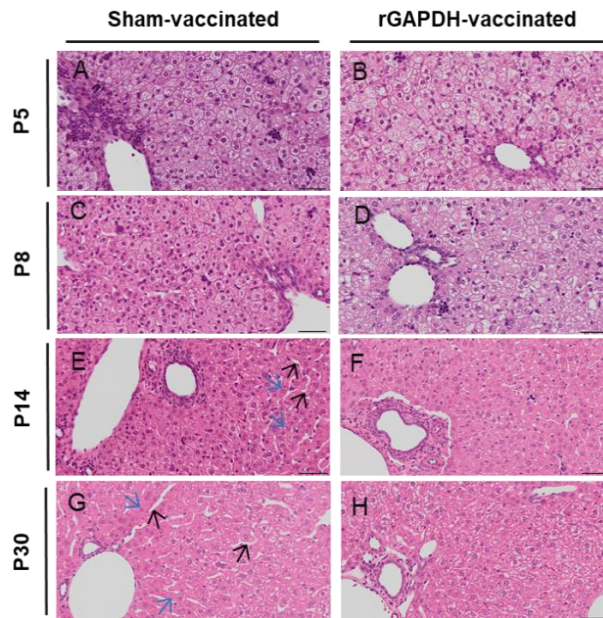
Figure 22 | Histological analysis of mice liver. Female BALB/c mice were immunized with GBS rGAPDH plus the Alum (rGAPDH-vaccinated) or only with the Alum (Sham-vaccinated). (A-H) H&E staining of liver tissue of pups born from Sham- and rGAPDH-vaccinated mothers at post-natal days 5 (A and B), 8 (C and D), 14 (E and F) and 30 (G and H). Representative data from 2 to 5 independent litters [n=6 (P5 and P8 Sham-vaccinated); n=9 (P5 rGAPDH-vaccinated); n=12 (P8 rGAPDH-vaccinated); n=5 (P14 Sham-vaccinated and P30 rGAPDH-vaccinated); n=3 (P14 rGAPDH-vaccinated); n=2 (P30 Sham-vaccinated)]. Original magnification 20x. Scale bars are 50 μm. Black arrows indicate sinusoids and blue arrows identify hepatocyte cords.

The assembly of microbial communities within the gastrointestinal tract during early life plays a critical role in endocrine and metabolic pathways. Moreover, there is now mounting evidence, in humans and rodents, for the role of the gut microbiota in non-alcoholic fatty liver disease (NAFLD) / non-alcoholic steatohepatitis (NASH) (140, 143, 144). Thus, we next questioned whether early life hepatic alterations could have a long-term impact on liver metabolism. For that purpose, the progeny of both Sham- and rGAPDH-vaccinated mothers were submitted to a high-fat diet (HFD) immediately upon weaning, for 30 consecutive days. HFD is a dietary regimen rich in animal saturated fat (45% lard), known to induce a metabolic disorder. The body weight gain of both males and females were monitored.

At the end of the experiment, the body weight fold change was calculated for both groups of mice. Females born from rGAPDH-vaccinated mothers presented a significant increase in body weight fold change when compared to the Sham-vaccinated female progeny (Figure 23A). The male offspring born from rGAPDH-vaccinated dams presented the same tendency, although not statistically significant (Figure 23A). Moreover, analysis of the percentage of body weight gained weekly showed that from week 2 till the end of the experiment, females born from rGAPDH-vaccinated mothers displayed a significant increase when compared to the Sham-vaccinated group (Figure 23B). Once again, males from the rGAPDH-vaccinated group also presented higher weight gain percentage every week, albeit not statistically significant (Figure 23B).
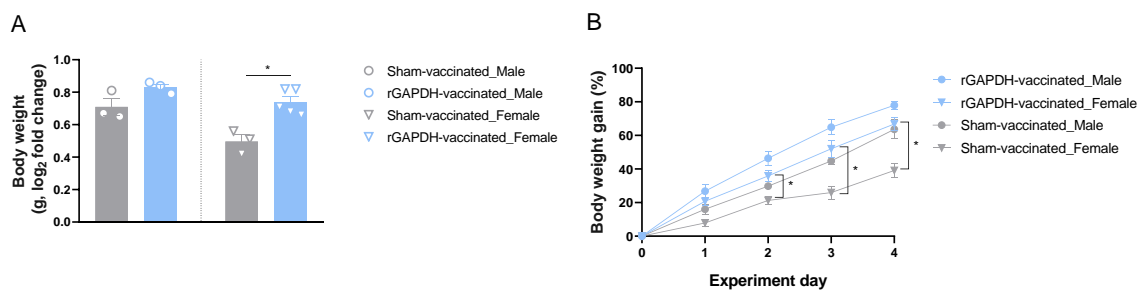
Figure 23 | Maternal rGAPDH-vaccination suggests predisposition of their progeny to weight gain during young adulthood. Female BALB/c mice were immunized with GBS rGAPDH plus the Alum (rGAPDH-vaccinated) or only with the Alum (Sham-vaccinated). The offspring of both groups were fed with a high-fat diet for 30 days, immediately upon weaning. (A) Weight gain fold change at the end of the experiment. (B) Weekly percentage of body weight gain. Bars (A) and symbols (B) indicate the mean ± SEM and represents 1 independent experiment [n= 3 (Sham-vaccinated group, males and females); n=5 (rGAPDH-vaccinated, females); n=3 (rGAPDH-vaccinated, males)]. Comparisons by Mann-Whitney test. *$P < 0.05$.

In order to determine if the diet induced an inflammatory response, the splenic immune cells were examined by flow cytometry, at the end of the experiment. Relatively to the male born from rGAPDH-vaccinated progenitors, no differences were found in both myeloid and lymphoid populations (Figures 24 and 25). Regarding female mice, the rGAPDH-vaccinated group showed a significant increase in the number of neutrophils (Figure 24B) and a decrease in the frequency of Ly6C^{high} monocytes. No differences were found in the remaining myeloid populations nor in the lymphoid cells (Figures 24 and 25).
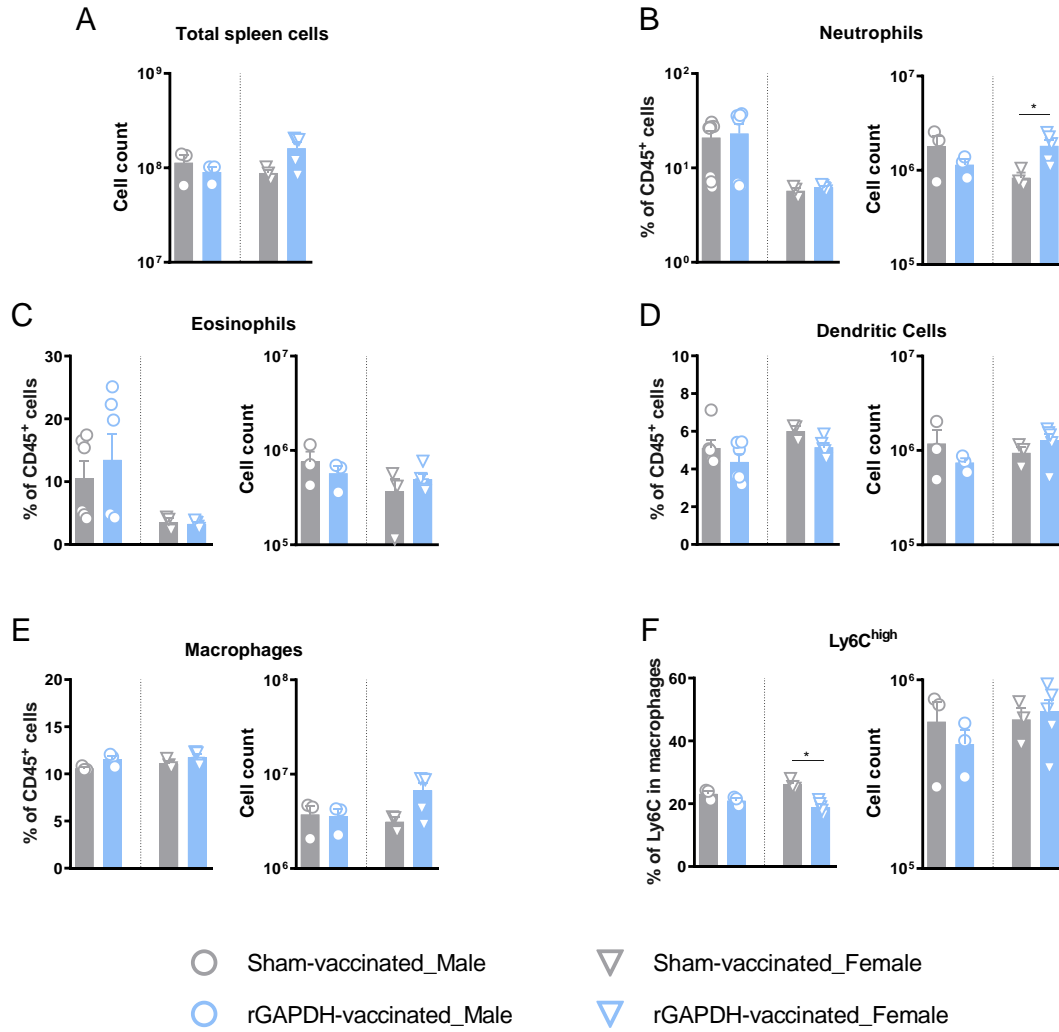
Figure 24 | Female rGAPDH-vaccinated offspring fed with high-fat diet is slightly affected in myeloid populations. Mice born from Sham- and rGAPDH-vaccinated mothers were fed with a high-fat diet for 30 days, immediately upon weaning. Animals were sacrificed and the spleen excised for flow cytometry analysis of myeloid populations. (A) Absolute number of total spleen cells. (B-H) Frequency and number of indicated myeloid populations. Each symbol indicates an individual mouse. Bars indicate the mean + SEM and represents 1 independent experiment [n= 3 (Sham-vaccinated group, males and females); n=5 (rGAPDH-vaccinated, females); n=3 (rGAPDH-vaccinated, males)]. Comparisons by Mann-Whitney test. *$P < 0.05$.
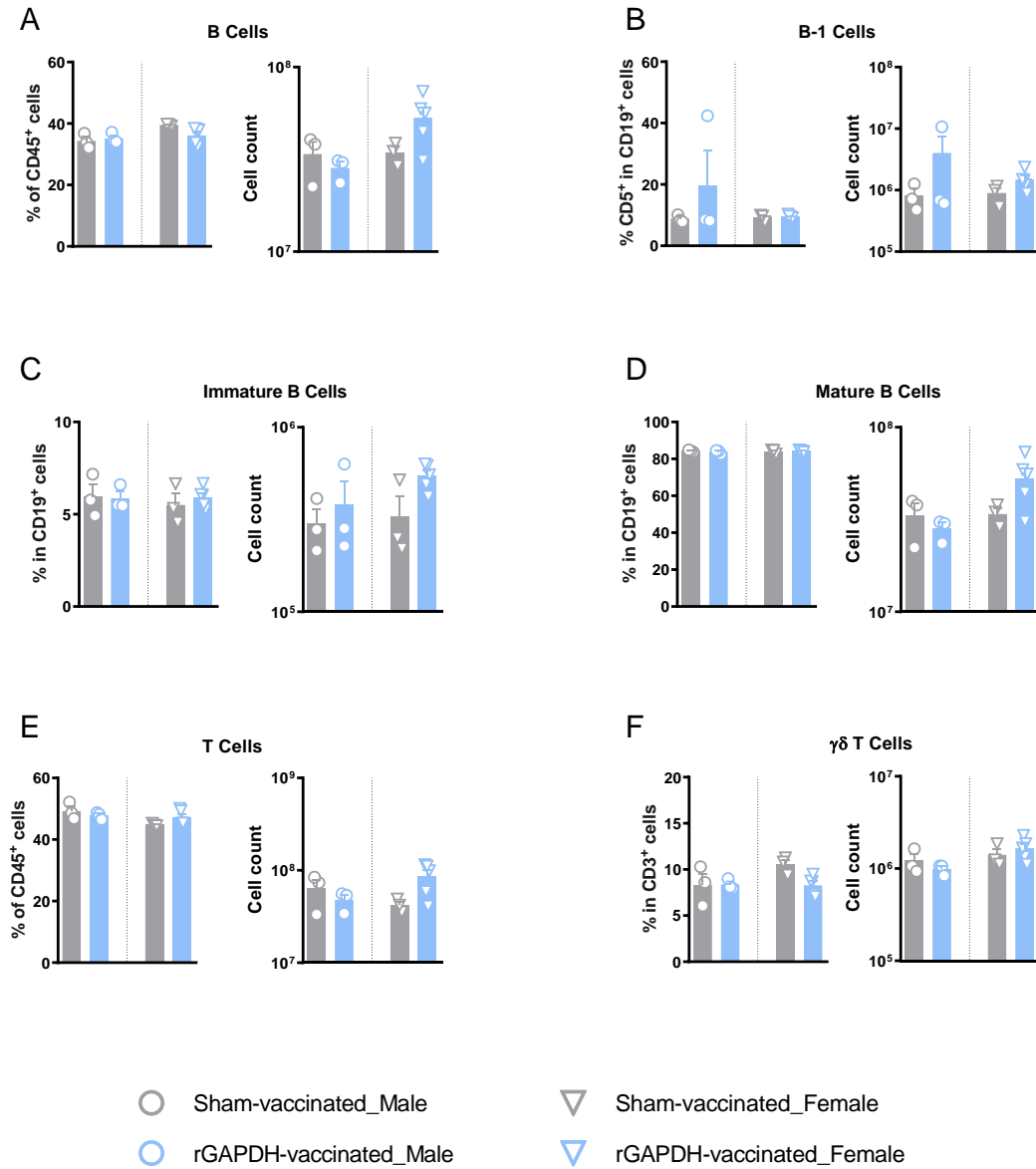
Figure 25 | Splenic lymphoid populations after mice high-fat diet. Mice born from Sham- and rGAPDH-vaccinated mothers were fed with a high-fat diet for 30 days, immediately upon weaning. Animals born from rGAPDH- and Sham-vaccinated mothers were sacrificed after 30 days of high-fat diet and the spleen excised for flow cytometry analysis of lymphoid populations. (A-F) Frequency and number of indicated lymphoid populations. Each symbol indicates an individual mouse. Bars indicate the mean + SEM and represents 1 independent experiment [n= 3 (Sham-vaccinated group, males and females); n=5 (rGAPDH-vaccinated, females); n=3 (rGAPDH-vaccinated, males)]. Comparisons by Mann-Whitney test.

Liver metabolic disturbances may lead to increased gut permeabilization, allowing bacterial liver colonisation (145, 146). To evaluate if mice born from rGAPDH-vaccinated mothers presented this phenomenon, we investigated the bacterial load in the liver of animals from both groups, at the end of the HFD protocol. As shown in Figure 26, no statistically significant differences were observed between groups.
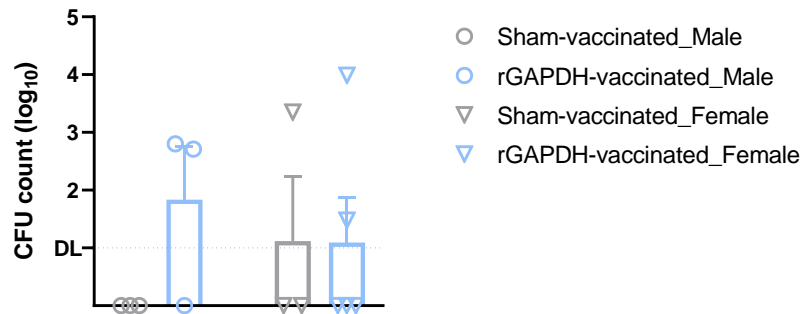
Figure 26 | Liver colonisation. Animals born from Sham- and rGAPDH-vaccinated mothers were sacrificed after 30 days of high-fat diet and the liver excised for bacterial colonisation assessment. Each symbol indicates an individual mouse. Bars indicate the mean + SEM and represents 1 independent experiment [n=3 (Sham-vaccinated group, males and females); n=5 (rGAPDH-vaccinated, females); n=3 (rGAPDH-vaccinated, males)]. Comparisons by Mann-Whitney test.

Furthermore, we measured serum levels of alanine aminotransferase (ALT), a hallmark of liver health (147). No significant differences were found between both groups (Figure 27).
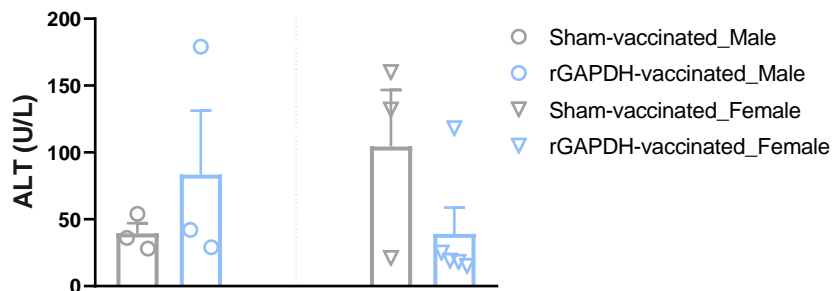
Figure 27 | ALT serum levels. Animals born from Sham- and rGAPDH-vaccinated mothers were sacrificed after 30 days of high-fat diet and the blood collected for ALT serum levels measurement. Each symbol indicates an individual mouse. Bars indicate the mean ± SEM and represents 1 independent experiment [n= 3 (Sham-vaccinated group, males and females); n=5 (rGAPDH-vaccinated, females); n=3 (rGAPDH-vaccinated, males)]. Comparisons by Mann-Whitney test.

Overall, mice born from rGAPDH-vaccinated dams seem to present a higher predisposition to weight gain when fed with a fat-enriched diet, especially females.

Discussion

Group B *Streptococcus* (GBS) remains the leading cause of neonatal sepsis and meningitis worldwide (2, 3), despite the current use of *intrapartum* antibiotic prophylaxis (IAP) to women at risk of transmission (5, 30, 31). IAP has been enormously beneficial and has vastly reduced the risk for early-onset sepsis in neonates (5, 30, 31). However, while its advantages should be recognized, its overuse and/or misuse is of serious concern and great efforts should be done to develop a vaccine to protect neonates and reduce IAP use. Our group has identified the extracellular form of GBS GAPDH as a valuable universal vaccine candidate against GBS infection (29). In a mouse model of GBS infection, maternal vaccination with recombinant rGAPDH or passive immunization with anti-rGAPDH IgG antibodies did confer neonatal protection (148).

However, GAPDH is a highly conserved bacterial enzyme and our group showed that antibodies raised against GBS rGAPDH, produced through maternal vaccination, recognize two intestinal neonatal commensals. Moreover, unpublished results indicate that maternal rGAPDH vaccination negatively impacts the splenic (Pinho B, BSc Thesis) and mucosal (Mesquita P, BSc Thesis) immune system, as well as the development of the central nervous system (Geraldo R, BSc Thesis) of their offspring.

Emerging data showed the importance of the intestinal bacterial microbiome in regulating and shaping haematopoiesis - the developmental programming of the immune system (81-84). Microbial components can access distal sites, such as the bone marrow, where they may regulate the proliferation and differentiation of progenitors and their differentiated cells (68). Thus, we hypothesized that if anti-rGAPDH IgG antibodies cross-reacts with neonatal intestine commensal bacteria, interfering with the early gut colonisation, maternal vaccination could impact the regulation of neonatal haematopoiesis. In this work, we studied the extension of maternal rGAPDH vaccination impact in their offspring, within haematopoietic organs, during the first week of life.

Haematopoiesis starts at gestational phase, where the liver is the major haematopoietic site (45-48). Haematopoietic cells then migrate to the spleen and bone marrow near birth, meaning that, during the first days of life, the liver and spleen are still haematopoietic organs (49). In this work, we demonstrated an altered haematopoiesis in pups born from rGAPDH-vaccinated mothers, that appear five days after birth. In the bone marrow, decreased frequency in both haematopoietic stem cells and multipotent progenitors (LSK), as well as in GMP, in the rGAPDH-vaccinated progeny, were observed. Regarding the spleen, decreased frequencies of LSK, CLP and MEP were observed in pups born from rGAPDH-vaccinated mothers when compared to those born from Sham-vaccinated females. In the liver, although the haematopoietic alterations

were subtle, we observed a slight increase in the number of myeloid progenitors (CMP and GMP) in the progeny of rGAPDH-vaccinated mothers. One week after birth, all three organs showed increased percentage of GMP, when compared to the Sham-vaccinated offspring. Moreover, at this stage, bone marrow of pups born from rGAPDH-vaccinated mothers presented increased frequency of CMP and MEP, whereas the spleen showed decreased number of LSK. Additionally, the values reached at P8 in the rGAPDH-vaccinated offspring were similar to those observed five days after birth in the Sham-vaccinated progeny, which suggests that rGAPDH-vaccinated offspring reach control levels later in time, presenting a delay in the haematopoiesis.

Haematopoiesis is a highly regulated mechanism, and during the neonatal period the spleen, bone marrow, and liver cooperatively contribute to haematopoietic homeostasis. Indeed, despite the decreased frequency of GMP observed in the bone marrow of P5 animals born from rGAPDH-vaccinated mothers, this did not reflect in a decrease in mature myeloid cells in the spleen and liver. On the opposite, five days after birth, we found increased frequency and number of dendritic cells, macrophages and inflammatory Ly6C^{high} monocytes in both liver and spleen of pups born from rGAPDH-vaccinated mothers. The heightened frequency of Ly6C^{high} monocytes further persisted in P8 pups. These data favour the idea that all haematopoietic organs can be compensating each other. This is further supported by the absence of differences in mature circulating cells, despite the observed differences within the organs. Notwithstanding, we did not evaluate the phenotypic and functional profile of circulating cells, nor evaluated the presence of immune cells at early stages of development, such as transitional B cells, or recent thymic emigrants, which are known to be increased in newborns (59, 61, 62). Moreover, based on our experiments, we cannot determine which organ is contributing to the overall mature populations. It is known that upon maturation in the bone marrow, differentiated leukocytes egress into the blood circulation, in a process dependent on the expression of extracellular matrix components, chemokines and their receptors, adhesion molecules, and other (149). We did not study mature populations in the bone marrow, and these might not be affected. Moreover, chemokines and chemokine receptors, or other adhesion molecules were not studied. It has been shown that during the first week of life, the liver might be the major haematopoietic organ and is the origin of bone marrow and spleen HSC (49). Thus, it is reasonable to speculate that the higher numbers of CMP and GMP found in the liver are contributing to the increased numbers of the mature macrophages, dendritic cells and inflammatory monocytes, observed in the rGAPDH progeny at both P5 and P8. However, it is also possible that cells from Sham- or rGAPDH-vaccinated offspring have different abilities to

traffic to peripheral organs. Experiments using chimeric mice or competitive trafficking assays could allow uncovering this paradigm.

Little is known about neonatal haematopoiesis, and, to or knowledge, only one study on the effect of neonatal gut dysbiosis in early life haematopoiesis was published. In that study, perinatal antibiotic exposure led to a reduced and altered microbiota, which, in turn, increased bone marrow CMP and decreased GMP (82). The animal age at which these experiments were performed is not mentioned, making the comparison with our work not possible. However, the evidence that gut dysbiosis impacts neonatal haematopoiesis is in agreement with our findings. We do not know the specific alterations and the extension of the impact of maternal rGAPDH-vaccination on their offspring gut microbiota, but faecal samples of pups from both Sham- and rGAPDH-vaccinated mothers at different time points were already sent to LGC Genomics GmbH for analysis. Microbiota characterization will be performed through next-generation sequencing techniques, targeting the sequencing of 16S rRNA. Thus, in the near future, we will be able to understand more accurately the effect of maternal vaccination on gut colonisation of their progeny. Regarding adults, few studies have also already reported an association with altered/depleted microbiota and modified haematopoiesis in both antibiotic-treated or germ-free (GF) mice (81, 83, 84). Antibiotic treated mice showed decreased bone marrow cell counts of LSK and CLP (83), similarly to what was observed in our study in the bone marrow and spleen of neonates at P5. GF mice also presented diminished bone marrow LSK cell count and, in addition, showed decreased frequency and number of GMP (81, 84), once again in accordance to our findings in the bone marrow five days after birth, highlighting the importance of gut colonisation to the immune system balance. These studies also report differences in the myeloid and lymphoid mature cells, namely a decrease in bone marrow granulocytes and B cells, in accordance with the diminished haematopoietic progenitors (81-84). Regarding the liver and spleen of both GF and antibiotic-treated adult mice, a reduction in the percentages and numbers of macrophages, monocytes and neutrophils was reported (81).

Independently of the cell origin and haematopoietic regulation, our results suggest a neonatal window, between P5 and P8, to a higher inflammatory state. Accordingly, studies from our laboratory showed that eight-day-old pups born from rGAPDH-vaccinated mothers presented significantly higher serum levels of the inflammatory cytokines IFN-γ and IL-6 than pups born from Sham-vaccinated progenitors (Andrade EB, *unpublished data*). This inflammatory state might be due to the altered gut microbiota in pups born from rGAPDH-vaccinated dams. Knowing the link between bacteria and inflammation, and that microbiome abnormalities can impair the barrier

functions of the intestinal mucosa, leading to enhanced mucosa permeability and subsequent translocation of bacteria, it will be interesting to evaluate if these pups have increased bacteria colonisation in the liver. It is reasonable to speculate that higher bacterial levels in these animals would lead to increased activation of pattern recognition receptors, and subsequently to the induction of inflammatory responses. Also, it has been recently reported that monocyte myeloid-derived suppressor cells (M-MDSC), are upregulated in newborns and are important for the regulation of newborns' T-cell suppressive capacity and antimicrobial activity (150). These cells are characterized as CD11b$^+$Ly6G$^-$Ly6C^{high} (151), similarly to our strategy to define the Ly6C^{high} population. Functional studies of M-MDSC isolated from pups born from Sham- and rGAPDH-vaccinated mothers will confirm their suppressive role in our experimental model. Moreover, newborn accumulation of MDSC has been linked to lactoferrin, an immunoregulatory component of milk (150, 152, 153). Thus, it is possible that rGAPDH-vaccination might be altering the components of maternal breast milk, interfering in normal infant cellular development. An alternative hypothesis is that higher levels of observed Ly6C^{high} monocytes could be in particular MDSCs, important for the control of inflammation, associated with a possible bacterial translocation from the gut in these animals.

Regarding the lymphoid compartment, liver presented increased frequency and number of B cells as well as CD5$^+$ B-1 cells. Moreover, the total number of these cells were also significantly increased in the spleen, at P5, despite no differences in the frequency of CLP in the BM and CLP number in the liver and spleen. Gut microbiota is also known to play an active role in spleen development and maturation (154). The splenic environment stimulates neutrophils to become B-helper neutrophils (NBH cells), activating B cells through the expression of BAFF, APRIL, CD40L and IL-21 (155). Neutrophil-activated B cells lead to the production of immunoglobulins, somatic hypermutation and class switch recombination (156). However, these neutrophils are known to be dependent on the commensals (157). Therefore, neonatal gut colonisation may be crucial for the interaction between neutrophils and B cells in the spleen. In order to understand B-cell activation in the rGAPDH-vaccinated progeny, we will perform a full characterization of splenic B cells, namely marginal zone (MZ) B cells, B-1a, B-1b and transitional B cells. Levels of circulating Igs will also be studied, to determine the splenic B-cells activation state.

Altogether, our work provided a kinetic haematopoietic study in early life, showing the impact of a possible altered/depleted gut microbiota due to maternal vaccination with

GAPDH in the immune system development of their offspring, during the first week of life.

Innate immune cells are the first responders to infection, mediating early pathogen control and coordinating downstream immune reactions. Given the increased frequency and number of phagocytic and inflammatory cells, found at P5, in both liver and spleen from pups born from rGAPDH-vaccinated females, we questioned if these alterations would confer protection to neonatal infections. Moreover, at this stage, maternal vaccination increased neonatal liver B and CD5^{+} B-1 cells, both in frequency and number, which, albeit the immaturity/poor functionality of these populations at this stage of development (57, 62-64), seem to corroborate the hypothesis that neonates born from rGAPDH-vaccinated mothers could be protected against *E. coli* infection. Thus, we orally infected post-natal day 4 pups with a K1 *E. coli* strain, as a model of enteric infections. Both groups presented low mortality rate, as well as equivalent body weight at 3 days post-infection. Nevertheless, neonates born from the rGAPDH-vaccinated group were able to effectively clear the bacteria from circulation, in contrast with the observed in the Sham-vaccinated progeny. Interestingly, no differences were found in organ colonisation between Sham- and rGAPDH-vaccinated offspring. Given that this group presented increased phagocytic populations, this result might suggest that rGAPDH-vaccinated progeny might have impaired antimicrobial activity, justifying why they are not able to control bacterial dissemination as we first thought. To confirm this hypothesis, we will evaluate macrophage antimicrobial activity, by isolating and culture macrophages from P5-P8 pups from both groups, followed by bacterial inoculation and CFU count.

In addition to not being protected against *E. coli* infection, previously unpublished work from our group showed that maternal vaccination with rGAPDH does not confer protection against GBS infection using a model in which the pathogen is transmitted to the offspring from vaginally colonised pregnant females, as it occurs in humans (Mestre A, BSc thesis; Marques C, BSc thesis). In fact, although not significant, rGAPDH-vaccinated offspring presented a higher mortality rate than the obtained in the control group (Mestre A, BSc thesis). Moreover, no differences were found in levels of bacterial colonisation in the lungs, brain, liver nor intestine of pups born from rGAPDH-vaccinated group, comparing to those born from Sham-vaccinated mothers, at post-natal days 1, 3 and 5 (Marques C, BSc thesis).

To more accurately understand if the observed haematopoietic changes in the rGAPDH-vaccinated offspring alter susceptibility to other relevant neonatal pathogens,

we will use another model of infection. According to WHO, *Streptococcus pneumoniae* is the most common causative agent of pneumonia in children (158). Moreover, it has been described that commensal bacteria are pivotal for the neonatal resistance to this infection (159). Thus, we will infect pups using the intra-tracheal inoculation route, as a model to mimic infant pneumonia. The neonatal challenge will be performed at P4, similarly to what was with *E. coli*, and at P5, where higher immunological differences appeared in neonates born from rGAPDH-vaccinated progenitors. Furthermore, susceptibility to *Listeria monocytogenes* will also be tested, given the decreased erythroid progenitors found in the spleen and the fact that mature cells from this lineage are known to contribute for neonatal immunosuppression (160). Although we did not study the erythroid cells, fewer progenitors in the spleen of rGAPDH-vaccinated progeny might lead to limited mature cells. In this case, it might be translated in reduced susceptibility to this pathogen.

Early life dysbiosis not only influences susceptibility to infections and sepsis, but also a broad range of metabolomic disorders in adulthood, including obesity (106, 108). Hepatic metabolism is an important step in the final liver development (142). Histopathological analysis of the liver throughout the first month of life suggests that pups born from rGAPDH-vaccinated offspring present an altered/delayed liver development. The normal structure of neonatal liver is different from the adults, presenting high cellularity and no organization (142). The liver architecture becomes more similar to adults with time, with increased sinusoids and hepatocyte cords (142). Our work showed that, at P14 and 30, liver from pups born from rGAPDH-vaccinated mothers presented higher disorganization when compared to age-matched controls, suggesting a delay in the development of the liver. Moreover, livers of pups born from rGAPDH-vaccinated mothers presented differences that might be related to metabolism. One week after birth, pups from Sham-vaccinated progeny presented less or smaller vacuoles, while rGAPDH-vaccinated group showed a vacuolation profile similar to the observed at P5. Although in less extension, the same differences were still observed between groups, at P14. Vacuolation within hepatocytes during the neonatal period is common, given the enriched lipid diet during lactation (142). However, it is expected to decrease over time, as observed in the progeny of Sham-vaccinated mothers. To confirm that the observed steatosis in pups born from rGAPDH-vaccinated mothers is due to lipid accumulation, Oil Red O staining will be performed. Dysbiosis has been associated with several liver disorders. To shed light if the observed alterations in the liver have a long-term impact, we submitted the progeny of Sham- and rGAPDH-vaccinated mothers to a high-fat diet (HFD) immediately upon weaning, for 30 days. Despite being preliminary, as only one

litter from each group was analysed, our results show that animals born from rGAPDH-vaccinated progenitors, specifically females, have increased weight gain upon week 2, which might suggest an altered metabolism. To better understand the effects of the HFD, food intake should be monitored to confirm if the weight gain is due to altered metabolism or higher caloric intake. Moreover, the weight gain of animals with normal diet must be determined. No major differences were found in splenic myeloid nor lymphoid populations in the spleen of either Sham- or rGAPDH-vaccinated offspring, suggesting the absence of inflammation, a common consequence of HFD. Likewise, serum levels of ALT, an indicator of liver health (147) did not differ between groups, within gender. However, other parameters, as inflammatory cytokines, must be quantified. We were unable to confirm the hypothesis that gut permeability could be altered in pups born from rGAPDH-vaccinated mothers, as it has been previously described in animals submitted to HFD (161).

In conclusion, this work provided an exhaustive characterisation of the impact of maternal rGAPDH vaccination on the haematopoiesis of their progeny. Both progenitors and mature populations were affected from day 5 to 8 after birth, mainly presenting an overall delay in the progenitor's development and increased myeloid populations in the liver and spleen in pups born from rGAPDH-vaccinated offspring (summarised in figure 28). Moreover, we showed that maternal vaccination might also affect liver development and metabolism, being detected not only a delay in the liver structural organization but also an increased lipid accumulation.
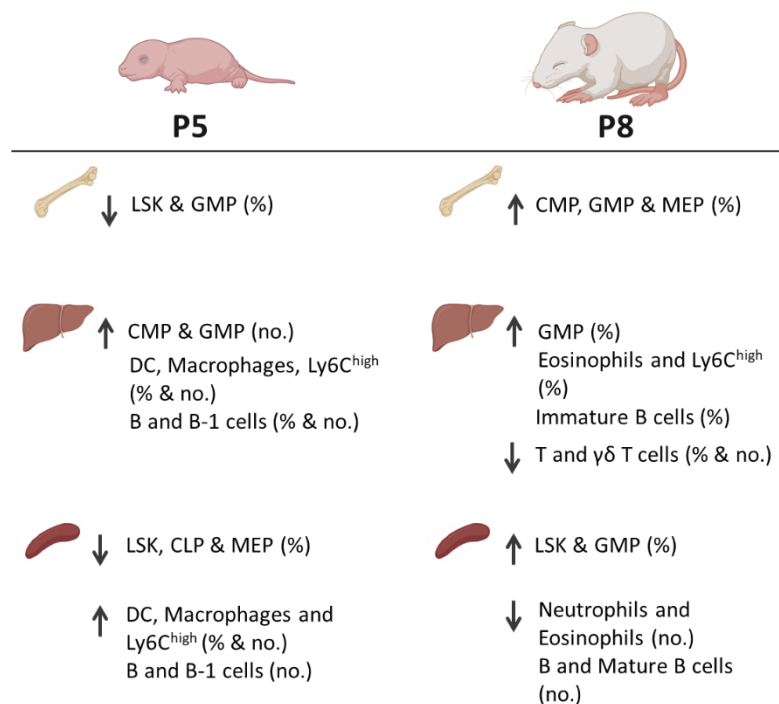
Figure 28 | Summary of haematopoietic alterations in the pups born from rGAPDH-vaccinated mothers. Arrows represent differences when compared to the animals born from Sham-vaccinated mothers.

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