Faculty of Engineering of the University of Porto

Study the accessibility of IL-10 locus in a murine model of Neonatal Sepsis

Tiago Pereira Rodrigues

Dissertation for the conclusion of the integrated Master in Bioengineering
Major Molecular Biotechnology

Advisor: Pedro Madureira

2017

“Intelligence is the ability to adapt to change”

Stephen Hawking

Abstract

Sepsis remains one of the leading causes of neonatal mortality and morbidity in the world. Along the years, researchers have believed that the development of sepsis was due to an exacerbated inflammatory response. However, recent studies have identified counter mechanisms that inhibit the progression of inflammation and can, in turn, dysregulate the immune system to a point of suppression. These evasion-like mechanisms are engaged by bacterial endo and exotoxins that promote, upon recognition by Pattern Recognition Receptors on host immune cells, the release of anti-inflammatory cytokines with a downregulation purpose.

Previous results have unveiled a common virulence mechanism shared by different bacteria that caused an early immunosuppression in the neonatal host. Namely, we found that neonatal susceptibility to Group B Streptococcus (GBS) is due to a natural tendency of neonates to produce high amounts of interleukin(IL)-10, a strong immunosuppressor molecule. We also discovered that GBS exploits this natural tendency of new-born by producing an extracellular from of bacterial glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Moreover, that $CD5^+$ B1 cells are the main producers of IL-10 upon recognition of bacterial GAPDH and that Toll-like receptor (TLR)2 is the cellular receptor responsible for the recognition of bacterial GAPDH. Consequently, we found that TLR2-deficient mice ($TLR2^{-/-}$) are significantly more resistant to GBS-induced sepsis than wild type mice.

Although GBS is one of the leading agents of neonatal sepsis, other gram-positive coccus such as *Streptococcus pneumoniae* and *Staphylococcus aureus*, or Gram-negative bacteria as *Escherichia coli* and *Klebsiella pneumoniae* are also very frequently isolated from cases of severe neonatal infections. In fact, altogether these bacteria are responsible for approximately 95% of all the infections occurring in the neonatal period, with similar clinical pathologies.

Thus, we hypothesized that different bacterial pathogens (GBS, *S. pneumoniae*, *S. aureus, E. coli* and *Klebsiella pneumoniae*) are using the same strategy to infect the neonatal host, inducing early IL-10 production through TLR2 signalling. Thus, the innate commitment of new-borns to produce high IL-10 levels would be the main reason for their susceptibility to bacterial infections.

In order to evaluate the role of IL-10 production in the susceptibility of neonatal mice to infections caused by the bacteria referred above, the survival of $TLR2^{-/-}$ mice pups was compared with the one of wild-type controls after bacterial challenge in a 12-day period. We observed that $TLR2^{-/-}$ pups presented significantly higher survival rates upon infection with all the tested bacteria. Moreover, the administration of anti-IL-10 monoclonal antibodies (mAb) before bacterial challenge also significantly increased survival in comparison with pups that received control mAb. These results, indicate that indeed different bacterial pathogens use TLR2-mediated IL-10 production as a form to escape from host immune system.

Altogether, the results present herein also point to the fact that an anti-inflammatory cytokine - IL-10 - is associated with the susceptibility to neonatal bacterial sepsis, a condition associated with an exacerbated inflammatory response. As extensive data in the literature also highlights the role of IL-10 in inhibiting the inflammatory response, we hypothesize that IL-10 production could be auto-regulated by negative feedback mechanism induced by IL-10 itself. In order to confirm this, we compare the ability of mice bone-marrow derived macrophages (BMM) to produce IL-10 in response to different TLR-agonists in *in vitro* cultures in the presence or absence of IL-10. For that BMM were incubated with recombinant mice IL-10 (rIL-10), or medium alone 12 hours before stimulus with TLR4 or TLR2 agonists. IL-10 was quantified in culture supernatants 12h after stimulus with TLR agonists LPS and PMA3CSK4. In both cases, pre-

treatment with rIL-10 significantly decreased the ability of this cells to produce IL-10 upon stimulus. Thus, this result indicates that IL-10 exerts a self-regulatory mechanism that will limit the ability to produce IL-10 in response to TLR ligands for a period of at least 24 hours. This could also explain why a peak of IL-10 production mediated by TLR2 signalling soon upon infection will limit the ability of the host to counter-balance the exacerbated inflammatory response observed in the cases of neonatal bacterial sepsis.

Resumo

A sépsis continua a ser uma das principais causas de mortalidade e morbidade neonatal no mundo. Ao longo dos anos, os investigadores acreditavam que o desenvolvimento da sépsis era devido a uma resposta inflamatória exacerbada. No entanto, estudos recentes identificaram mecanismos contrários que inibem a progressão da inflamação e podem, por sua vez, desregular o sistema imunológico até um ponto de supressão. Estes mecanismos de evasão são desencadeados por endo- e exotoxinas bacterianas que promovem, após reconhecimento por Recetores de Reconhecimento de Padrões em células imunes do hospedeiro, a libertação de citocinas anti-inflamatórias com o objetivo de promover a *downregulation*.

Resultados anteriores revelaram um mecanismo comum de virulência que é partilhado por diferentes bactérias que causam imunossupressão precoce no hospedeiro neonatal. Ou seja, descobrimos que a suscetibilidade neonatal a *Streptococcus* do Grupo B (GBS) é devido a uma tendência natural do recém-nascidos para produzir elevadas quantidades da interleucina (IL) -10, uma forte molécula imunossupressora. Nós também descobrimos que a GBS explora essa tendência natural dos recém-nascidos, produzindo uma forma extracelular da proteína bacteriana gliceraldeído-3-fosfato desidrogenase (GAPDH). Além disso, que as células $CD5^+$ B1 são as principais produtoras de IL-10 após o reconhecimento da GAPDH bacteriana e que o recetor Toll-like (TLR) 2 é o recetor celular responsável pelo reconhecimento da GAPDH bacteriana. Consequentemente, descobrimos que os ratinhos deficientes em TLR2 ($TLR2^{-/-}$) são significativamente mais resistentes à sépsis induzida por GBS do que os ratos *wild type*. Embora a GBS seja um dos principais agentes da sépsis neonatal, outras bactérias gram-positivas como a *Streptococcus pneumoniae* e a *Staphylococcus aureus*, ou bactérias Gram-negativas como *Escherichia coli* e *Klebsiella pneumoniae* também são frequentemente isoladas de casos de infeções neonatais graves. Na verdade, estas bactérias são responsáveis por aproximadamente 95% de todas as infeções que ocorrem no período neonatal, com patologias clínicas similares. Assim, a hipótese que se colocou foi que diferentes patogénios bacterianos (GBS, *S. pneumoniae*, *S. aureus*, *E. coli* e *Klebsiella pneumoniae*) utilizam a mesma estratégia para infetar o hospedeiro neonatal, induzindo a produção inicial de IL-10 através da sinalização por TLR2. Assim, o compromisso inato dos recém-nascidos de produzir altos níveis de IL-10 seria a principal razão para a sua maior suscetibilidade a infeções bacterianas.

Para avaliar o papel da produção de IL-10 na suscetibilidade de murganhos recém-nascidos a infeções causadas pelas bactérias acima referidas, a sobrevivência de ratinhos recém-nascidos $TLR2^{-/-}$ foi comparada com a de recém-nascidos *wild type*, durante 12 dias após o estímulo bacteriano. Observamos que os recém-nascidos $TLR2^{-/-}$ apresentaram taxas de sobrevivência significativamente maiores após a infeção com todas as bactérias testadas. Além disso, a administração de anticorpos monoclonais anti-IL-10 (mAb) antes da estimulação bacteriana também aumentou significativamente a sobrevivência em comparação com filhotes que receberam o mAb de controlo. Estes resultados indicam que, de fato, diferentes patogénios bacterianos usam a produção de IL-10 mediada por TLR2 como forma de escapar do sistema imunológico do hospedeiro.

Claramente, os resultados aqui apresentados também apontam para o fato de que uma citocina anti-inflamatória - IL-10 - está associada à suscetibilidade à sépsis bacteriana neonatal, condição associada a uma resposta inflamatória exacerbada. Está extensivamente descrito na literatura o papel da IL-10 na inibição da resposta inflamatória, por isso a hipótese que se colocou foi que a produção de IL-10 poderia ser auto-regulada por um mecanismo de feedback negativo induzido pela própria IL-10. Para confirmar esta hipótese, comparamos a capacidade de macrófagos derivados de medula óssea (BMM) para produzir IL-10 em resposta a diferentes agonistas de TLR em culturas *in vitro* na presença ou ausência de IL-10. Para isso, BMM foram

incubados com IL-10 recombinante (rIL-10), ou apenas meio de cultura, 12 horas antes do estímulo com agonistas TLR4 ou TLR2. IL-10 foi quantificada mo sobrenadante de cultura 12h após estímulo com agonistas de TLR - LPS e PMA3CSK4. Em ambos os casos, o pré-tratamento com rIL-10 diminuiu significativamente a capacidade destas células de produzir IL-10 após o estímulo. Assim, este resultado indica que a IL-10 exerce um mecanismo de auto-regulação que irá limitar a capacidade de produzir IL-10 em resposta a ligandos de TLR durante um período de pelo menos 24 horas. Isso também pode explicar por que um pico da produção de IL-10 mediada pela sinalização TLR2 logo após a infecção limitará a capacidade do hospedeiro para contrabalançar a resposta inflamatória exacerbada observada nos casos de sépsis bacteriana neonatal.

Acknowledgments

Este trabalho foi o culminar de um percurso académico de sucesso, mas atribulado. As pessoas que descrevo em baixo desempenharam um papel fulcral em todo o meu percurso. A todas elas, o meu maior e sincero obrigado.

Às minhas irmãs e à minha mãe. São o melhor exemplo de mulher que eu conheço. Cresceram comigo, educaram-me e suportaram-me em todos os momentos. Juntos passamos por muitas adversidades durante a nossa vida e, juntos, ultrapassamos todos os obstáculos.

Aos meus tios e avó por me terem proporcionado uma educação de excelência.

À Sofia, pelo amor incondicional e pelo apoio sem limite. A sua dedicação às pessoas é contagiante e eu não fui exceção. Com uma paciência de santa, ajudou-me sempre que precisei, mesmo quando não dizia que precisava.

Aos meus amigos, em especial à Cristiana, a Eliana, o João e o Miguel. O facto de termos passado pelas mesmas situações juntos, tornou-nos mais fortes e resilientes. Não teria escolhido melhores pessoas com quem quisesse existir.

À Tuna de Tecnologia de Saúde do Porto. Prima-se por se considerar uma escola de fazer homens, mas acima de tudo é e sempre será a minha segunda família. Com ela passei dos melhores momentos da minha vida. Experiências que me fizeram crescer, esforçar e ser criativo. Foram os que me proporcionaram as melhores histórias e aos quais eu devo e deverei sempre o homem que sou hoje.

Às Malhas pela diversão e momentos extravagantes. Sempre na companhia de grandes amigos, farão parte dos melhores momentos que passei no centro histórico do Porto.

Ao meu orientador, por ter acreditado nas minhas capacidades e nunca ter desistido de mim e aos meus colegas de trabalho, pela paciência e pelos ensinamentos.

À Faculdade de Engenharia da Universidade do Porto pelas condições que providenciou para a conclusão deste curso.

E aos meus professores por motivarem-nos cada vez mais a sermos melhores.

Este trabalho é dedicado ao meu pai.

Index

Abstract iii

Resumo vi

Acknowledgments ix

Figure Index xii

Abreviations and Symbols xiii

Chapter 2 14

Introduction 14
- Neonatal bacterial sepsis 14
- Epidemiology of early onset bacterial sepsis 15
- Treatment/prevention 16
  - Intrapartum Antibiotics Prophylaxis (IAP) 16
  - Vaccination 16
- Methods for differential diagnosis of sepsis in new-borns 17
- Sepsis biomarkers in neonates 17
  - C-reactive protein (CRP) 17
  - Procalcitonin 18
  - IL-6 18
  - IL-10 18
- Regulation of IL-10 production and expression 19
- Neonatal immune system 20
  - Innate immune system 20
  - Adaptive immune system 21
- Previous results 22
- IL-10 production induced by TLR2 recognition inhibits neutrophil recruitment 23
- Auto-regulation by IL-10 23
- Aim of work 24

Materials and Methods 24

Results 26

Discussion 31

Chapter 3 36

Bibliography 36

Supplementary data 46

Figure Index

FIGURE 1. Neonatal B1 cells are the major producers of IL-10 upon stimulus by bacterial GAPDH. 22

FIGURE 2 . Abrogation of TLR2 signalling in neonatal mice increases resistance against bacterial infections. 26

FIGURE 3. IL-10 blocking results in increase protection of neonatal mice against bacterial infections 27

FIGURE 4. Exogenous IL-10 inhibits downstream production of IL-10 in vivo. 28

FIGURE 5. BMMs treated with rIL-10 show less production of IL-10 at early timepoints. 29

FIGURE 6. Treatment with rIL-10 inhibits downstream production of IL-10 in bacterial challenged BMMs. 30

FIGURE 7. BMMs treated with rIL-10 do not alteration in the production of TNF-α. 30

FIGURE 8. BMMs treated with rIL-10 show no alteration in the production of IL-6. 31

FIGURE 9 IL-10 neutralization reduces bacterial burden in new-born. 46

Abreviations and Symbols

ACOG	American College of Obstetrics and Gyneology
APC	Antigen presenting cell
BMM	Bone-marrow derived macrophages
CBC	Complete blood count
CD	Cluster of differentiation (36, 40, 80, 86)
CDC	Centre for Disease Control (U.S.)
CRP	C-reactive protein
DC	Dendritic cell
E. coli	*Escherichia coli*
ECDC	European Centre for Disease Prevention and Control
EMA	European Medicine Agency
EOS	Early Onset Sepsis
ERK	Extracellular-signal regulated kinase
FoxP3	Forkhead box P3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBS	Group B *Streptococcus*
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IAP	Intra-partum antibiotic prophylaxis
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin (1, 2, 6, 10, 12, 27)
LNA	Locked nucleic acid probe
LOS	Late Onset Sepsis
LPS	Lipopolysaccharide
Mac-1	Macrophage-1 antigen
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex (class I/class II)
MRSA	Methicillin-resistant *Staphylococcus aureus*
NET	Neutrophil extracellular trap
NF-kB	Nuclear Factor – Kappa B
NKC	Non-killer cell
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PCT	Procalcitonin
PNA	Peptide nucleic acid probe
PRR	Pattern recognition receptor
rGAPDH	Recombinant GAPDH
SNP	Single nucleotide polymorphisms
TGF-B	Transforming Growth factor beta
Th	Helper T cell
TLR	Toll-like receptor (2, 4)
TNF-α	Tumor necrosis factor α
TRAF3	TNF receptor associated factor
Treg	Regulator T cell
VLBW	Very-low birth weight (related to infants)
WHO	World Health Organization

Chapter 2

Introduction

Neonatal bacterial sepsis

Neonatal sepsis emerges as one of the most life-threatening healthcare problems, remaining one of the leading causes of morbidity and mortality among newborns (Camacho-Gonzalez, Spearman, & Stoll, 2013). It is a clinical syndrome characterized by an exacerbated inflammatory response due to infection (Dellinger et al., 2013). According to World Health Organization (WHO), neonatal sepsis is responsible for more than one million deaths worldwide per year, ranking the top ten causes of infant death in the USA. Current medicine has evolved to reduce complications in preterm infants through advances in neonatal intensive care and prophylaxis, however, sepsis still affects millions of individuals with an increasing mortality rate (Heron et al., 2009; Lagu et al., 2012).
In 2013, of the estimated 6.3 million deaths of children around the world, 2.8 million corresponded to neonatal deaths, of which 15% (420,000) were due to sepsis (global, regional, and national causes of child mortality in 2000-13) (ECDC, 2009). Also, in 2013, the Centres for Disease Control (CDC) confirmed 2 million reports of infection with resistant bacteria in the USA with an associated cost to the healthcare system of $21-34 billion per year (CDC, 2013). One year later, the WHO reported nearly 4 million neonatal deaths of which 25% were due to bacterial and 42% of these deaths occurred in the first weeks of life. Furthermore, 30-50% of the surviving newborns suffered from cognitive impairment and seizures (WHO, 2014).

Neonates have an increased susceptibility to bacterial infections and for most of which there is no available vaccine (K. Edmond & Zaidi, 2010). Usually, most of the infections associated with neonatal bacterial sepsis occur in the first week of life due to vertical transmission of bacteria from colonized mothers.

While *in* utero, bacteria can ascend from the vaginal tract to the amniotic fluid with or without rupture of membranes (K. Edmond & Zaidi, 2010). Consequently, the fetus becomes infected while inside the womb. The labor is the second critical point for pathogen invasion. At this stage, several traumatic events occur on the mother's placenta exposing the fetus to all kind of bacteria that colonize the genital tract of the mother. Thus, during the passage through the birth channel the babies can inhale/swallow commensal bacteria of the mother's genital tract (K. M. Edmond et al., 2012; Stoll et al., 2011).

Epidemiology of early onset bacterial sepsis

Early-onset neonatal sepsis (EOS) is defined based on the age of onset of infection in preterm infants with <72h of life and is usually distinguished by bacteraemia, meningitis and pneumonia (Edwards & Gonik, 2013; Hornik et al., 2012). The development of EOS is commonly associated with maternal bacterial colonization, chorioamnionitis, premature or prolonged rupture of membranes, maternal urinary infection and preterm delivery (Control & Report, 2016). EOS is typically caused by vertical transmission of microorganisms that colonize the mother's genitourinary tract. The most common pathogen found in EOS in preterm babies is Group B *Streptococcus* (GBS), followed by the Gram-negative bacteria *Escherichia coli* and *Klebsiella pneumoniae*. Among the cases of EOS in preterm babies, *E. coli* is the most common pathogen. Late onset sepsis (LOS) occurs after 3 postnatal days and affects predominantly VLBW infants that survived EOS. Transmission of pathogens, in the case of LOS, is commonly originated from breakage of natural barriers, necrotizing enterocolitis, prolonged use of antibiotics, exposure to the hospital environment or through contact with the community (Cohen-Wolkowiez et al., 2009; Gonzalez, Mercado, Johnson, Brodsky, & Bhandari, 2003). *Staphylococcus spp.* are predominantly associated with LOS, accounting for more than 70% of incidences (Stoll et al., 2002).

Staphylococcus aureus is also very frequent in cases of LOS in preterm babies (Lukacs & Schrag, 2012; Van Den Hoogen, Gerards, Verboon-Maciolek, Fleer, & Krediet, 2009). Despite medical advances, VLBW are still at elevated risk of sepsis accounting for 20% of VLBW infant deaths. In fact, VLBW infants are especially vulnerable to infections due to the immaturity of the neonatal immune system, prolonged stay in hospitals and frequent invasive procedures (Memar, Alizadeh, Varshochi, & Kafil, 2017). VLBW infants that survive EOS or LOS are susceptible to developing neuronal or lung-associated pathologies (Neurodevelopment et al., 2008; Polin, 2008; Stoll et al., 2002).

In the case of EOS, GBS has been identified as the most common cause of infection in new-born. With 10 different serotypes identified, GBS is a gram-positive encapsulated bacterium that commonly colonize the maternal genitourinary tract. This gram-positive diplococcus is an encapsulated commensal bacterium that colonizes the genitourinary tract of approximately 30% of the women. Despite this commensal nature under certain circumstances, it may become a life-threatening agent with the ability to induce sepsis. During pregnancy, GBS preferably settles in mucous membrane sites with no symptomatic signs. Consequently, absence of treatment and the mucosal environment provide a suitable milieu for rapid colonization and growth (K. M. Edmond et al., 2012; Hornik et al., 2012). As the newborn immune system is not fully developed, both the innate and adaptive immune system are not able to withstand invasive infections. GBS has been revealed as the most common etiologic agent of EOS and principal cause of meningitis, septicemia and pneumonia.

E. coli is a rod-shaped gram-negative bacterium that is commonly detected in the maternal urogenital tract (Tsai, Chen, Wang, Chen, & Chen, 2012). As the second most common cause of neonatal sepsis in new-born, *E. coli* presents a consistent genetic diversity which enhances the development of neonatal sepsis and meningitis (Kaczmarek, Budzyńska, & Gospodarek, 2012; Xie, Kim, & Kim, 2004). Several studies alert for the rapid increase of sepsis induced gram-negative associated to the frequency of maternal antibiotic prophylaxis although a direct link has yet to be established (Schrag et al., 2006).

As far as LOS is concerned, the predominant cause is *S. aureus*. Commonly present in nosocomial cases of infection, *S. aureus* is a gram-positive bacterium characterized by its ability to up-regulate virulence factors upon host immune response (Naber, 2009). Furthermore, it has been described an increased appearance of penicillin and methicillin resistant strains (Styers, Sheehan, Hogan, & Sahm, 2006).

Treatment/prevention

Among the pharmaceutical drugs used to treat sepsis in neonates, the most common are beta-lactams such as oxacillin, cefotaxime and ampicillin; extended-spectrum beta-lactams and carbapenem meropenem. All these antimicrobial agents work to inhibit peptidoglycan synthesis on the bacterial wall. Other antimicrobial classes inhibit cell wall synthesis (vancomycin) or protein synthesis (aminoglycosides) (Jacqz-aigrain, Zhao, Sharland, & Anker, 2013).

- **Intrapartum Antibiotics Prophylaxis (IAP)**

One of the recommendations of the CDC and American College of Obstetrics and Gynaecology (ACOG) is the use of IAP in mothers colonised with GBS, whose babies are at increased risk of developing GBS-induced diseases (Lambert et al., 2009). Based on the screening on prenatal GBS, this method has improved risk-based IAP and lowered GBS rates to only 0.66/1000 live births (Stafford, Stewart, Sheffield, & Wendel, 2012). Chemoprophylaxis with penicillin is currently the recommended therapy for mothers with prenatal GBS-positive cultures or for mothers with unknown GBS status (Control & Report, 2016). Treatment recommendations for women with significant penicillin allergy currently include clindamycin or vancomycin depending on the patient’s drug susceptibility. The development of antibiotic resistance which precedes an increasing neonatal infection has been the major focus of concern (Manning et al., 2003). Since IAP has become the standard of care for cases at increased risk of developing neonatal infections, it has been seen an increase incidence of infections with *E. coli* allied to a higher resistance to ampicillin and penicillin (Ecker, Donohue, Kim, Shepard, & Aucott, 2012). In fact, the generalised use of IAP caused a shift in the predominance of the primary cause of disease in preterm infant population. In the early 1990s GBS was the leading cause of neonatal infection and, in low-income countries, remains a major healthcare problem. However, recent reports dated from 2012, affirm that most of the current neonatal infections in high-income countries are due to Gram-negative microorganisms (Shane & Stoll, 2015; Stoll et al., 1996).

- **Vaccination**

The clear majority of neonatal deaths caused by bacterial infections occur in low-income countries. The lack of a strong healthcare system and low or none hygienic conditions pose a major threat to neonatal health (Black et al., 2010). IAP can in fact prevent EOS from GBS, however, the frequent use of antibiotics renders the appearance of antibiotic-resistant bacteria (Moore, Schrag, & Schuchat, 2003). Moreover, IAP has no impact on the cases of LOS. Maternal vaccination has demonstrated an important mechanism to protect new-borns by providing protective antibodies. In fact, currently there are several maternal vaccines implemented in the healthcare system that successfully protect infants from tetanus toxoid and *H. influenza* infections (Demicheli, Barale, & Rivetti, 2005; Zaman et al., 2008).
Currently known, GBS has 5 types of capsular polysaccharides (from a total of 10 different serotypes) that account for 95% of neonatal disease, specifically serotypes Ia, Ib, II, III and V. (Zaleznik et al., 1996).
It is usually argued that neonatal susceptibility to GBS is mainly due to lack of specific antibodies against GBS capsular polysaccharides (Carol J Baker et al., n.d.). This poses a major difficulty in developing a GBS vaccine due to its several serotypes. GBS serotypes tend to vary with the country. US vaccination against neonatal infections caused by GBS may not be as successful in Europe or Asia due to serotype difference. Thus, we can determine that a possible solution is to develop a vaccine based on GBS transversal antigens. Therefore, the vaccine would not be dependent on the serotype of the bacteria. Instead, it would take advantage of a conserved antigen to be recognized as a pathogen (WHO/IVD, 2003). As the major risk of neonatal infection is due to maternal colonization of the genitourinary tract, maternal immunization stands as the best approach to prevent neonatal sepsis (Demicheli et al., 2005). Furthermore, vaccination presents higher benefits comparing to IPA: lower risks of

development of resistant GBS strains; decreased number of EOS cases caused by *E. coli* and other gram-negative infections and the ability to reach a larger group of pregnant women.
Current vaccination status against bacterial sepsis is focused against GBS but it presents several limitations. Despite the variety of approaches, current developed GBS vaccines present low immunogenicity (C. J. Baker, Marcia, & Rench, 1998), are serotype specific (Paoletti & Kasper, 2002) or induce cross-reactivity with other vaccines (Paoletti & Madoff, 2002). Also, it has not been described a successful vaccine against neonatal infections through *E. coli, K. pneumoniae* and MRSA. Further investigation is needed to develop a vaccine that can prevent bacterial neonatal sepsis through maternal immunization.

Methods for differential diagnosis of sepsis in new-borns

Current diagnosis techniques involve blood and urine cultures, blood cell count and lumbar puncture for cell count and culture, antigen detection and C-reactive protein. (Johnson et al., 2015). Also, other biomarkers of acute-phase reaction are often being used to assist diagnosis of sepsis in infants.
The most common methods for infant diagnosis of sepsis is a complete blood cell count (CBC). Considering that low white blood cells and neutrophil counts are associated with severe risk of infection, CBC poses as an important diagnostic method. In a CBC, the main goal is to assess white blood cells presence, mature neutrophil counts and its ratio to immature neutrophils. However, neutrophil maturation and absolute numbers are age dependent. At the first 12h of age, neutrophils reach a peak but after 3 days neutrophils fall to 1/3 of its first count (Behrman, Rosenfeld, Browne, & Ph, 1979). Combined with total leukocyte count, haematological tests fail to accurately differentiate septic infants from new-borns with other non-infectious disease. Overall, diagnosis through CBC approach is unreliable and needs a more specific substitute for the correct diagnosis of neonatal bacterial sepsis.

Sepsis biomarkers in neonates

Isolation of bacteria from blood culture is the gold standard in cases of infection for sepsis diagnosis. However, the results usually take more than 24h to appear. In the meantime, the risk of mortality grows exponentially (Critical & Medicine, 2017). Even when blood cultures are negative, sepsis prognosis cannot be excluded once isolation of bacteria from blood may reflect asymptomatic bacteraemia or contamination (Kellogg & Ferrentino, 2017). Besides cell cultures, other techniques have evolved to fill the gaps between diagnostic methods. Non-culture dependent methods include proteomics, *in situ* hybridization, polymerase chain reaction (PCR) (Brozanski, Jones, Krohn, & Jordan, 2006), mass spectroscopy and gene arrays (Shah & Padbury, 2014).

- **C-reactive protein (CRP)**

This acute-phase reactant is the most extensively studied and most available for laboratory tests concerning neonatal sepsis diagnosis. It was discovered by Tillet and Francis in 1930 when analysing the serum of adults diagnosed with pneumococcal pneumonia (Tillett & Francis, 1930). CRP is composed of 5 identical polypeptide subunits organized in a pentameter shape and is produced in the liver after stimulation with IL-1, IL-6 and TNF-α (Blackwell, Snodgrass, Madimenos, & Sugiyama, 2010; Pepys, 1982). The binding sites of each subunit allow CRP to recognize and bind to phospholipid and phosphocholine components of damaged cell walls and nuclear antigens resulting in the formation of CRP-ligand complexes. In turn, these complexes can activate the complement system, bind to innate immune cells and thus stimulate an inflammatory response (Epstein, 1999). In an acute-phase inflammatory response, CRP levels can rise as much as 1000-fold within 4-6 hours of infection and reach its peak after 24-48h (Ehl,

Gering, Bartmann, Josef, & Pohlandt, 1997). In the case of bacterial infections, CRP levels range from 150 to 350 mg/L while the normal levels of CRP in healthy preterm infants vary between 2 to 5 mg/L (Jaye & Waites, 1997). Preterm infants have sparse number of CRP which increases in case or haemolysis or other injuries like chorioamnionitis corroborating the presence of infection (Murphy & Weiner, 2017; Schmutz, Henry, Jopling, & Christensen, 2008). However, CRP analysis lacks some references and key features which would consider this analysis as a gold standard. In any case, a clinical laboratory test needs to present high sensitivity and specificity. In the case of neonatal sepsis, the tests use for diagnosis must accurately indicate abnormal results in every infant with sepsis and normal results to non-infected infants (Jaye & Waites, 1997). Although CRP test has a high specificity (~94%), it lacks on sensitivity (~67%) which poses a disadvantage as a diagnostic tool. Time is critical to achieve the highest sensitivity when using CRP as a clinical tool. As CRP levels rise between 12-24h, after the onset of infection symptoms, an early or late test will yield a low sensitivity in the detection of CRP (Joan, 2003).

- **Procalcitonin**

Another extensively studied acute-phase reactant is procalcitonin (PCT). This protein contains 116 amino acids and is the precursor of calcitonin. It can arise from endopeptidase-cleaved preprocalcitonin, in neuroendocrine cells of the lung and the intestine or in the liver in response to inflammatory stimuli (Maruna, Nedělníková, & Gürlich, 2000) or in the liver similarly to CRP (Oberhoffer et al., 1999). In healthy individuals, PCT levels are residual (~0.1mg/mL) but in cases of bacterial infections, PCT levels can rise above 1 mg/mL in the first 3-4h, reaching its peak after 6h of infection (Miedema et al., 2011). Furthermore, in the case of sepsis, studies have shown that higher levels of PCT are associated with elevated risk of severe sepsis and high mortality prognosis (Jain et al., 2014). However, similarly to CRP, PCT levels may be elevated due to non-infectious conditions. Infants with perinatal asphyxia, pneumothorax or hemodynamic failure also present elevated levels of PCT that are not distinguishable from septic new-borns up to 48h after manifestation of infectious symptoms (Lapillonne, Basson, Monneret, Bienvenu, & Salle, 1998; Monneret et al., 1997).

- **IL-6**

Upon infection, serum IL-6 level increase significantly (Article, 2000; Cernada et al., 2012). Although it has a high percentage of positive predictive value (87-100%), IL-6 half-life is very low which makes it difficult to be detected.
Thus, many of the clinical diagnostic approaches that are currently in use have low predictable value for the diagnosis of sepsis in due time. Many of the signs of sepsis are nonspecific which can also be observed in other non-infectious conditions and therefore clinical diagnosis is difficult. Furthermore, bacteraemia can be asymptomatic and occur without the presence of observable signs (Buckler et al., 2010; Ottolini, Lundgren, Mirkinson, Cason, & May, 2003). Finally, we can conclude that current diagnostic methods can only assist in the decision of treatment discontinuation but cannot be used as differential diagnostic in neonatal sepsis.

- **IL-10**

IL-10 is an anti-inflammatory cytokine that is mainly produced in macrophages, neutrophils, dendritic cells, $CD4^+$ T cells and B cells with the main function of controlling hyperinflammation (Sabat et al., 2010). In 2010, Edward F. Bell et al, determined the levels of neutrophilic CD64, PCT and IL-10 in 49 new-borns with neonatal sepsis. When compared to the other studied biomarkers for sepsis, IL-10 showed the highest specificity (84%) and sensitivity (92%) (Zeitoun, Gad, Attia, Abu Maziad, & Bell, 2010). Furthermore, Andaluz-Ojeda et al (2012), recruited 29 patients diagnosed in the first 24h after admission in ICU with severe sepsis or septic shock and profiled 17 immune mediators from the patients' serum. Their results showed that IL-10 was the only immunosuppressive cytokine present in elevated levels in the plasma of deceased

patients (Andaluz-Ojeda et al., 2012). Other studies also shown that patients with severe sepsis had IL-10 increased levels in the plasma which posed a correlation to the severity of the disease and death (Hotchkiss et al., 2016). However, IL-10 has not been thoroughly studied as a biomarker for neonatal sepsis diagnosis so further research is needed.

Regulation of IL-10 production and expression

The evolution of the immune system has become an important mechanism for the host survival against multiple pathogenic microorganisms. However, the challenge is to respond with enough intensity and duration to eradicate the pathogen with minimal damage to host tissue. Balanced mechanisms inherent to the immune system play a vital role on the control of the immune response and reactivity to self, limiting host damage. The production of anti-inflammatory cytokines or interleukins (IL) is one of the most important pathways that restrain the inflammatory response. These cytokines act on different steps of the immune machinery preventing inflammatory and/or autoimmune pathologies. Sepsis syndrome acts as an acute response to a variety of stimuli or noxious insults to the organism. In this case, bacterial infection represents a challenge to the development of immunomodulatory therapeutic approaches (Van der Poll & Van Deventer, 1999). Host response to infection must be a balanced-based immune expression to be an effective antimicrobial defence preventing organ failure and death. In fact, in models of severe systemic infection, neutralization of TNF or IL-1 expression resulted in reduced mortality (Fischer et al., 1992). On the other hand, in experiments with pneumonia as an induced localized infection, local activity of proinflammatory cytokines revealed important antibacterial response (Echtenacher, Mannel, & Hultner, 1996).

In this scenario, IL-10 assumes a vital role in restraining the excess of inflammation. IL-10 was first described in 1989 as an immune mediator secreted by mouse Th2 cell clones with the ability to inhibit the synthesis of IL-2 and IFN-γ in Th1 cell clones (Fiorentino, Bond, & Mosmann, 1990). Human IL-10 is a 35kD homodimer composed of two non-covalently bonded monomers in a V-shaped orientation. Monomers are composed of six helices and a disulphide bridge which provides structural strength and it is crucial for its biological activity (Windsor et al., 1993). IL-10 gene is located in the chromosome 1 and encodes a 178 amino acid protein that is secreted after cleavage of the 18 amino acid long signal peptide (Josephson, Logsdon, & Walter, 2001; Kim, Brannan, Copeland, Jenkinst, & Tariq, 2016). IL-10 plays an important role in the down-regulation of cell activation-associated co-receptors on antigen-presenting cells such as CD40, CD80, CD86 and MHC-II as well as in the inhibition of the expression of integrins, such as Mac-1 (Menezes et al., 2009) and inhibition of oxidative explosion driven by neutrophils (Dang et al., 2006). Moreover, this cytokine participates on the suppression of T cell proliferation and IL-2, IL-6 and IFN-γ production, on the maintenance of FoxP3 expression on Treg cells (Murai et al., 2009) and the suppression of NK cell function (Scott, Hoth, Turina, Woods, & Cheadle, 2006).

On the other hand, induction of IL-10 production by microbial pathogens is a common mechanism of host evasion that has been described in bacteria (Sing et al., 2005), fungi (Vecchiarelli et al., 1996) and virus (Kotenko, Saccani, Izotova, Mirochnitchenko, & Pestka, 2000). One of the most well-known examples is the viral infection through Epstein-Barr virus (EBV). EBV is a herpesvirus responsible for asymptomatic infections. Early after infection, EBV induces B cells to produce a viral IL-10 homolog (vIL-10) which is encoded by the BCRF1 gene (Jochum, Moosmann, Lang, Hammerschmidt, & Zeidler, 2012; Samanta, Iwakiri, & Takada, 2008). vIL-10 binds to the IL-10R and enhances B cell viability by supressing the production of pro-inflammatory cytokines during acute infection. This way, T cell and NK cell response is

inhibited which in turn favours EBV dissemination (Lindquester, Greer, Stewart, & Sample, 2014).
Today is known that a broad variety of leukocytes produce IL-10. However, which cell is responsible for IL-10 increased concentration in a particular situation is dependent on (i) the stimuli, (ii) the affected tissue and (iii) the time-point in an immune response (Saraiva & O'Garra, 2010). Innate and adaptive immune cells present different pathways for IL-10 production with emphasis to the role of different receptors and protein kinases. Cells from innate immunity like monocytes, myeloid dendritic cells and macrophages, secrete IL-10 via activation of TLR-4 (Fiorentino et al., 1990), NF-kB p65/p50 (Driessler, Venstrom, Sabat, Asadullah, & Schottelius, 2004), TRAF3 (Häcker et al., 2006) and ERK kinase (Liu, Cao, Huizinga, Hafler, & Toes, 2014) upon bacterial endogenous/exogenous mediators like LPS. Neutrophils revealed, alongside other myeloid cells, a significant IL-10 production when isolated from burnt patients (Kasten, Muenzer, & Caldwell, 2010; Laichalk et al., 1996). This altered phenotype may be induced by signalling mechanisms mediated by GM-CSF and TNF-α and consequently p38 MAPK phosphorylation which is known to control IL-10 production (Chen, Huang, Lee, Hsu, & Lu, 2006; Foey et al., 1998). Innate cells like macrophages also secrete IL-10 via CD36 and MAP kinase signalling in the clearance of apoptotic cells (Chung et al., 2007). In contrast, the adaptive immune cells demonstrate different transcription factors and other mediators that integrate the IL-10 production mechanism. T cells, in particular Th1 and Th2 cells, have shown to produce similar amounts of IL-10 (Jankovic et al., 2007; Saraiva et al., 2009) mostly through activation of ERK1 and ERK2 MAP kinases. It has been described that IL-10 expression and production is influenced by a series of factors like IL-12, interferon-regulatory factor 4 and IL-27 (Garcia et al., 2009; Saraiva et al., 2009). Also, in Treg cells is described the expression of IL-10 which is affected by IL-2 and TGF-β. On the other hand, retinoic acid, involved in Treg development, enhance Foxp3 expression but inhibits IL-10 (Maynard et al., 2009; Tsuji-Takayama et al., 2008). In 2014, Bi-Sheng Liu et al, showed that TLR activation in human B cells leads to potent IL-10 induction. After TLR signalling, the activation of ERK and STAT3 is responsible for the production of this cytokine (Liu et al., 2014).
All these reports agree that the regulation of IL-10 expression on a genetic point of view would increase the feasibility to modulate this cytokine phenotype. The ability to analyse in real time the events that occur before IL-10 production would provide a solid method for early onset sepsis diagnosis in newborns. Consequently, the application of a well-timed therapy could decrease preterm and term infants' mortality and morbidity.

Neonatal immune system

- **Innate immune system**

Innate immune cells recognize pathogens via pattern recognition receptors (PRRs). PRRs are expressed on the cell surface or intracellularly (cytosolic or associated with organeles' membrane) and play an important role in the detection of pathogens. These receptors can be divided into four groups: Toll-like receptors which are mainly associated with the recognition of bacteria, parasites and viruses (Akira, Uematsu, & Takeuchi, 2006); C-type lectin receptors that mainly detect fungi (Geijtenbeek & Gringhuis, 2009); NOD-like receptors that commonly bind to intracellular bacteria (Inohara, Chamaillard, McDonald, & Nuñez, 2005) and RIG-I-like receptors which are involved in the recognition of viruses nuclear molecules (RNA and DNA) (Nayak, Roth, & Mcgavern, 2014). All the known PRRs are distinct according to their function, localization in the cell and specificity.
TLRs are expressed on the cell surface or within intracellular vesicles and are stimulated by the presence of pathogen-associated molecular patterns (PAMPs) such as cell wall/membrane components (LPS, peptidoglycans, flagelin), or intracellular components (single or double

stranded RNA or DNA) (Kawai & Akira, 2011). In general, each TLR has a specific ligand required for stimulation, and more than one TLR can be stimulated simultaneously allowing for concerted responses to be produced (Trinchieri & Sher, 2007). Following TLR stimulation, second messenger-specific intracellular signaling cascades are activated that result in gene expression, cytokine/chemokine production and cellular activation (Kawai & Akira, 2011). Distinctions between neonatal and adult innate and adaptive immune function likely contribute to the susceptibility of newborns to infection. The neonatal innate immune response has been considered "immature", as functional impairments in phagocytosis and other bactericidal activity as compared to adults have been noted in neonatal innate immune effector cells, such as neutrophils and macrophages (Wynn et al., 2008). Neonatal leukocytes also demonstrate decreased responsiveness to agonists of classic PRRs, such as LPS signals through TLR4 (Sadeghi et al., 2007; Yan et al., 2004). Neutrophils are, whether in adult or neonatal infections, the primary responders to pathogen-induced inflammation (Adkins, Bu, & Cepero, 2015; Adkins, Bu, Guevara, & Alerts, 2015). The neutrophil is not only able to phagocytose and clear bacteria but it is also able to release anti-microbial proteins and peptides such as lactoferrin and bacterial/permeability-increasing protein upon activation at infected sites. Nevertheless, there is a reduced pool of neutrophils in neonates. Besides, neonatal neutrophils have reduced ability to form neutrophil extracellular traps (NETs) which is important to restrain the spreading of extracellular bacteria. Also, several studies point out the reduced chemotaxis of neonatal neutrophils (Hazeldine et al., 2014). As an important mechanism of migration to the infected tissues, neonatal neutrophils show a deficient adhesion to the endothelium compared to adult neutrophils. Adhesion molecules such as β_2 integrins Mac-1 and LFA-1 have demonstrated abnormal expression and functional dynamics in new-born neutrophils which hinders neutrophil ability to adhere and transmigrate to the infected tissues (Abughali, Berger, & Tosi, 1994; Carr, 2000).
Monocytes and Dendrtic Cells express lower levels of MHC-II molecules which contributes to a reduced APC function. Compared to adults, neonates have a decreased Th1-polarizing/pro-inflammatory response to PRR agonists. In fact, neonatal cytokine response is often Th2 and Th17 polarized. These phenotype leads to increased production of IL-6 and IL-10 (Sadeghi et al., 2007; Vosters et al., 2010).

- **Adaptive immune system**

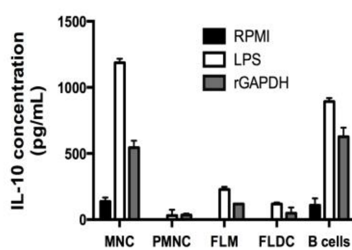
There are several phenotypic and functional differences between neonate and adult adaptive immune system. It is believed that new-born susceptibility to infections is partly due to the absence of immunological memory. Also, the number of immune cells are lower than in adults which affects the possibility of a strong immunological response. Although several studies have postulated that outnumbered neonatal immune cells have a similar performance to adult cells of the adaptive immune system (Adkins, Leclerc, & Marshall-Clarke, 2004).
Studies in neonatal $CD4^+$ T cells have demonstrated that production of IL-4, IL-5 and IL-10 is mostly Th2 originated and that this Th2-polarization decreases the production of IFN-γ and IL-2 (Zaghouani, Hoeman, & Adkins, 2009). Regarding $CD8^+$ T cells, neonatal mouse blood cord studies have showed a deficiency in the function of these cells (Mccarron & Reen, 2010). Other phenotypically different T cells have been extensively studied in neonates. Literature mentions that neonates also lack Th17 cells in number (De Roock et al., 2013) and that Treg cells have an important role in the control of maternal alloreactivity in the developing fetus (Teles, Zenclussen, & Schumacher, 2013). In a study with RAG-1-defecient murine neonates (lacked an adaptive immune system), the survival rate was not altered after severe polymicrobial infection compared to the wild type group (Wynn et al., 2008). The pattern of cytokine production following microbial stimulus is also altered in newborns, which can also compromise the correct activation and differentiation of CD4+ T cells (Zhu, Paul, Zhu, & Paul, 2014)
Another important cell of the adaptive immune system are the B cells. In new-born, these cells lack antigenic exposure conferring a partly developed surface Ig repertoire. Although in low

concentrations in neonates, they seem to be the primary source of the response against *S. pneumoniae* through the production of T-cell indenpendent antibodies (Haas, Poe, Steeber, & Tedder, 2005). Several intrinsic features may be the cause of the decreased production of antibodies namely: B cell immaturity, low B cell repertoire of Igs or decreased strength of BCR signaling (Basha, Surendran, & Pichichero, 2014). Functionally, B cells can be divided in B1 and B2. B1 cells express $CD5^{+}$ at the cell surface and are the primary response against bacterial and fungal infections after birth (Griffin, Holodick, & Rothstein, 2011). Furthermore, it is known that B1 cells have fewer somatic mutations and serve as the front line of defense in neonates (Griffin & Rothstein, 2011). Also, besides comprising 40% of the neonatal peripheral blood, have the ability to secrete IL-10 and TGF-ß and promote a Th2 response (Sanz et al., 2010).

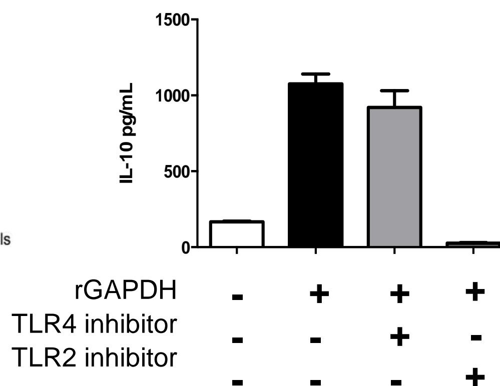
Previous results

It is already known that GAPDH is present not only in the cytosol but also on the surface (Alvarez, Blaylock, & Baseman, 2003) and it can even be secreted (Berner, 2004) by different bacteria. Madureira et al, demonstrated that GAPDH was present in NEM316 culture supernatants without biological mark of isocitrate dehydrogenase (cytosolic biomarker of bacterial lysis). Furthermore, they report that recombinant GAPDH (rGAPDH) and GAPDH extracted from S. agalactiae induces B cell and T cell activation with higher predominance of B lymphocytes *in vitro* studies. It is widely appreciated that different microbial antigens like lipopolysaccharide (LPS) and extracellular enzymes can activate polyclonal B cell activation (Lima, Bandeira, Portnoi, & Ribeiro, 1992; Madureira, 2004) and contribute for the evasion of the host immune system. As alike, GAPDH-induced B cell activation seems to promote *S. agalactiae* escape from host immune response. More importantly, the group reported the presence of prominent levels of IL-10, an immunosuppressive cytokine, in mice treated with rGAPDH. Therefore, IL-10 plays a vital role in the immunosuppression of the host infected with *S. agalactiae* and the presence of GAPDH upon bacterial challenge is the main cause of susceptibility to infection. In fact, our group discovered that GBS secretion of GAPDH induces early production of IL-10 in a murine model for neonatal sepsis. We also demonstrated that early production of IL-10 due to secretion of GAPDH hinders host immune response through inhibition of neutrophil recruitment to the infected organs (Madureira et al., 2011).

A



C



B

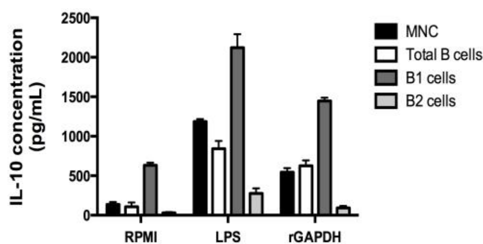


FIGURE 1. Neonatal B1 cells are the major producers of IL-10 upon stimulus by bacterial GAPDH.

(A) Spleen mononuclear cells (MNC), neutrophils from peripheral blood (PMNC), macrophages (FLM) or dendritic cells (FLDC) derived from the liver and B cells purified from the spleen of new-born mice were stimulated *in vitro* with 0,5 µg/mL of LPS, 25 µg/mL of rGAPDH or medium alone (RPMI) for 12h at 37ºC with 5% CO_2. In all conditions $5x10^5$ cells/well were used. (B) $2,5x10^5$ of mononuclear cells, total B cells, B1 cells or B2 cells from the spleen of new-born mice were stimulated *in vitro* with 0,5 µg/mL of LPS, 25 µg/mL of rGAPDH or with medium alone (RPMI) for 12h at 37ºC with 5% CO_2. After incubation, IL-10 concentration was quantified in the supernatants. Data depicted in the figure represent mean + SEM of at least two independent experiments. (C) $2,5x10^5$ of B1 cells purified from the spleen of newborn mice were stimulated *in vitro* with 25 µg/mL of rGAPDH in the presence of TLR2 or TLR4 inhibitors as indicated for 12h at 37ºC with 5% CO_2. After incubation IL-10 concentration was quantified in the supernatants.

Previous unpublished results from the group showed that GAPDH-induced bacterial virulence is transversal to other bacteria and that neonatal immune response produces elevated amounts of IL-10 upon bacterial challenge through GAPDH secretion (Madureira et al., 2011). Furthermore, we found that B cells are the major source of GAPDH-induced IL-10 production. In this study, different leukocyte populations were purified from neonatal mice and challenged in vitro with rGAPDH. In fact, other studies have showed that leukocytes from new born mice and human neonates are responsible for the production of elevated levels of IL-10 upon infection (Wynn et al., 2011; Zhang et al., 2007). Ultimately, B cells might be the main cell population that produce considerable amounts of IL-10 when compared to the other leukocyte populations. However, B cells can be divided in B1 and B2 cells. So, to assess which B cell subset is the main contributor to IL-10 elevated concentration, both were incubated with rGAPDH. It was observed that B1 cells retained the ability to produce IL-10 while B2 cells only produced minimal amounts of the same cytokine (Figure 1B). Further studies were engaged to understand how GAPDH influences IL-10 production in B1 cells. Results showed that upon bacterial challenge, GAPDH interacts with TLR2 (Figure 1C).

IL-10 production induced by TLR2 recognition inhibits neutrophil recruitment

As mentioned earlier, the neonatal immune system is on a very early stage of development. The absence of antigen presentation drives the newborn to rely on his innate immune cells like neutrophils, dendritic cells and macrophages. We have seen that TLR are able to recognize pathogenic ligands and that its basal expression is similar in neonates and adults (Levy, 2005). In 2013, Elva et al. clarified the importance of TLR2 in the neonatal sepsis induced by GBS infection. The main goal was to investigate whether TLR2-induced IL-10 production plays a role in the process of sepsis resulted from the impairment of neutrophils. As several reports already showed, in cases of severe sepsis and septic shock, there is evidence of a defective migration of neutrophils to the area of infection (Benjamim, Ferreira, & Cunha, n.d.; Darini et al., 2002). TLR2 or IL-10 blocking demonstrated to be pivotal on the increased survival of infected mice due to timed neutrophil migration (Andrade et al., 2013; Madureira et al., 2011). In fact, mice treated with anti-IL-10R mAb showed higher survival rates compared to wild type mice after GBS infections. Thus, it was demonstrated that production of IL-10 upon recognition of GBS through TLR2 is of major importance for bacterial virulence.

Auto-regulation by IL-10

Clearly many mechanisms play a key role on the regulation of expression of IL-10. It is important to understand whether IL-10 expression is autoregulated by cells and what consequences underlie this mechanism. In previous work, Knolle P.A. et al, explained that IL-10 release in the microenvironment as well as downregulation of IL-10 cytokine would interfere with the outcome of the immune response to a pathogen. This research was based on the analysis of the kinetics of IL-10 and its regulation in Kupffer cells (KC). Their findings proven that upon bacterial endotoxin challenge, KC exhibit high IL-10 mRNA fold levels than the control population. Furthermore, ELISA assays showed concordant quantification of IL-10 in the cell culture supernatant. In addition, they explained that IL-10 release is transcriptionally regulated

as mRNA inhibitor actinomycin D blocked the induction of IL-10 mRNA in endotoxin stimulated KCs. This result suggests that IL-10 production and release from KC is dependent of a new protein synthesis. Finally, they addressed the regulation of IL-10 expression through endogenous and exogenous IL-10 protein. In both assays, it was shown that IL-10 mRNA levels in KC are autoregulated by endo- or exogenous IL-10 (Knolle et al., 1998).

Aim of work

Immunotherapies against sepsis-inducing bacteria are lacking in sensitivity and specificity. Consequently, prevalence of the disease remains elevated and mortality does not show progress of ceasing.

The main objective of this work is to evaluate if neonatal susceptibility to different bacterial pathogens is associated with TLR2-mediated IL-10 production.

Specific aims of this study include:

- Evaluate if TLR2 deficient neonatal mice ($TLR2^{-/-}$) are more resistant to infection caused by MRSA, *K. pneumoniae* and *E. coli*.
- Evaluate if the blockade of IL-10 increases protection in neonatal mice against infections caused by GBS, MRSA, *K. pneumoniae* and *E. coli*.
- Evaluate a possible mechanism of self-regulatory mechanism induced by IL-10.

Materials and Methods

Mice

Six- to eight-week-old male and female, C57BL/6, and TLR2-deficient C57BL/B6 ($TLR2^{-/-}$) mice were purchased from The Jackson Laboratory. Animals were kept at the animal facilities of the I3S during the time of the experiments. Mice were housed in Techniplast ventilated polycarbonate cages under positive pressure with hard-wood bedding and provided with Mucedola Diet and fresh tap water, ad libitum, throughout the study. All animals were housed in environmentally controlled cages with 40 air changes per hour. The temperature was maintained at 21–23 °C and the relative humidity at 55 ± 10% with a 12-h light/dark cycle. All animals were quarantined for a week prior to study initiation. Each study animal was assigned a unique number and identified by ear notches.

Bacteria

The bacteria used in this study are listed in the table below. All the strains are clinical isolates obtained from the blood or cerebral-spinal fluid of infected babies who were admitted at Centro Hospitalar do Porto. The Microbiology Department of Hospital Geral de Santo António provided all the bacterial strains. GBS and S. pneumoniae were grown in Todd-Hewitt broth or agar (Difco Laboratories) containing 0.001 mg/mL of colistin sulphate and 0.5µg/mL of oxalinic acid (Streptococcus Selective Supplement, Oxoid) and E. coli and Staphylococcus aureus were cultured on Todd-Hewitt broth or agar medium. Bacteria were grown at 37º C.

Bacteria	***Strain***
Group B Streptococcus (GBS)	NEM316
Staphylococcus aureus	M513261H4
Escherichia coli	M506837H2
Klebsiella pneumoniae	M505592UR

Antibody treatments

For IL-10 signaling blocking, 100µg of anti-IL-10R mAb (1B1.3a, BD Pharmingen) was administered intraperitoneally and control animals received the same amount of isotype-matched control antibody.

Neonatal mouse model of bacterial infection

Neonatal (48h-old) BALB/c, C57BL/6 wild-type (WT) or TLR2-deficient C57BL/B6.129-Tlr2$^{tm1Kir/J}$ (TLR2$^{-/-}$) mice were infected subcutaneously (sc) with the indicated inoculum of the bacteria in a maximum volume of 40 µl. Newborns were kept with their mothers during the entire time of the experiment. Survival curves were determined over a 12-day experimental period.

Antibody treatment

Antibody treatment was performed in newborns 10h prior to infection with indicated bacteria. Newborn mice were treated with 200ug sc of InVivoMab anti-mouse IL-10.

Bone-marrow-derived macrophages cultures

Young adult C57Bl/6 mice were euthanized through cervical dislocation. After disinfection of abdominal and lower members area, femur and tibiae were removed with autoclaved surgery material. Remaining surrounding tissue was removed and after cutting off epiphysis, bone marrow cells were flushed to a 50mL polystyrene tube with the help of a syringe filled with 5mL/bone of ice cold RPMI 1640 using a 26G needle. Cells were reserved for 5 min for sedimentation then were filtered through glass-wool and then were centrifuged at 1200rpm for 10min at 4ºC. The pellet was resuspended in 10mL of complete medium (RPMI-1640 medium, 10% heat activated fetal bovine serum, 1% penicillin-streptomycin provided from GIBCO, 25mM HEPES, 0,05mM of b-mercaptoethanol and 1ng/mL of M-CSF (acquired from Immunotools)) and cells were incubated on a Petri dish overnight at 37ºC with 5% of CO2 concentration. Non-adherent cells were recovered to a 50mL polystyrene tube and Petri dish was washed 3x with 5mL of ice cold RPMI-1640. Cells were then centrifuged at 1200rpm for 10 min at 4ºC. Supernatant was discarded and pellet was resuspended with 1mL of RPMI-1640 for cell count with trypan blue on an optical microscope (Leica DMLB). Cell concentration was adjusted to 1x10^5 cells/well and were incubated on a 96-well culture plate at 37ºC with 5% CO2. Medium was changed on the fourth day with pre-warmed complete medium. With exception for centrifugation and cell count, all procedure was performed on a Technipast BS48 Biosafety cabinet laminar flow chamber. OECD principles of good laboratory practice (ENV/MC/CHEM(98)17) and general requirements for the competence of testing and calibration laboratories (ISO 17025) were followed.

rIL-10 assays

Recombinant IL-10 was purchased from Immunotools and was prepared to the concentration advised in the manufacturer's instructions. 7-day-old bone marrow differentiated macrophages were stimulated with 10ng/mL of rIL-10 to evaluate rIL-10 kinetic. After 12h, cells were incubated for 4h or 18h with bacterial stimuli (LPS, PAM3CSK4, GAPDH GBS) at 37ºC with 5% CO2. Supernatant was collected for quantification analysis.

Cytokine quantification

TNF-α, IL-6 and IL-10 recovered from newborn and cell cultures were quantified by ELISA (R&D Systems), according to the manufacturer's instructions.

Statistical analysis

Two-way ANOVA was used to analyse difference between groups. Survival studies were analysed using the log-rank test. A P value <0,05 was considered statistically significant.

Results

TLR2 deficient (TLR2$^{-/-}$) new-born mice are more resistant to bacterial infection

Previous results from the group demonstrated the importance of IL-10 production mediated by TLR2 signalling for the susceptibility of neonatal mice to GBS infections. Taking in account that we found that extracellular GAPDH from GBS is the microbial factor responsible for inducing TLR2-mediated early IL-10 production, and considering that GAPDH is very conserved amongst different bacterial pathogens associated with neonatal sepsis, we hypothesised that different bacteria can use the same mechanism to evade the neonatal host.
To confirm that, TLR2$^{-/-}$ C57Bl/6 pups were infected with MRSA, *K. pneumoniae* and *E. coli*. WT C57Bl/6 were used as controls and survival rate was analysed 12 days post-infection (Figure 2). Compared to the control group, survival of TLR2$^{-/-}$ mice was markedly increased when infected with the different bacteria.

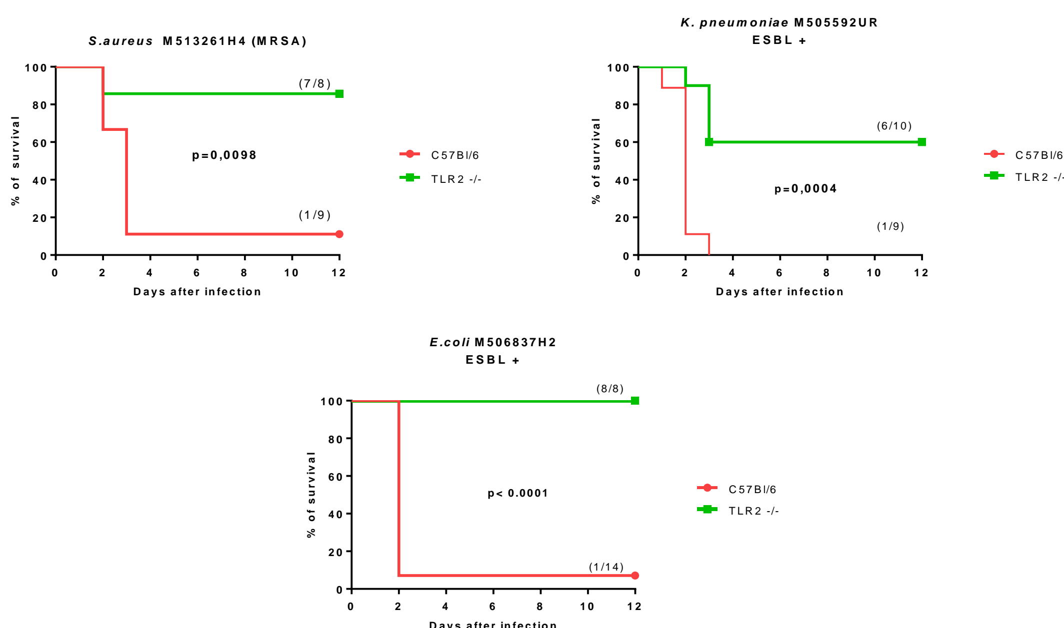


FIGURE 2 . Abrogation of TLR2 signalling in neonatal mice increases resistance against bacterial infections. Survival curves of WT or TLR2$^{-/-}$ neonatal mice infected s.c. 48h after birth with 10^6 CFU of penicillin-resistant strain of *S. pneumoniae*, 10^5 CFU of ESBL producing strains of *E. coli* and *K. pneumoniae* and 10^6 CFU of MRSA. Results represent data pooled from five independent experiments. The numbers between parenthesis represent the number of animals that survived the different infectious challenges versus the total number of infected animals. Statistical differences (P values) are indicated.

Blocking IL-10 inhibits bacteria colonization and increases survival rate

We have seen that neonatal susceptibility to infections with GBS is mainly due to the production of high levels of IL-10 in an early stage of infection. Taking into account that different bacterial pathogens are frequently associated with the same clinical pathophysiology, we hypothesize

that IL-10 early production mechanism is transversal to other sepsis-inducing bacteria. New-born mice were treated with anti-il-10 mAb before subcutaneous inoculation of GBS NEM316 or methicillin-resistant *Staphylococcus aureus* (MRSA). Organ CFUs were analysed 2h, 7h and 22h after infection. Administration of anti-IL-10 mAb reduced the colonization of GBS in the affected tissues. Pups treated with anti-IL-10 mAb showed less CFU count in the liver and the lung after 7h of GBS NEM316 infection compared to wild type. At 22h, the difference is not significant. *Staphylococcus aureus* results were not conclusive (Supplementary data. Figure 9). To understand if this mechanism was transversal to both gram-negative and gram-positive bacteria, pups received anti-IL-10 treatment and then were infected with GBS NEM316, *S. aureus*, *E. coli* and *Klebsiella pneumoniae*. Mice treated with anti-IL-10 mAb and infected with *E. coli* and *K. pneumoniae* accounted for a survival rate of 58% and 50% respectively (figure 3. A, 3.C). As for the gram-positive bacteria experiments, 86% survived GBS NEM316 challenge and 75% resisted infection induced by *S. aureus* (figure 3.B, 3.D).

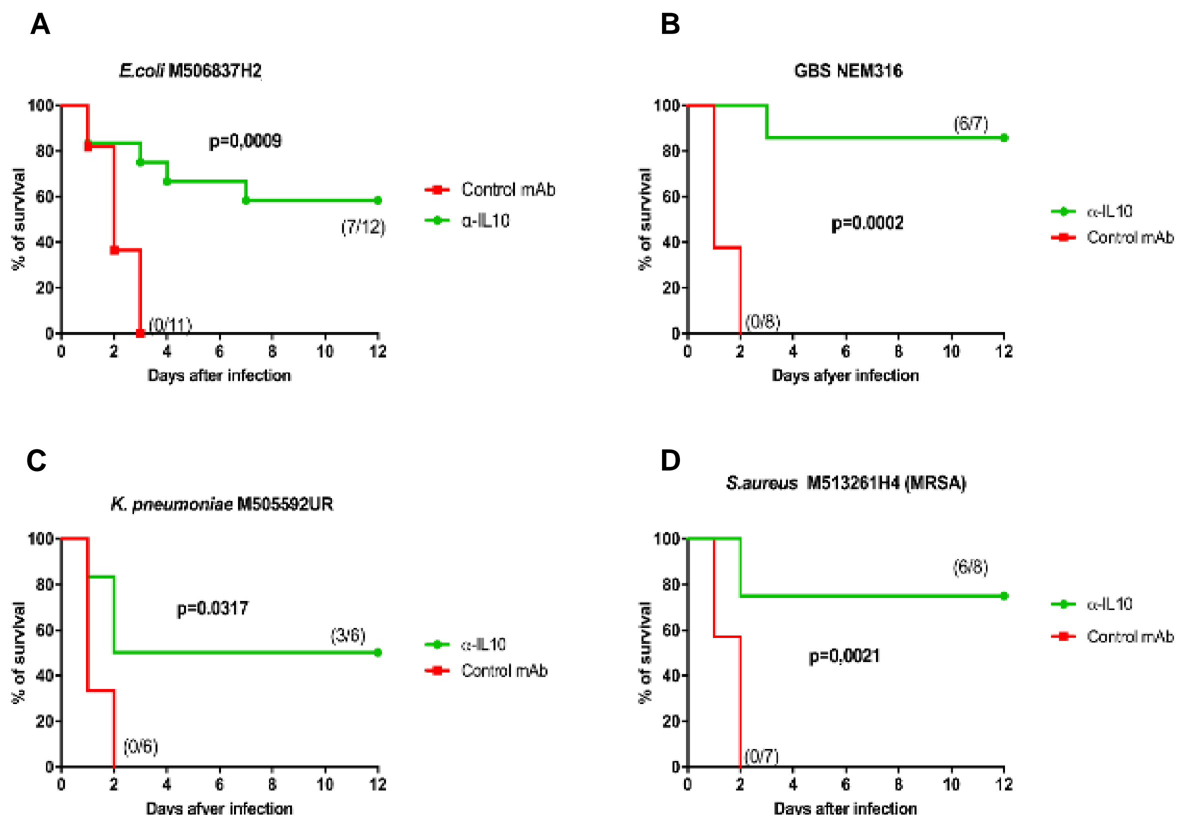


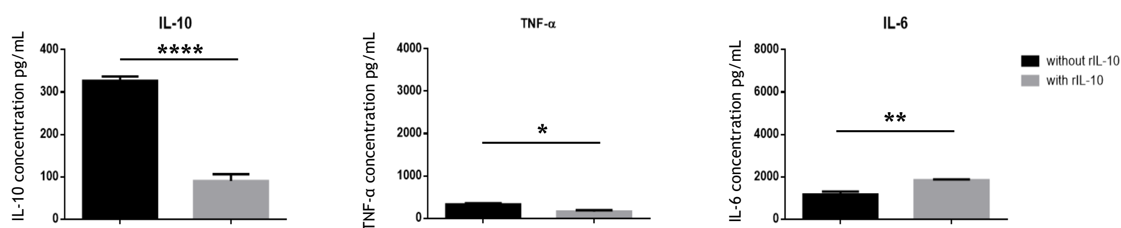
FIGURE 3. IL-10 blocking results in increase protection of neonatal mice against bacterial infections
(A-D) New-born mice (<36h) were injected i.p. with anti-IL-10 mAb or isotype control IgG (200ug) and 12h later were challenged s.c. with 10^6 NEM316 GBS CFU, 10^5 of extended-spectrum becta-lactamase (ESBL) producing strains of *E. coli* and *K. pneumoniae* or 10^6 of methicillin-resistant *S. aureus* (MRSA). Results represent data from six independent experiments. The numbers between parenthesis indicate the number of animals that survived the different infectious challenges versus the total number of infected animals. Statistical differences (P values) are indicated.

Exogenous IL-10 regulates downstream production of IL-10 in new-born mice

All the results presented above indicate an important role for IL-10 production in the susceptibility to neonatal sepsis caused by distinct bacteria. Thus, it is interesting to notice that the exacerbated inflammatory response observed in sepsis is primarily caused by a lack of responsiveness from the neonate immune system caused by the production of an anti-inflammatory cytokine, IL-10.

However, and due to the marked anti-inflammatory activity of IL-10, it was supposed that the IL-10 production induced by bacterial GAPDH would also inhibit or limit the production of inflammatory cytokines. So, we hypothesised that the early peak of IL-10 production observed upon bacterial GAPDH signalling in bacterial infection can limit the ability of the immune cells to produce IL-10. If true, this would mean that early IL-10 production is not only limiting the function of innate response by the neonates but is also hindering the control of the inflammatory response at early time points of infections.
To evaluate the behaviour of treatment with rIL-10 *in vivo*, newborn mice were treated with rIL-10 for 20h and then were inoculated with PAM3CSK4 (TLR2 agonist) and LPS (TLR4 agonist) for 4h. Levels of IL-10, TNF-α and IL-6 were quantified after serum collection. Production of IL-10 was reduced in mice treated with rIL-10 prior to infection comparing to WT. Both TNF-α and IL-6 levels remained with no statistical difference.

LPS



PAM3CSK4

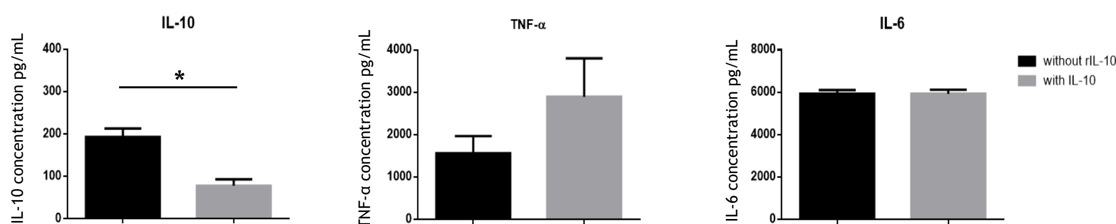


FIGURE 4. Exogenous IL-10 inhibits downstream production of IL-10 *in vivo*.

(A, B) New-born C57Bl/6 mice treated with 25 ng/pup for 20h and WT were injected with 3 ug/pup of TLR2-agonist PAM3CSK4 or 3 ug/pup of TLR4-agonist LPS for 4h. Serum was collected and levels of IL-10, TNF-α and IL-6 were measured by enzyme-linked immunosorbant assay. Statistical differences (P values) between treated and WT animals are indicated.

Exogenous rIl-10 regulates macrophage immune response after bacterial toxin challenge in *in vitro*.

Results herein showed that IL-10 displays a crucial role in the regulation of the immune response. Blockade of IL-10 enhances survival rate of bacterial infected new-born mice and exogenous IL-10 regulates IL-10 production *in vivo*. Previous work demonstrated that immune cells are the main producers of IL-10. In fact, data from the group corroborates the notion that new-born have high number of B1 cells and that these are the main producers of IL-10 upon bacterial challenge (figure 1B).
To understand how IL-10 influences immune cells, bone-marrow-derived macrophages were treated for 12h with rIL-10 and then were stimulated with LPS (TLR4 agonist), PAM3CSK4 (TLR2 agonist) and GAPDH+GBSf. As observed in figure 5, cells that were treated with rIL-10 showed less production of IL-10 after 12h of stimulation with LPS, PAM3CSK4 and GAPDH+GBSf. Non-

treated and treated cells that were challenged with GAPDH+GBSf, show an observable difference in the production of IL-10 which is in concordance with previous data.

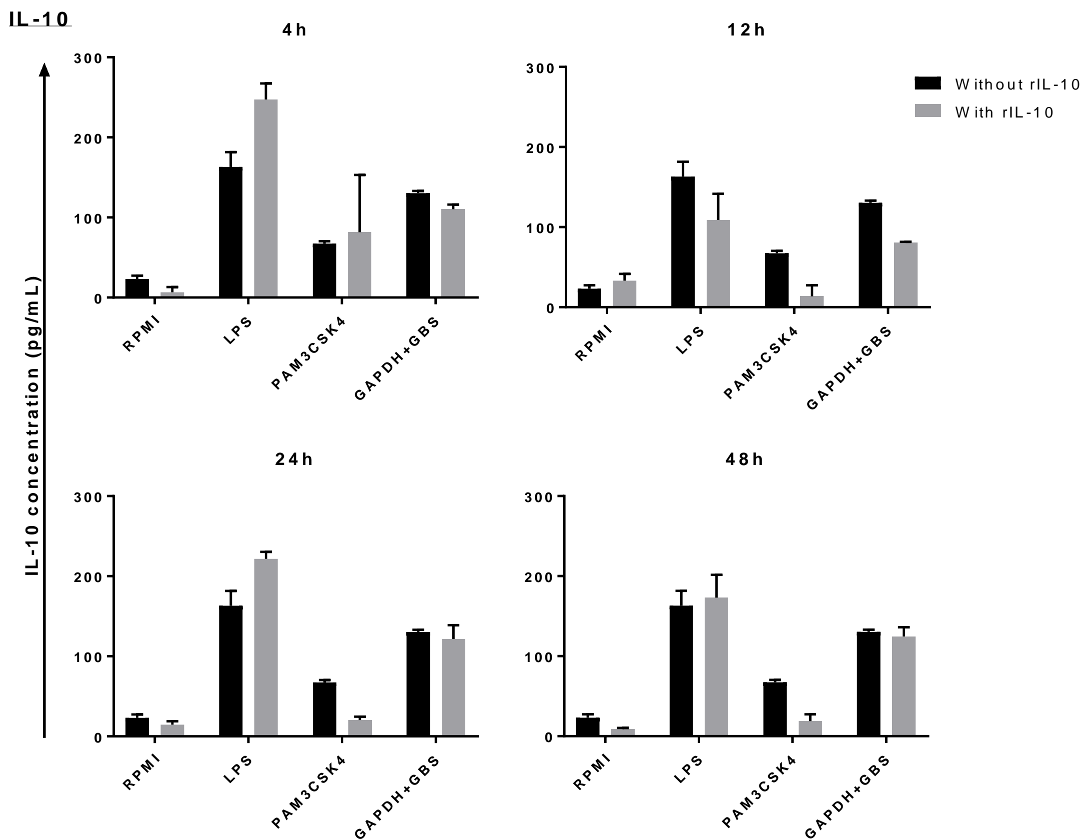


FIGURE 5. BMMs treated with rIL-10 show less production of IL-10 at early timepoints.

Quantification of IL-10 in BMM when treated with TLR agonists with or without previous rIL-10 treatment. BMM were harvested as mentioned in materials and methods. Cells were plated at a concentration of *$1x10^5$* cells/well. Treated cells were incubated with 10 ng/mL of rIL-10 for 12h at 37ºC and 5% CO_2. Stimulation was performed 12h after treatment at a concentration of: 0,5 µg/mL of LPS, 1 µg/mL of PAM3CSK4, 25 µg/mL (GAPDH) + 10/1 (GBSf). Quantification of IL-10 and statistical analysis was determined as described in materials and methods.

BMM show lower production of IL-10 after bacterial challenge with GBS NEM316, *E. coli* and MRSA when previously incubated with rIL-10.

Cusumano et al. demonstrated that administration of rIL-10 before GBS infection in neonatal resulted in increased survival. The authors concluded that IL-10 protect the neonatal mice from the excess of inflammatory response characteristic of neonatal bacterial sepsis. However, this is in clear contrast with our results, which demonstrate that blocking IL-10 production increases survival upon bacterial challenge. However, in the study from Cusumano et al. the protective effect of IL-10 administration is only observed if administrated at least 12h before GBS challenge.
According to what is demonstrated above, what the authors were doing was to inhibit IL-10 production at the time of infection.
To analyse the effect of different sepsis-inducing bacteria on the production of IL-10, BMMs were treated or not with rIL-10 for a 12h period and then infected with GBS NEM316, *E. coli* and MRSA. After 2h and 48h of infection there was no significant difference between BMMs treated with or without rIL-10. However, between 12h and 24h post-infection, IL-10 quantification was significantly lower in culture treated with exogenous IL-10. We have already seen that GBSf-infected cells that were treated with rIL-10, produced less IL-10 between 12h

and 24h post-infection. Interestingly, the same happens when cells are challenged with other sepsis-inducing bacteria (figure 5).

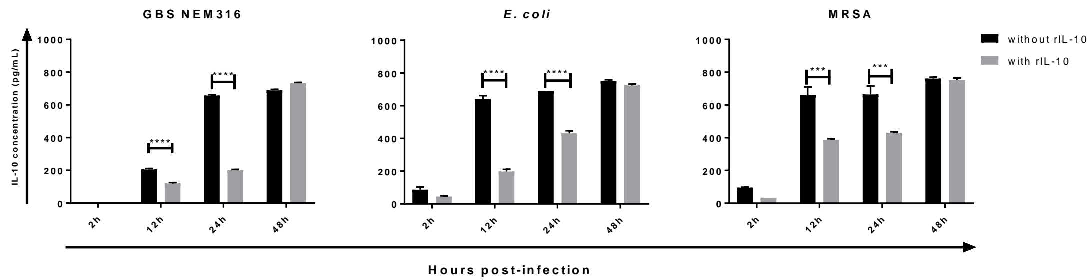


FIGURE 6. Treatment with rIL-10 inhibits downstream production of IL-10 in bacterial challenged BMMs.

Quantification of IL-10 in adult C57Bl/6 BMM cell cultures with/without rIL-10 treatment before infection with GBS NEM316, *E. coli* and MRSA. Adult murine BMM were harvested as described (see materials and methods). Cells were incubated in a 96-well plate at a concentration of $1x10^5$ cells/well. Exogenous rIL-10 was administered at a concentration of 10 ng/mL for 12h prior to infection with $8,425x10^5$ CFU/mL of GBS NEM316, $1,14x10^6$ CFU/mL of *E. coli* and $2,5x10^6$ CFU/mL of *S. aureus*. IL-10 protein concentration from culture supernatant was quantified by ELISA assay.

To address the relevance of IL-10 prior to infection in the production/expression of pro-inflammatory cytokines, BMMs were stimulated with LPS, PAM3 CSK4, and GAPDH+GBS 12h after treatment with or without rIL-10. As shown on figure 7, pre-treatment with exogenous IL-10 did not produce any difference in TNF-α or IL-6 levels in any condition. Production of these cytokines was constant with no significant difference (figure 7, 8).

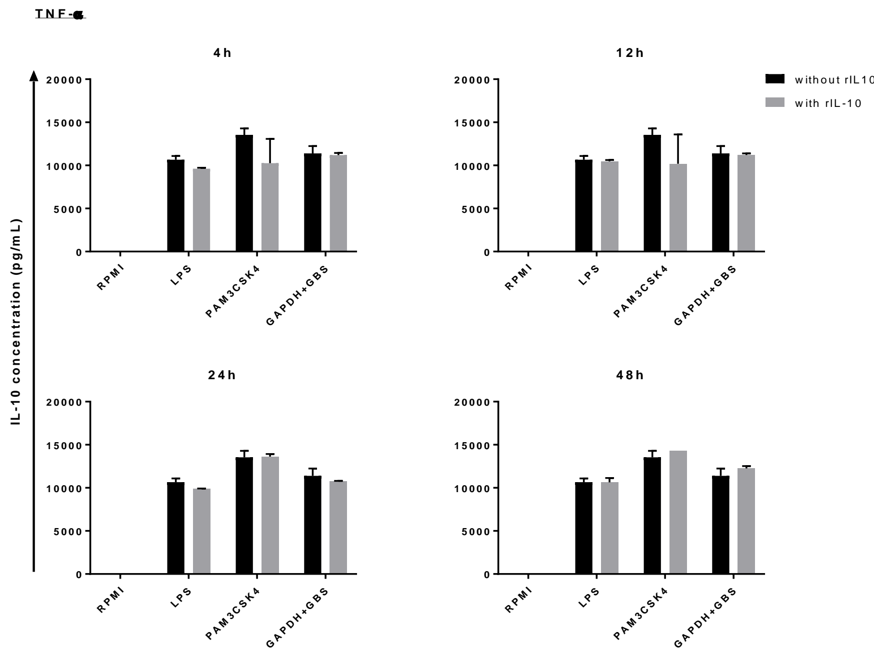


FIGURE 7. BMMs treated with rIL-10 do not alteration in the production of TNF-α.

Quantification of TNF-α in bmm when treated with different TKR agonists with bacterial toxins with or without rIL-10 treatment. BMM were harvested as mentioned in materials and methods. Cells were plated at a concentration of $1x10^5$ cells/well. Treated cells were incubated with 10 ng/mL of rIL-10 for 12h at 37ºC and 5% CO_2. Stimulation was performed 12h after treatment at a concentration of: 0,5 µg/mL of LPS, 1 µg/mL of PAM3CSK4, 25 µg/mL (GAPDH) + 10/1 (GBSf). Serum was collected at the indicated timepoints. Quantification of IL-10 and statistical analysis was determined as described in materials and methods

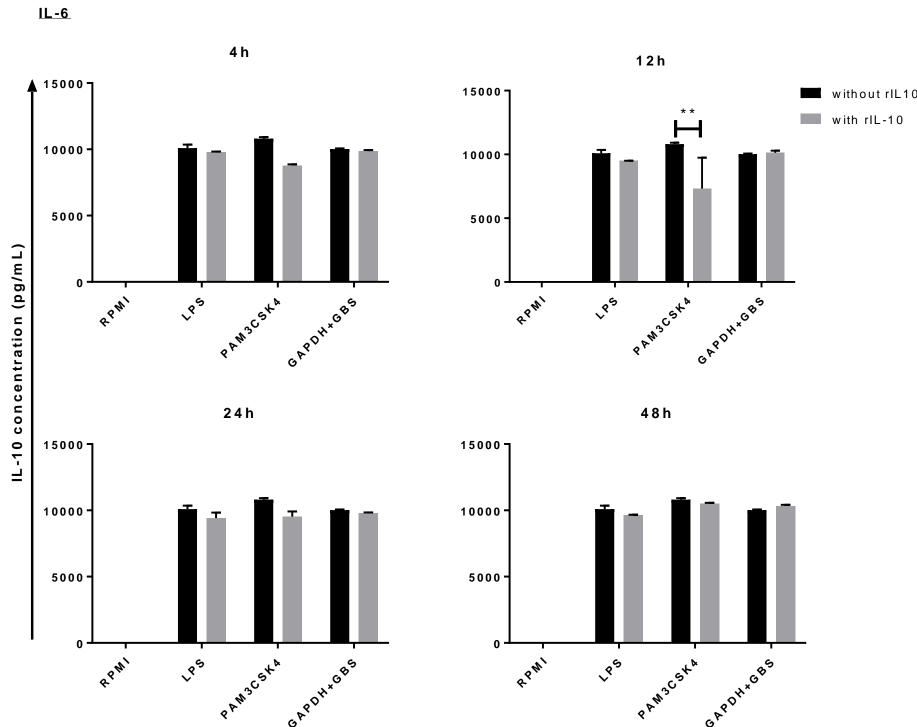


FIGURE 8. BMMs treated with rIL-10 show no alteration in the production of IL-6.

Quantification of IL-6 in BMMs when treated with TLR agonists with or without rIL-10 treatment. BMM were harvested as mentioned in materials and methods. Cells were plated at a concentration of *1x10⁵* cells/well. Treated cells were incubated with 10 ng/mL of rIL-10 for 12h at 37ºC and 5% CO_2. Stimulation was performed 12h after treatment at a concentration of: 0,5 µg/mL of LPS, 1 µg/mL of PAM3CSK4, 25 µg/mL (GAPDH) + 10/1 (GBSf). Serum was collected at the indicated timepoints. Quantification of IL-10 and statistical analysis was determined as described in materials and methods

Discussion

More than 20 years ago, Sorenson et al, published a cohort investigation where it is clear a distinct relationship between genetics and susceptibility to infections. Close to 900 adopted children born between 1924 and 1926 were followed alongside their adopted and biological parents until 1982. Interestingly, infection-related death of the biological parents before 50 years old, was indicative of a 5,8% chance of infection mortality in descendance. However, when adopted parents died of infection, no correlation was evident to the adopted descendance. They concluded that there must be a strong association between genetics and susceptibility to infection (Thorkkild, Sorenson, Stat., & Per Kragh Andersen, 1988). Consequently, researchers have been interested in the link between genetic science and sepsis predisposition. Recent work has been focused on genome-wide association studies and gene polymorphisms. Gene polymorphisms are substitutions, deletions or insertions of nucleotides at a chromosome site. The most common are single nucleotide polymorphisms (SNP) as they occur in 1 every 1000 base pairs of DNA. The latter can result in serious protein expression/production alteration. As science progressed, many SNPs have been found in some genes that altered the outcome of inflammatory responses, coagulation and other important immune responses to sepsis (Arcaroli, Fessler, & Abraham, 2005; Holmes, Russell, & Walley, 2012; Waterer & Wunderink, 2003). Some SNPs have been extensively studied to understand

the influence in the outcome of an infection. For example, replacement of aspartic acid with glycine is described as polymorphism that is associated with reduced expression and function of TLR4 *in vitro* (Schwartz, 2001). *In vivo* studies show that this polymorphism is associated with higher risk of septic shock and poor outcome (Agnese et al., 2002; Lorenz, Mira, Frees, & Schwartz, 2002). However, it is not associated with susceptibility of meningococcal septic shock in children (Karoly et al., 2007). In other TLRs have also been found SNPs. In the case of gene polymorphisms in TLR2, which is the gold standard receptor for Gram-positive bacteria, some have been found as deleterious in children and adults infected. Moreover, cytokine production can also be affected by SNPs although results may be controversial. For example, it has been described 2 allelic variants for the TNF-α gene. The TNF2 allele, which is altered by a SNP, has been related to higher expression of TNF-alpa and increased susceptibility to septic shock and mortality in adults and meningococcal septic shock in children (Nadel, Newport, Booy, & Levin, 1996). Finally, an SNP of the bactericidal permeability increasing protein gene has also been associated with increased mortality rates from septic shock in children (Michalek et al., 2007). Several authors unveiled that IL-10 phenotype and function depends on the stimuli, the recognition of said stimuli, and the timing of the stimuli. The kind of microorganism will influence the pathway of IL-10 production due to the different manners of host invasion and recognition. Upon recognition of the foreign pathogen, a series of signalling pathways initiate. Despite the common goal (IL-10 production), the different pathways of recognition and signal transduction will influence the readiness and intensity of IL-10 expression/production. We have seen that the major innate immune cells produce IL-10 upon recognition of the pathogen.

As a potent anti-inflammatory enforcer, prominent levels of IL-10 can inhibit the production of proinflammatory cytokines from Th cells. As seen in BALB/c infected with *Mycobacterium avium*, an early production of IL-10 is correlated with failure of BALB/c mice to control the infection. When IL-10 was neutralized, mice showed resistance to pathogen and reduced severity of the disease (Roque et al., 2007). However, it is not clear whether the production of IL-10 is a cause or a consequence of pathogen proliferation. On the one hand, infection resolution is dependent of effective but controlled immune responses. If the host immune response is unchecked, an exacerbated inflammation takes place and may develop inflammatory disorders, autoimmune diseases and even some cancers. On the other hand, pathogens take advantage of IL-10 expression to evade the host immune system and facilitate persistent infection (Couper, Blount, & Riley, 2008).

Previous work by the group demonstrated that GAPDH is a highly conserved protein present in GBS (Madureira et al., 2011). They showed that sepsis-inducing bacteria take advantage of extracellular GAPDH to escape the host immune system. In a western-blot analysis of total extracellular products of cell cultures of *E. coli*, *S. pneumoniae* and *S. aureus* using purified rabbit IgG against GBS-GAPDH as a primary antibody, was evident the presence of a band that was identified as GAPDH through mass-spectrometry analysis. There is straightforward evidence that extracellular GAPDH is transversal to all the bacteria mentioned. Nonetheless, whether bacteria were using GAPDH as a mean of host evasion was still unclear. Knowing that TLR2 is the cellular receptor for GAPDH ligand, we compared the survival rate of $TLR2^{-/-}$ new-born mice with WT mice when infected with clinical isolates *E. coli*, *K. pneumoniae* and MRSA. The latter were not able to resist bacterial challenge as nearly all succumbed in the first two days of bacterial challenge. Interestingly, $TLR2^{-/-}$ infected pups showed remarkedly higher resistance to bacteria. In correlation to previous results one can assume that these bacteria are not able to successfully evade the host immune system due to absent recognition of GAPDH in $TLR2^{-/-}$ mice.

It is also widely known that, neonates or adults admitted in ICU with severe sepsis, present high levels of IL-10. Furthermore, it has already been published that the level of IL-10 in the serum is positively correlated to the severity of the disease and the mortality rate (Andaluz-Ojeda et al., 2012). Group findings also showed an association between IL-10 elevated

production in leukocytes when stimulated with rGAPDH. Importantly, these results also demonstrate that when TLR2 is inhibited in leukocytes stimulated with rGAPDH, IL-10 production is completely abrogated. Altogether, these results indicate not only that GAPDH-dependent bacterial infection leads to the early production of IL-10 but also, that this mechanism is related to recognition of bacterial GAPDH through TLR2.

In this work, we show that injection of anti-IL-10 mAb prior to bacterial infection results in augmented survival rate in newborn mice when challenged with GBS and MRSA. Cusumano et al. published that BALB/c mice (<24h old) pre-treated with anti-IL-10 mAb at 6h before GBS infection prevented increased levels of IL-10. However, their findings also showed that survival rate and colony count was not significantly different which is in contrast with our results (Cusumano et al., 1996).

Our hypothesis is that blockade of the IL-10, hinders the bacterial evasion mechanism to the host immune system. Given the importance of IL-10 to bacterial evasion through host immunosuppression, the reduction of this cytokine compromised the pathogen replication and colonization. Upon bacterial challenge, the immune cells were instructed to produce IL-10, however, due to initial inhibition, this production was in a less extent. Therefore, the immune response was more efficient in reducing bacterial burden and tissue damage.

Clearly there is an association between IL-10 and neonatal susceptibility to bacterial infections induced by GAPDH. Yet, the cellular mechanism inherent to this relationship is still under-researched.

All the data presented herein pointed to the question: is IL-10 autoregulated by immune cells in the context of bacterial GAPDH-induced infection? Knolle et al, revealed that IL-10 production and secretion in KC challenged with bacterial endotoxin is due to a new protein synthesis (Knolle et al., 1998). According to published literature, injection of exogenous IL-10 would diminish immune cells' ability to produce elevated levels of IL-10 after bacterial infection. To understand how exogenous IL-10 would influence immune cells, BMM were treated with rIL-10 12h before stimulation with LPS, PAM3 CSK4 and 'GAPDH plus GBS' (figure 5). In fact, reduction of IL-10 production was observed in cells treated with rIL-10, 12h before stimulation with 'GAPDH plus GBS'. We can therefore hypothesize that rIL-10 incubation prior to infection has an inhibitory effect in the mechanism of IL-10 production due to TLR2-dependent GAPDH recognition.

It is known that different bacteria are responsible for the prevalence of sepsis in both infants and adults. Data showed that the mentioned bacteria (GBS, *E. coli*, MRSA) take advantage of GAPDH to induce immunosuppression in the host caused by elevated levels of IL-10. Adult BMMs treated with exogenous IL-10 show (figure 6) lower levels of IL-10 production after 12h and 24h of infection with the mentioned bacteria. These results corroborate the theory that the same mechanism of infection is transversal to all the tested bacteria. Besides the anti-inflammatory function, IL-10 has also been described as immunosuppressive if overproduced, posing an advantage for bacteria that induce IL-10 production as an evasion mechanism. As we can see, treatment with rIL-10 prior to infection, inhibit downstream production by the macrophages in early timepoints of infection. Also, our results support the notion that macrophages are a strong source of early IL-10 production, and that timed incubation with exogenous IL-10 results in a downstream reduction of IL-10 secretion by macrophages. This cytokine is able to induce a pre-inhibition of its own production after bacterial infection. This way, upon bacterial challenge, the induction of IL-10 production in initial stages is abrogated. Consequently, immune cells are stimulated to produce pro-inflammatory cytokines which in turn will be essential for bacterial clearance and resolution of infection.

An unchecked production of IL-10 leads to appearance of autoimmune diseases, sepsis and tissue damage caused by exacerbated inflammation. In this work, we demonstrate that pre-incubation with rIL-10 does not affect significantly TNF-α or IL-6 production. We have seen that cells treated with rIL-10 will produce less IL-10 after infection. Consequently, IL-10's anti-inflammatory function is diminished resulting in normal production and secretion of TNF-

α and IL-6 in early stages. More data is needed to understand if after 48h of infection, levels of these cytokines are affected. One of the most important functions of IL-10 is the regulation of the immune response. In its absence, cells continuously produce immune mediators which are important in the initial stages of infection. However, the exaggerated production of pro-inflammatory cytokines results in self-damage if the infection is not resolved. Therefore, it would important to analyse TNF-α and IL-6 levels in cells treated or not with rIL-10 in later timepoints of infection.

In the case of IL-10 it has been described that neutrophils, macrophages, dendritic cells, T cells and B cells are all possible sources for its production in bacterial infections. Also, GAPDH has been found as a virulence-associated protein that induces IL-10 production in CD5+ B1 cells through TLR2 recognition. However, neonates rely mostly on their innate immune system as the adaptive immune system is underdeveloped. The fact that B cells predominantly produce higher levels of IL-10 through TLR2 recognition than innate immune cells in and early onset infection, is yet to be understood. Comparison between these cell subsets will be important to understand IL-10 production mechanism and its influence in the host immune response to bacterial challenge.

The immunosuppressive role of IL-10 in B1 cells has already been demonstrated. Interestingly, peritoneal B1 (B-1P) cells can autoregulate their own expression of IL-10. In this study, B-1P cells showed a weaker response to TLR stimulation due to rapid induction of IL-10. The authors explained that, compared to other B cells, B-1P cells produced higher amounts of IL-10 as early as 6h after LPS or CpG stimulation and showed reduced cell proliferation. After IL-10 effects were neutralized, B-1P proliferation was increased when induced by LPS and CpG (Sindhava, Woodman, Stevenson, & Bondada, 2010). These findings corroborate previous reports that characterize an inhibitory effect of high doses of exogenous IL-10 on human leukemic CD5+ B-cells (Tangye et al., 2015). Moreover, the authors describe a B cell subset that expresses CD5+, a common T cell marker, as the main producers of IL-10 (constitutively and after TLR signalling) (Sindhava et al., 2010). Anne O'Garra et al, suggested that the ability of B cells to secrete high concentration of IL-10 is inherent to a subpopulation called Ly-1 (B1) cells. In this study peritoneal B cells were stimulated with LPS showed significant production of IL-10 (O'garra et al., 1992). Also it has been shown that B1 cells more abundant in newborns than in adult mice (Sun, Deriaud, Leclerc, & Lo-Man, 2005).

Our research group results demonstrated that a specific B-cell subset, CD5+ B cells, are the main producers of IL-10 upon TLR2 recognition of bacterial GAPDH in a neonatal infection model. Moreover, that the elevated concentration of IL-10 induced by CD5+ B cells-GAPDH, influence host immunosuppression and consequent response to invading bacteria. Yet some questions remain unanswered: do B cells produce higher levels of IL-10 than other innate or adaptive immune cells? Is TLR2 signalling sufficient to produce enough IL-10 that would lead to immunosuppression? What are the epigenetic and chromatin modifications that promote IL-10 gene expression in B cells after GAPDH recognition? After IL-10 endogenous production, what mechanisms are involved in the autocrine function in these cells? Future work is needed to fully understand this autoregulatory mechanism in these cells.

For the past few years, scientists have been intrigued with the capabilities of genetic information and how it can be assessed. Their aim relies on the achievement of genetic therapies or readouts that can help cure diseases. So, the concept of transferring genetic information to polypeptides enable the possibility of designing short nucleic acid fragments (oligonucleotides) for application in biomolecular tools and work as therapeutic agents (Crick, 1970). Past work provided information regarding synthetic oligonucleotides and their useful inhibition of RNA translation in Rous sarcoma virus (Stephenson & Zamecnik, 1978) and gene mapping (Paterson, Robertst, & Kuff, 1977). Through the advances of modern science, researchers learnt to synthesize and develop various new chemically modified oligonucleotides with ease. It has already been described the use peptide nucleic acid/locked nucleic acid probes (PNA/LNA) to identify bacteria through fluorescent hybridization *in situ*

(FISH). PNA molecules emerge as a versatile synthetic pseudo peptide with an uncharged backbone of N-(2-aminoethyl) glycine motifs repeats linked by peptide bonds instead of the ordinary phosphodiester backbone. Nielsen et al, initially proposed their binding to dsDNA by Hoogsteen-like hydrogen bond (Nielsen, Egholm, Berg, & Buchardt, 1991). On the other hand, LNAs, also known as 2-O,4-C-methylene bridge nucleic acid, have been explored for their antisense applications being widely associated as RNA analogues (Lundin et al., 2013). Like PNAs, these molecules have been used in molecular diagnostics and theranostics. LNAs also promote stabilized duplexes with RNA and DNA by increased Tm due to conformational rigidity (Braasch & Corey, 2001)

For future work, the main attractive feature of these molecules is their ability to interfere with gene transcription and translation. Several reviews have been published that remark the potential of antisense and antigene therapy of PNAs. A series of papers have been published regarding the antigene and antisense advantages of PNAs. The general strategies reported include: gene activation regulation (Amit-Avraham et al., 2015), exon skipping enhancement (O'Donovan et al., 2014) and antibacterial antisense targeted to translation initiation (Ghosal & Nielsen, 2012). Antisense PNAs show great promise towards gene silencing. It has been shown that TNA targeting using antisense PNA can accurately downregulate P. falciparum gene expression silencing essential genes that participate in infection (Kolevzon, Nasereddin, Naik, Yavin, & Dzikowski, 2014). Regarding the broad applications of PNA/LNA probes, there is an increasing interest to apply this technique for thorough exploration of the IL-10 gene. A detailed investigation on the pathways that lead to IL-10 gene expressing will promote the comprehension on the influence of the timing of IL-10 production and how it influences the immune response.

Chapter 3

Bibliography

Abughali, N., Berger, M., & Tosi, M. F. (1994). Deficient total cell content of CR3 (CD11b) in neonatal neutrophils. *Blood*, *83*(4), 1086–1092.
Adkins, B., Bu, Y., & Cepero, E. (2015). Exclusive Th2 Primary Effector Function in Spleens but Mixed Th1/Th2 Function in Lymph Nodes of Murine Neonates. https://doi.org/10.4049/jimmunol.164.5.2347
Adkins, B., Bu, Y., Guevara, P., & Alerts, E. (2015). Murine Neonatal CD4 + Lymph Node Cells Are Highly Deficient in the Development of Antigen-Specific Th1 Function in Adoptive Adult Hosts. https://doi.org/10.4049/jimmunol.169.9.4998
Adkins, B., Leclerc, C., & Marshall-Clarke, S. (2004). Neonatal adaptive immunity comes of age. *Nature Reviews Immunology*, *4*(7), 553–564. https://doi.org/10.1038/nri1394
Agnese, D. M., Calvano, J. E., Hahm, S. J., Coyle, S. M., Corbett, S. A., Calvano, S. E., & Lowry, S. F. (2002). Human Toll-Like Receptor 4 Mutations but Not CD14 Polymorphisms Are Associated with an Increased Risk of Gram-Negative Infections. *The Journal of Infectious Diseases*, *186*(10), 1522–1525. https://doi.org/10.1086/344893
Akira, S., Uematsu, S., & Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell*, *124*(4), 783–801. https://doi.org/10.1016/j.cell.2006.02.015
Alvarez, R. A., Blaylock, M. W., & Baseman, J. B. (2003). Surface localized glyceraldehyde-3-phosphate dehydrogenase of Mycoplasma genitalium binds mucin, *48*, 1417–1425. https://doi.org/10.1046/j.1365-2958.2003.03518.x
Amit-Avraham, I., Pozner, G., Eshar, S., Fastman, Y., Kolevzon, N., Yavin, E., & Dzikowski, R. (2015). Antisense long noncoding RNAs regulate var gene activation in the malaria parasite Plasmodium falciparum. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(9), E982-91. https://doi.org/10.1073/pnas.1420855112
Andaluz-Ojeda, D., Bobillo, F., Iglesias, V., Almansa, R., Rico, L., Gandía, F., … Bermejo-Martin, J. F. (2012). A combined score of pro- and anti-inflammatory interleukins improves mortality prediction in severe sepsis. *Cytokine*, *57*(3), 332–336. https://doi.org/10.1016/j.cyto.2011.12.002
Andrade, E. B., Alves, J., Madureira, P., Oliveira, L., Ribeiro, A., Cordeiro-da-Silva, A., … Ferreira, P. (2013). TLR2-induced IL-10 production impairs neutrophil recruitment to infected tissues during neonatal bacterial sepsis. *Journal of Immunology*, *191*(9), 4759–4768. https://doi.org/10.4049/jimmunol.1301752
Arcaroli, J., Fessler, M. B., & Abraham, E. (2005). GENETIC POLYMORPHISMS AND SEPSIS. *Shock*, *24*(4), 300–312. https://doi.org/10.1097/01.shk.0000180621.52058.e1
Article, C. M. E. R. (2000). Cytokines as markers of bacterial sepsis in newborn infants : a review, *19*(9), 879–887.
Baker, C. J., Marcia, M. D., & Rench, B. S. (1998). Immunization of pregnant women with a polysaccharide vaccine of group B Streptococcus. *The New England Journal of Medicine*, *319*(18), 1180–1185.

Baker, C. J., Paoletti, L. C., Wessels, M. R., Guttormsen, H., Rench, M. A., Hickman, M. E., & Kasper, D. L. (n.d.). Safety and Immunogenicity of Capsular Polysaccharide – Tetanus Toxoid Conjugate Vaccines for Group B Streptococcal Types Ia and Ib, 142–150.
Basha, S., Surendran, N., & Pichichero, M. (2014). Immune responses in neonates. *Expert Review of Clinical Immunology*, *10*(9), 1171–1184. https://doi.org/10.1586/1744666X.2014.942288
Behrman, E., Rosenfeld, C. R., Browne, R., & Ph, D. (1979). AND NEONATAL The neonatal blood count in health and disease . L Reference values for neutrophilic cells, *95*(1), 89–98.
Benjamim, C. F., Ferreira, S. H., & Cunha, F. Q. (n.d.). Role of Nitric Oxide in the Failure of Neutrophil Migration in Sepsis, 214–223.
Berner, R. (2004). Identification and immunoreactivity of proteins released from Streptococcus agalactiae, 818–824. https://doi.org/10.1007/s10096-004-1229-y
Black, R. E., Cousens, S., Johnson, H. L., Lawn, J. E., Rudan, I., Bassani, D. G., … Mathers, C. (2010). Global, regional, and national causes of child mortality in 2008: a systematic analysis. *The Lancet*, *375*(9730), 1969–1987. https://doi.org/10.1016/S0140-6736(10)60549-1
Blackwell, A. D., Snodgrass, J. J., Madimenos, F. C., & Sugiyama, L. S. (2010). Life history, immune function, and intestinal helminths: Trade-offs among immunoglobulin E, C-reactive protein, and growth in an Amazonian population. *American Journal of Human Biology*, *22*(6), 836–848. https://doi.org/10.1002/ajhb.21092
Braasch, D. a, & Corey, D. R. (2001). Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. *Chemistry & Biology*, *8*(1), 1–7. https://doi.org/10.1016/S1074-5521(00)00058-2
Brozanski, B. S., Jones, J. G., Krohn, M. J., & Jordan, J. A. (2006). Use of polymerase chain reaction as a diagnostic tool for neonatal sepsis can result in a decrease in use of antibiotics and total neonatal intensive care unit length of stay, (October), 688–692. https://doi.org/10.1038/sj.jp.7211597
Buckler, B., Bell, J., Sams, R., Cagle, W., Bell, S. A., Allen, C., … Bhatia, J. (2010). Unnecessary Workup of Asymptomatic Neonates in the Era of Group B Streptococcus Prophylaxis, *2010*. https://doi.org/10.1155/2010/369654
Camacho-Gonzalez, A., Spearman, P. W., & Stoll, B. J. (2013). Neonatal Infectious Diseases. Evaluation of Neonatal Sepsis. *Pediatric Clinics of North America*. https://doi.org/10.1016/j.pcl.2012.12.003
Carr, R. (2000). Neutrophil production and function in newborn infants. *British Journal of Haematology*, *110*(1), 18–28. https://doi.org/10.1046/j.1365-2141.2000.01992.x
CDC. (2013). Antibiotic resistance threats in the United States, 2013. *Current*, 114. https://doi.org/CS239559-B
Cernada, M., Badía, N., Modesto, V., Alonso, R., Mejías, A., Golombek, S., & Vento, M. (2012). Cord blood interleukin-6 as a predictor of early-onset neonatal sepsis, 203–207. https://doi.org/10.1111/j.1651-2227.2011.02577.x
Chen, L.-W., Huang, H.-L., Lee, I.-T., Hsu, C.-M., & Lu, P.-J. (2006). Thermal Injury-Induced Priming Effect of Neutrophil Is Tnf-?? and P38 Dependent. *Shock*, *26*(1), 69–76. https://doi.org/10.1097/01.shk0000209531.38188.18
Chung, E. Y., Liu, J., Homma, Y., Zhang, Y., Brendolan, A., Saggese, M., … Ma, X. (2007). Interleukin-10 expression in macrophages during phagocytosis of apoptotic cells is mediated by homeodomain proteins Pbx1 and Prep-1. *Immunity*, *27*, 952–964. https://doi.org/10.1016/j.immuni.2007.11.014
Cohen-Wolkowiez, M., Moran, C., Benjamin, D. K., Cotten, C. M., Clark, R. H., & Smith, P. B. (2009). Early and late onset sepsis in late preterm infants. *The Pediatric Infectious Disease Journal*, *28*(12), 1052–1056. https://doi.org/10.1097/INF.0b013e3181acf6bd
Control, D., & Report, M. W. (2016). Prevention of Perinatal Group B Streptococcal Disease, *51*(Cdc), 1–22.
Couper, K. N., Blount, D. G., & Riley, E. M. (2008). Infection IL-10: The Master Regulator of Immunity to IL-10: The Master Regulator of Immunity to Infection. *J Immunol References*, *180*, 5771–5777. https://doi.org/10.4049/jimmunol.180.9.5771
Crick, F. (1970). Central dogma of molecular biology. *Nature*, *227*(5258), 561–3. https://doi.org/10.1038/227561a0
Critical, P., & Medicine, C. (2017). International pediatric sepsis consensus conference : Definitions for sepsis and organ dysfunction in pediatrics *, 2–3.

https://doi.org/10.1097/01.PCC.0000149131.72248.E6
Cusumano, V., Genovese, F., Mancuso, G., Carbone, M., Fera, M. T., & Teti, G. (1996). Interleukin-10 protects neonatal mice from lethal group B streptococcal infection. *Infection and Immunity*, *64*(7), 2850–2. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=174154&tool=pmcentrez&rendertype=abstract
Dang, P. M. C., Elbim, C., Marie, J. C., Chiandotto, M., Gougerot-Pocidalo, M. A., & El-Benna, J. (2006). Anti-inflammatory effect of interleukin-10 on human neutrophil respiratory burst involves inhibition of GM-CSF-induced p47PHOX phosphorylation through a decrease in ERK1/2 activity. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *20*(9), 1504–1506. https://doi.org/10.1096/fj.05-5395fje
Darini, A. L. C., Inoue, R. Y., Silva, J. S., Ferreira, S. H., Cunha, F. Q., Paulo, Ä., … Paulo, Ä. (2002). Involvement of NO in the failure of neutrophil migration in sepsis induced by Staphylococcus aureus.
De Roock, S., Stoppelenburg, A. J., Scholman, R., Hoeks, S. B. E. A., Meerding, J., Prakken, B. J., & Boes, M. (2013). Defective TH17 development in human neonatal T cells involves reduced RORC2 mRNA content. *Journal of Allergy and Clinical Immunology*, *132*(3), 1–6. https://doi.org/10.1016/j.jaci.2013.04.014
Dellinger, R. P., Levy, M. M., Rhodes, A., Annane, D., Gerlach, H., Opal, S. M., … Moreno, R. (2013). Surviving sepsis campaign: International guidelines for management of severe sepsis and septic shock, 2012. *Intensive Care Medicine*, *39*(2), 165–228. https://doi.org/10.1007/s00134-012-2769-8
Demicheli, V., Barale, a, & Rivetti, a. (2005). Vaccines for women to prevent neonatal tetanus. *Cochrane Database of Systematic Reviews (Online)*, (5), CD002959. https://doi.org/10.1002/14651858.CD002959.pub2
Driessler, F., Venstrom, K., Sabat, R., Asadullah, K., & Schottelius, A. J. (2004). Molecular mechanisms of interleukin-10-mediated inhibition of NF-k k k k B activity: a role for p50. *Clin Exp Immunol*, *135*, 64–73. https://doi.org/10.1046/j.1365-2249.2004.02342.x
ECDC. (2009). *The bacterial challenge : time to react*. *Reproduction* (Vol. 6 July 201). https://doi.org/10.2900/2518
Echtenacher, B., Mannel, D. N., & Hultner, L. (1996). Critical protective role of mast cells in a model of acute septic peritonitis. *Nature*. https://doi.org/10.1038/381075a0
Ecker, K. L., Donohue, P. K., Kim, K. S., Shepard, J. A., & Aucott, S. W. (2012). The impact of group B streptococcus prophylaxis on late-onset neonatal infections. *Journal of Perinatology*, *33*(3), 206–211. https://doi.org/10.1038/jp.2012.76
Edmond, K. M., Kortsalioudaki, C., Scott, S., Schrag, S. J., Zaidi, A. K., Cousens, S., & Heath, P. T. (2012). Group B streptococcal disease in infants aged younger than 3 months: Systematic review and meta-analysis. *The Lancet*, *379*(9815), 547–556. https://doi.org/10.1016/S0140-6736(11)61651-6
Edmond, K., & Zaidi, A. (2010). New approaches to preventing, diagnosing, and treating neonatal sepsis. *PLoS Medicine*, *7*(3), 1–8. https://doi.org/10.1371/journal.pmed.1000213
Edwards, M. S., & Gonik, B. (2013). Preventing the broad spectrum of perinatal morbidity and mortality through group B streptococcal vaccination. *Vaccine*, *31*(S4), D66–D71. https://doi.org/10.1016/j.vaccine.2012.11.046
Ehl, S., Gering, B., Bartmann, P., Josef, H., & Pohlandt, F. (1997). C-reactive protein: A useful marker for guiding duration of antibiotic therapy in suspected neonatal septicaemia? *Pediatrics*, *99*, 216. https://doi.org/10.1542/peds.99.2.216
Epstein, F. H. (1999). Acute-phase Proteins and Other Systemic Responses to Inflammation. *The New England Journal of Medicine*, 448–454.
FIORENTINO, D., BOND, M., & MOSMANN, T. (1990). Two Types of Mouse T Helper Cell. *Citeseer*, *170*(December 1989). Retrieved from http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:TWO+TYPES+OF+MOUSE+T+HELPER+CELL#5
Fischer, E., Marano, M. A., Van Zee, K. J., Rock, C. S., Hawes, A. S., Thompson, W. A., … Moldawer, L. L. (1992). Interleukin-1 receptor blockade improves survival and hemodynamic performance in Escherichia coli septic shock, but fails to alter host responses to sublethal endotoxemia. *Journal of Clinical Investigation*, *89*(5), 1551–1557. https://doi.org/10.1172/JCI115748

Foey, A. D., Parry, S. L., Williams, L. M., Feldmann, M., Foxwell, B. M. J., & Brennan, F. M. (1998). Regulation of Monocyte IL-10 Synthesis by Endogenous IL-1 and TNF-α: Role of the p38 and p42/44 Mitogen-Activated Protein Kinases. *The Journal of Immunology*, *160*(2), 920–928.
Garcia, C. a, Wang, H., Benakanakere, M. R., Barrett, E., Kinane, D. F., & Martin, M. (2009). c-jun controls the ability of IL-12 to induce IL-10 production from human memory CD4+ T cells. *Journal of Immunology (Baltimore, Md. : 1950)*, *183*(7), 4475–4482. https://doi.org/10.4049/jimmunol.0901283
Geijtenbeek, T. B. H., & Gringhuis, S. I. (2009). Signalling through C-type lectin receptors: shaping immune responses. *Nature Reviews Immunology*, *9*(7), 465–479. https://doi.org/10.1038/nri2569
Ghosal, A., & Nielsen, P. E. (2012). Potent antibacterial antisense peptide-peptide nucleic acid conjugates against Pseudomonas aeruginosa. *Nucleic Acid Therapeutics*, *22*(5), 323–334. https://doi.org/10.1089/nat.2012.0370
Gonzalez, B. E., Mercado, C. K., Johnson, L., Brodsky, N. L., & Bhandari, V. (2003). Early markers of late-onset spesis in premature neonates: clinical, hematological and cytokine profiles. *Journal of Perintal Medicine*, *31*, 60–68.
Griffin, D. O., Holodick, N. E., & Rothstein, T. L. (2011). Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20 $^{+}$ CD27 $^{+}$ CD43 $^{+}$ CD70 $^{-}$. *The Journal of Experimental Medicine*, *208*(1), 67–80. https://doi.org/10.1084/jem.20101499
Griffin, D. O., & Rothstein, T. L. (2011). A small CD11b $^{+}$ human B1 cell subpopulation stimulates T cells and is expanded in lupus. *The Journal of Experimental Medicine*, *208*(13), 2591–2598. https://doi.org/10.1084/jem.20110978
Haas, K. M., Poe, J. C., Steeber, D. A., & Tedder, T. F. (2005). B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S. pneumoniae. *Immunity*, *23*(1), 7–18. https://doi.org/10.1016/j.immuni.2005.04.011
Häcker, H., Redecke, V., Blagoev, B., Kratchmarova, I., Hsu, L.-C., Wang, G. G., … Karin, M. (2006). Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature*, *439*(7073), 204–207. https://doi.org/10.1038/nature04369
Hazeldine, J., Harris, P., Chapple, I. L., Grant, M., Greenwood, H., Livesey, A., … Lord, J. M. (2014). Impaired neutrophil extracellular trap formation: A novel defect in the innate immune system of aged individuals. *Aging Cell*, *13*(4), 690–698. https://doi.org/10.1111/acel.12222
Heron, M., Hoyert, D. L., Murphy, S. L., Xu, J., Kochanek, K. D., & Tejada-Vera, B. (2009). National vital statistics reports. *National Vital Statistics Reports*, *57*(14).
Holmes, C. L., Russell, J. A., & Walley, K. R. (2012). critical care review Genetic Polymorphisms in Sepsis and Septic Shock *. https://doi.org/10.1378/chest.124.3.1103
Hornik, C. P., Fort, P., Clark, R. H., Watt, K., Benjamin, D. K., Smith, P. B., … Cohen-Wolkowiez, M. (2012). Early and late onset sepsis in very-low-birth-weight infants from a large group of neonatal intensive care units. *Early Human Development*, *88*(SUPPL.2), S69–S74. https://doi.org/10.1016/S0378-3782(12)70019-1
Hotchkiss, R. S., Moldawer, L. L., Opal, S. M., Reinhart, K., Turnbull, I. R., & Vincent, J.-L. (2016). Sepsis and septic shock. *Nature Reviews. Disease Primers*, *2*, 16045. https://doi.org/10.1016/j.celrep.2016.01.043.Improved
Inohara, N., Chamaillard, M., McDonald, C., & Nuñez, G. (2005). NOD-LRR PROTEINS: Role in Host-Microbial Interactions and Inflammatory Disease. *Annual Review of Biochemistry*, *74*(1), 355–383. https://doi.org/10.1146/annurev.biochem.74.082803.133347
Jacqz-aigrain, E., Zhao, W., Sharland, M., & Anker, J. N. Van Den. (2013). Seminars in Fetal & Neonatal Medicine Use of antibacterial agents in the neonate : 50 years of experience with vancomycin administration. *Seminars in Fetal and Neonatal Medicine*, *18*(1), 28–34. https://doi.org/10.1016/j.siny.2012.10.003
Jain, S., Sinha, S., Sharma, S. K., Samantaray, J. C., Aggrawal, P., Vikram, N., … Khan, N. (2014). Procalcitonin as a prognostic marker for sepsis: a prospective observational study. *BMC Research Notes*, *7*(1), 458. https://doi.org/10.1186/1756-0500-7-458
Jankovic, D., Kullberg, M. C., Feng, C. G., Goldszmid, R. S., Collazo, C. M., Wilson, M., … Sher, A. (2007). Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. *The Journal of Experimental Medicine*, *204*(2), 273–283. https://doi.org/10.1084/jem.20062175

Jaye, D., & Waites, K. (1997). Clinical applications of C-reactive protein in pediatrics. *Pediatrics Infectious Disease*, *16*, 735–746.
Joan, M. (2003). The Role of C-reactive Protein in the evaluation and management of infants with suspected sepsis. In *Beyond the Basics: Advanced Physiology and Care Concepts* (pp. 3–13).
Jochum, S., Moosmann, A., Lang, S., Hammerschmidt, W., & Zeidler, R. (2012). The EBV immunoevasins vIL-10 and BNLF2a protect newly infected B cells from immune recognition and elimination. *PLoS Pathogens*, *8*(5). https://doi.org/10.1371/journal.ppat.1002704
Johnson, C. E., Whitwell, J. K., Pethe, K., Saxena, K., Super, D. M., & Objectives, A. (2015). Term Newborns Who Are at Risk for Sepsis : Are Lumbar Punctures Necessary ?, *99*(4), 1–7.
Josephson, K., Logsdon, N. J., & Walter, M. R. (2001). Crystal structure of the IL-10/IL-10R1 complex reveals a shared receptor binding site. *Immunity*, *15*(1), 35–46. https://doi.org/10.1016/S1074-7613(01)00169-8
Kaczmarek, A., Budzyńska, A., & Gospodarek, E. (2012). Prevalence of genes encoding virulence factors among Escherichia coli with K1 antigen and non-K1 E. coli strains. *Journal of Medical Microbiology*, *61*(PART 10), 1360–1365. https://doi.org/10.1099/jmm.0.044263-0
Karoly, E., Fekete, A., Banki, N. F., Szebeni, B., Vannay, A., Szabo, A. J., … Reusz, G. S. (2007). Heat shock protein 72 (HSPA1B) gene polymorphism and toll-like receptor (TLR) 4 mutation are associated with increased risk of urinary tract infection in children. *Pediatric Research*, *61*(3), 371–374. https://doi.org/10.1203/pdr.0b013e318030d1f4
Kasten, K. R., Muenzer, J. T., & Caldwell, C. C. (2010). Neutrophils are significant producers of IL-10 during sepsis. *Biochemical and Biophysical Research Communications*, *393*(1), 28–31. https://doi.org/10.1016/j.bbrc.2010.01.066
Kawai, T., & Akira, S. (2011). Review Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. *Immunity*, *34*(5), 637–650. https://doi.org/10.1016/j.immuni.2011.05.006
Kellogg, J. A., & Ferrentino, F. L. (2017). Frequency of low level bacteremia in infants from birth to two months of age ., *2*, 7–8.
Kim, J. M., Brannan, C. I., Copeland, N. G., Jenkinst, N. A., & Tariq, A. (2016). Structure of the mouse IL-10 gene and chromosomal localization of the mouse and human genes . J M Kim , C I Brannan , N G Copeland , N A Jenkins , T A Khan and K W Moore Information about subscribing to The Journal of Immunology is online at : STRUCTURE O.
Knolle, P. A., Uhrig, A., Protzer, U., Trippler, M., Duchmann, R., Buschenfelde K.-, H. M. Z., & Gerken, G. (1998). Interleukin-10 expression is autoregulated at the transcriptional level in human and murine Kupffer cells. *Hepatology*, *27*(1), 93–99. https://doi.org/10.1002/hep.510270116
Kolevzon, N., Nasereddin, A., Naik, S., Yavin, E., & Dzikowski, R. (2014). Use of peptide nucleic acids to manipulate gene expression in the malaria parasite Plasmodium falciparum. *PLoS ONE*, *9*(1). https://doi.org/10.1371/journal.pone.0086802
Kotenko, S. V., Saccani, S., Izotova, L. S., Mirochnitchenko, O. V., & Pestka, S. (2000). Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). *Proceedings of the National Academy of Sciences*, *97*(4), 1695–1700. https://doi.org/10.1073/pnas.97.4.1695
Lagu, T., Rothberg, M. B., Shieh, M.-S., Pekow, P. S., Steingrub, J. S., & Lindenauer, P. K. (2012). Hospitalizations, costs, and outcomes of severe sepsis in the United States 2003 to 2007. *Critical Care Medicine*, *40*(3), 754–761. https://doi.org/10.1097/CCM.0b013e318232db65
Laichalk, L. L., Kunkel, S. L., Strieter, R. M., Danforth, J. M., Bailie, M. B., & Standiford, T. J. (1996). Tumor necrosis factor mediates lung antibacterial host defense in murine Klebsiella pneumonia. *Infection and Immunity*, *64*(12), 5211–5218.
Lambert, R. M., Baer, V. L., Wiedmeier, S. E., Henry, E., Burnett, J., & Christensen, R. D. (2009). Isolated elevated blood neutrophil concentration at altitude does not require NICU admission if appropriate reference ranges are used, *2002*, 822–825. https://doi.org/10.1038/jp.2009.3
Lapillonne, A., Basson, N., Monneret, G., Bienvenu, J., & Salle, B. L. (1998). Lack of specificity of procalcitonin for sepsis diagnosis in premature infants [6]. *Lancet*, *351*(9110), 1211–1212. https://doi.org/10.1016/S0140-6736(05)79165-0

Levy, O. (2005). to LPS and other Toll-like receptor agonists. https://doi.org/10.1179/096805105X37376
Lima, M., Bandeira, A., Portnoi, D., & Ribeiro, A. (1992). Protective Effect of a T-Cell-Dependent Immunosuppressive , Streptococcus intermedius, *60*(9), 3571–3578.
Lindquester, G. J., Greer, K. A., Stewart, J. P., & Sample, J. T. (2014). Epstein-Barr virus IL-10 gene expression by a recombinant murine gammaherpesvirus in vivo enhances acute pathogenicity but does not affect latency or reactivation. *Herpesviridae*, *5*(1), 1. https://doi.org/10.1186/2042-4280-5-1
Liu, B. S., Cao, Y., Huizinga, T. W., Hafler, D. A., & Toes, R. E. M. (2014). TLR-mediated STAT3 and ERK activation controls IL-10 secretion by human B cells. *European Journal of Immunology*, *44*(7), 2121–2129. https://doi.org/10.1002/eji.201344341
Lorenz, E., Mira, J. P., Frees, K. L., & Schwartz, D. A. (2002). Relevance of Mutations in the TLR4 Receptor in Patients With Gram-Negative Septic Shock. *Archives of Internal Medicine*, *162*(9), 1028. https://doi.org/10.1001/archinte.162.9.1028
Lukacs, S. L., & Schrag, S. J. (2012). Clinical sepsis in neonates and young infants, United States, 1988-2006. *Journal of Pediatrics*, *160*(6), 960–965.e1. https://doi.org/10.1016/j.jpeds.2011.12.023
Lundin, K. E., Højland, T., Hansen, B. R., Persson, R., Bramsen, J. B., Kjems, J., … Smith, C. I. E. (2013). *Biological Activity and Biotechnological Aspects of Locked Nucleic Acids*. *Advances in Genetics* (Vol. 82). https://doi.org/10.1016/B978-0-12-407676-1.00002-0
Madureira, P. (2004). Protection against systemic candidiasis in mice immunized with secreted aspartic proteinase 2, 334–342. https://doi.org/10.1111/j.1365-2567.2004.01819.x
Madureira, P., Andrade, E. B., Gama, B., Oliveira, L., Moreira, S., Ribeiro, A., … Ferreira, P. (2011). Inhibition of IL-10 production by maternal antibodies against group b streptococcus GAPDH confers immunity to offspring by favoring neutrophil recruitment. *PLoS Pathogens*, *7*(11), 1–14. https://doi.org/10.1371/journal.ppat.1002363
Manning, S. D., Foxman, B., Pierson, C. L., Tallman, P., Ascp, M. T., Baker, C. J., & Pearlman, M. D. (2003). Correlates of Antibiotic-Resistant Group B Streptococcus Isolated From Pregnant Women, *101*(1), 74–79.
Maruna, P., Nedělníková, K., & Gürlich, R. (2000). Physiology and genetics of procalcitonin. *Physiological Research*.
Maynard, C. L., Hatton, R. D., Helms, W. S., Oliver, J. R., Stephensen, C. B., & Weaver, C. T. (2009). Contrasting roles for all-trans retinoic acid in TGF-beta-mediated induction of Foxp3 and Il10 genes in developing regulatory T cells. *The Journal of Experimental Medicine*, *206*(2), 343–57. https://doi.org/10.1084/jem.20080950
Mccarron, M. J., & Reen, D. J. (2010). Neonatal CD8+ T-cell differentiation is dependent on interleukin-12. *Human Immunology*, *71*(12), 1172–1179. https://doi.org/10.1016/j.humimm.2010.09.004
Memar, M. Y., Alizadeh, N., Varshochi, M., & Kafil, H. S. (2017). Immunologic biomarkers for diagnostic of early-onset neonatal sepsis. *The Journal of Maternal-Fetal & Neonatal Medicine*, *7058*(August), 1–11. https://doi.org/10.1080/14767058.2017.1366984
Menezes, G. B., Lee, W.-Y., Zhou, H., Waterhouse, C. C. M., Cara, D. C., & Kubes, P. (2009). Selective down-regulation of neutrophil Mac-1 in endotoxemic hepatic microcirculation via IL-10. *Journal of Immunology (Baltimore, Md. : 1950)*, *183*(11), 7557–68. https://doi.org/10.4049/jimmunol.0901786
Michalek, J., Svetlikova, P., Fedora, M., Klimovic, M., Klapacova, L., Bartosova, D., … Hubacek, J. A. (2007). Bactericidal permeability increasing protein gene variants in children with sepsis. *Intensive Care Medicine*, *33*(12), 2158–2164. https://doi.org/10.1007/s00134-007-0860-3
Miedema, K. G. E., De Bont, E. S. J. M., Elferink, R. F. M. O., Van Vliet, M. J., Nijhuis, C. S. M. O., Kamps, W. A., & Tissing, W. J. E. (2011). The diagnostic value of CRP, IL-8, PCT, and sTREM-1 in the detection of bacterial infections in pediatric oncology patients with febrile neutropenia. *Supportive Care in Cancer*, *19*(10), 1593–1600. https://doi.org/10.1007/s00520-010-0987-6
Monneret, G., Labaune, J. M., Isaac, C., Bienvenu, F., Putet, G., & Bienvenu, J. (1997). Procalcitonin and C-reactive protein levels in neonatal infections. *Acta Paediatrica*, *86*, 209–212. https://doi.org/10.1111/j.1651-2227.1997.tb08870.x
Moore, M. R., Schrag, S. J., & Schuchat, A. (2003). Effects of intrapartum antimicrobial prophylaxis for prevention of group-B-streptococcal disease on the incidence and

ecology of early-onset neonatal sepsis. *Lancet Infectious Diseases*, *3*(4), 201–213. https://doi.org/10.1016/S1473-3099(03)00577-2
Murai, M., Turovskaya, O., Kim, G., Madan, R., Karp, C. L., Cheroutre, H., & Kronenberg, M. (2009). Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nature Immunology*, *10*(11), 1178–84. https://doi.org/10.1038/ni.1791
Murphy, K., & Weiner, J. (2017). Use of Leukocyte Counts in Evaluation of Early-onset Neonatal Sepsis, 1-2. https://doi.org/10.1097/INF.0b013e31822ffc17
Naber, C. K. (2009). *Staphylococcus aureus* Bacteremia: Epidemiology, Pathophysiology, and Management Strategies. *Clinical Infectious Diseases*, *48*(s4), S231–S237. https://doi.org/10.1086/598189
Nadel, S., Newport, M. J., Booy, R., & Levin, M. (1996). Variation in the tumor necrosis factor-α gene promoter region may be associated with death from meningococcal disease. *J.Infect.Dis.*, *174*(4), 878–880.
Nayak, D., Roth, T. L., & Mcgavern, D. B. (2014). Innate immunity to virus infection, *227*(2), 367–402. https://doi.org/10.1146/annurev-immunol-032713-120240.Microglia
Neurodevelopment, A., Infants, P., Sepsis, P., Enterocolitis, N., Abnormalities, W. M., & Imaging, M. R. (2008). Necrotizing Enterocolitis is Mediated by White Matter Abnormalities on.
Nielsen, P. E., Egholm, M., Berg, R. H., & Buchardt, O. (1991). Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science (New York, N.Y.)*, *254*(5037), 1497–1500. https://doi.org/10.1126/science.1962210
O'Donovan, L., Okamoto, I., Arzumanov, A. a, Williams, D. L., Deuss, P., & Gait, M. J. (2014). Parallel Synthesis of Cell-Penetrating Peptide Conjugates of PMO Toward Exon Skipping Enhancement in Duchenne Muscular Dystrophy. *Nucleic Acid Therapeutics*, *0*(0), 1–10. https://doi.org/10.1089/nat.2014.0512
O'garra, A., Chang, R., Go, N., Hastings, R., Haughton, G., & Howard, M. (1992). Ly???1 B (B???1) cells are the main source of B cell???derived interleukin 10. *European Journal of Immunology*, *22*(3), 711–717. https://doi.org/10.1002/eji.1830220314
Oberhoffer, M., Rs, I., Ns, S., Russwurm, S., Ne, E. S., Junker, U. D. O., & Reinhart, K. (1999). Procalcitonin expression in human peripheral blood mononuclear cells and its modulation by lipopolysaccharides and sepsis-related cytokines in vitro. *Journal of Laboratory and Clinical Medicine*, *134*(1), 49–55.
Ottolini, M. C., Lundgren, K., Mirkinson, L. J., Cason, S., & May, B. I. (2003). Utility of complete blood count and blood culture screening to diagnose neonatal sepsis in the asymptomatic at risk newborn, *22*(5), 430–434.
Paoletti, L. C., & Kasper, D. L. (2002). Conjugate vaccines against group B Streptococcus types IV and VII. *The Journal of Infectious Diseases*, *186*(1), 123–126. https://doi.org/10.1086/341073
Paoletti, L. C., & Madoff, L. C. (2002). Vaccines to prevent neonatal GBS infection. *Seminars in Neonatology*, *7*(4), 315–323. https://doi.org/10.1016/S1084-2756(02)90114-4
Paterson, B. M., Robertst, B. E., & Kuff, E. L. (1977). Structural gene identification and mapping by DNA * mRNA hybrid-arrested cell-free translation. *Proceedings of the National Academy of Sciences of the United States of America*, *74*(10), 4370–4374.
Pepys, M. B. (1982). C-reactive protein and the acute phase response. *Immunology Today*, *3*(2), 27–8. https://doi.org/10.1016/0167-5699(82)90052-4
Polin, R. A. (2008). Systemic infection and brain injury in the preterm infant. *Jornal de Pediatria*, *84*(3), 188–91. https://doi.org/doi:10.2223/JPED.1784
Roque, S., Nobrega, C., Appelberg, R., Correia-neves, M., Roque, S., Nobrega, C., … Correia-neves, M. (2017). IL-10 Underlies Distinct Susceptibility of BALB/c and C57BL/6 Mice to Mycobacterium avium Infection and Influences Efficacy of Antibiotic Therapy. https://doi.org/10.4049/jimmunol.178.12.8028
Sabat, R., Grütz, G., Warszawska, K., Kirsch, S., Witte, E., Wolk, K., & Geginat, J. (2010). Biology of interleukin-10. *Cytokine and Growth Factor Reviews*, *21*(5), 331–344. https://doi.org/10.1016/j.cytogfr.2010.09.002
Sadeghi, K., Berger, A., Langgartner, M., Prusa, A., Hayde, M., Herkner, K., … Fo, E. (2007). Immaturity of Infection Control in Preterm and Term Newborns Is Associated with Impaired Toll-Like Receptor Signaling, *195*, 296–302.
Samanta, M., Iwakiri, D., & Takada, K. (2008). Epstein-Barr virus-encoded small RNA induces

IL-10 through RIG-I-mediated IRF-3 signaling. *Oncogene*, *27*(30), 4150–60. https://doi.org/10.1038/onc.2008.75
Sanz, E., Munoz-A., N., Monserrat, J., Van-Den-Rym, A., Escoll, P., Ranz, I., … de-la-Hera, A. (2010). Ordering human CD34+CD10-CD19+ pre/pro-B-cell and CD19- common lymphoid progenitor stages in two pro-B-cell development pathways. *Proceedings of the National Academy of Sciences*, *107*(13), 5925–5930. https://doi.org/10.1073/pnas.0907942107
Saraiva, M., Christensen, J. R., Veldhoen, M., Murphy, T. L., Murphy, K. M., & O'Garra, A. (2009). Interleukin-10 Production by Th1 Cells Requires Interleukin-12-Induced STAT4 Transcription Factor and ERK MAP Kinase Activation by High Antigen Dose. *Immunity*, *31*(2–3), 209–219. https://doi.org/10.1016/j.immuni.2009.05.012
Saraiva, M., & O'Garra, A. (2010). The regulation of IL-10 production by immune cells. *Nature Reviews Immunology*, *10*(MARCH), 170–181. https://doi.org/10.1038/nri2711
Schmutz, N., Henry, E., Jopling, J., & Christensen, R. D. (2008). Expected ranges for blood neutrophil concentrations of neonates : the Manroe and Mouzinho charts revisited, 275–281. https://doi.org/10.1038/sj.jp.7211916
Schrag, S. J., Hadler, J. L., Arnold, K. E., Martell-Cleary, P., Reingold, A., & Schuchat, A. (2006). Risk Factors for Invasive, Early-Onset Escherichia coli Infections in the Era of Widespread Intrapartum Antibiotic Use. *Pediatrics*, *118*(2), 570–576. https://doi.org/10.1542/peds.2005-3083
Schwartz, D. A. (2001). The role of TLR4 in endotoxin responsiveness in humans. *Journal of Endotoxin Research*, *7*(5), 389–393. https://doi.org/10.1179/096805101101532972
Scott, M. J., Hoth, J. J., Turina, M., Woods, D. R., & Cheadle, W. G. (2006). Interleukin-10 suppresses natural killer cell but not natural killer T cell activation during bacterial infection. *Cytokine*, *33*(2), 79–86. https://doi.org/10.1016/j.cyto.2005.12.002
Shah, B. A., & Padbury, J. F. (2014). Neonatal sepsis: an old problem with new insights. *Virulence*, *5*(1), 170–8. https://doi.org/10.4161/viru.26906
Shane, A. L., & Stoll, B. J. (2015). Recent Developments and Current Issues in the Epidemiology , Diagnosis , and Management of Bacterial and Fungal Neonatal Sepsis, *1*(212).
Sindhava, V., Woodman, M. E., Stevenson, B., & Bondada, S. (2010). Interleukin-10 Mediated Autoregulation of Murine B-1 B-Cells and Its Role in Borrelia hermsii Infection, *5*(7). https://doi.org/10.1371/journal.pone.0011445
Sing, A., Reithmeier-Rost, D., Granfors, K., Hill, J., Roggenkamp, A., & Heesemann, J. (2005). A hypervariable N-terminal region of Yersinia LcrV determines Toll-like receptor 2-mediated IL-10 induction and mouse virulence. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(44), 16049–16054. https://doi.org/10.1073/pnas.0504728102
Stafford, I. A., Stewart, R. D., Sheffield, J. S., & Wendel, G. D. (2012). Efficacy of Maternal and Neonatal Chemoprophylaxis for Early-Onset Group B Streptococcal Disease, *120*(1), 123–129. https://doi.org/10.1097/AOG.0b013e3182592451
Stephenson, M. L., & Zamecnik, P. C. (1978). Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. *Proc Natl Acad Sci U S A*, *75*(1), 285–288. https://doi.org/10.1073/pnas.75.1.285
Stoll, B. J., Gordon, T., Korones, S. B., Shankaran, S., Tyson, J. E., Bauer, C. R., … Wright, L. L. (1996). Early-onset sepsis in very low birth weight neonates: A report from the National Institute of Child Health and Human Development Neonatal Research Network. *The Journal of Pediatrics*, *129*(1), 72–80. https://doi.org/10.1016/S0022-3476(96)70192-0
Stoll, B. J., Hansen, N., Fanaroff, A. a, Wright, L. L., Carlo, W. a, Ehrenkranz, R. a, … Poole, W. K. (2002). Late-onset sepsis in very low birth weight neonates: the experience of the NICHD Neonatal Research Network. *Pediatrics*, *110*(2 Pt 1), 285–291. https://doi.org/10.1542/peds.110.2.285
Stoll, B. J., Hansen, N. I., Sánchez, P. J., Faix, R. G., Poindexter, B. B., Van Meurs, K. P., … Higgins, R. D. (2011). Early onset neonatal sepsis: the burden of group B Streptococcal and E. coli disease continues. *Pediatrics*, *127*(5), 817–26. https://doi.org/10.1542/peds.2010-2217
Styers, D., Sheehan, D. J., Hogan, P., & Sahm, D. F. (2006). Laboratory-based surveillance of current antimicrobial resistance patterns and trends among Staphylococcus aureus: 2005 status in the United States. *Annals of Clinical Microbiology and Antimicrobials*, *5*, 2. https://doi.org/10.1186/1476-0711-5-2

Sun, C. M., Deriaud, E., Leclerc, C., & Lo-Man, R. (2005). Upon TLR9 signaling, CD5+ B cells control the IL-12-dependent Th1-priming capacity of neonatal DCs. *Immunity*, *22*(4), 467–477. https://doi.org/10.1016/j.immuni.2005.02.008

Tangye, S. G., Weston, K. M., Raison, R. L., Tangye, S. G., Weston, K. M., & Raison, R. L. (2015). Interleukin-10 Inhibits the In vitro Proliferation of Human Activated Leukemic CD5 B-Cells Interleukin- 10 Inhibits the In vitro Proliferation of Human Activated Leukemic C D S B-Cells, *8194*(November). https://doi.org/10.3109/10428199809057592

Teles, A., Zenclussen, A. C., & Schumacher, A. (2013). Regulatory T Cells are Baby's Best Friends. *American Journal of Reproductive Immunology*, *69*(4), 331–339. https://doi.org/10.1111/aji.12067

Thorkkild, I. A., Sorenson, D. M., Stat., G. G. N. C., & Per Kragh Andersen, L. S. (1988). Genetic and Environmental Influences on Premature Death in Adult Adoptees. *The New England Journal of Medicine*, *318*(12).

Tillett, W. S., & Francis, T. (1930). Serological Reactions in Pneumonia With a Non-Protein Somatic Fraction of Pneumococcus. *The Journal of Experimental Medicine*, *52*(4), 561–71. https://doi.org/10.1084/jem.52.4.561

Trinchieri, G., & Sher, A. (2007). Cooperation of Toll-like receptor signals in innate immune defence, *7*(March), 179–190. https://doi.org/10.1038/nri2038

Tsai, C. H., Chen, Y. Y., Wang, K. G., Chen, C. Y., & Chen, C. P. (2012). Characteristics of early-onset neonatal sepsis caused by Escherichia coli. *Taiwanese Journal of Obstetrics and Gynecology*, *51*(1), 26–30. https://doi.org/10.1016/j.tjog.2012.01.006

Tsuji-Takayama, K., Suzuki, M., Yamamoto, M., Harashima, A., Okochi, A., Otani, T., … Kibata, M. (2008). The production of IL-10 by human regulatory T cells is enhanced by IL-2 through a STAT5-responsive intronic enhancer in the IL-10 locus. *Journal of Immunology (Baltimore, Md. : 1950)*, *181*, 3897–3905. https://doi.org/10.4049/jimmunol.181.6.3897

Van Den Hoogen, A., Gerards, L. J., Verboon-Maciolek, M. A., Fleer, A., & Krediet, T. G. (2009). *Long-term trends in the epidemiology of neonatal sepsis and antibiotic susceptibility of causative agents*. *Neonatology* (Vol. 97). https://doi.org/10.1159/000226604

Van der Poll, T., & Van Deventer, S. J. H. (1999). Cytokines and anticytokines in the pathogenesis of sepsis. *Infectious Disease Clinics of North America*, *13*(2), 413–426. https://doi.org/10.1016/S0891-5520(05)70083-0

Vecchiarelli, A., Retini, C., Monari, C., Tascini, C., Bistoni, F., & Kozel, T. R. (1996). Purified capsular polysaccharide of Cryptococcus neoformans induces interleukin-10 secretion by human monocytes. *Infection and Immunity*, *64*(7), 2846–2849.

Vosters, O., Lombard, C., André, F., Sana, G., Sokal, E. M., & Smets, F. (2010). The interferon-γ and interleukin- 10 responses in neonates differ from adults , and their production remains partial throughout the first 18 months of life, 494–499. https://doi.org/10.1111/j.1365-2249.2010.04267.x

Waterer, G. W., & Wunderink, R. G. (2003). Science review : Genetic variability in the systemic inflammatory response, *7*(4). https://doi.org/10.1186/cc2164

WHO. (2014). Antimicrobial resistance. *Bulletin of the World Health Organization*, *61*(3), 383–94. https://doi.org/10.1007/s13312-014-0374-3

WHO/IVD. (2003). State of the art of new vaccines Initiative for Vaccine Research World Health Organization, (April), 1–74. Retrieved from www.who.int/vaccine_research/.../stateofart_excler.p...

Windsor, W. T., Syto, R., Tsarbopoulos, A., Zhang, R., Durkin, J., Baldwin, S., … Trotta, P. P. (1993). Disulfide bond assignments and secondary structure analysis of human and murine interleukin 10. *Biochemistry*, *32*(34), 8807–8815. https://doi.org/10.1021/bi00085a011

Wynn, J. L., Cvijanovich, N. Z., Allen, G. L., Thomas, N. J., Freishtat, R. J., Anas, N., … Bigham, M. T. (2011). The Influence of Developmental Age on the Early Transcriptomic Response of Children with Septic Shock, *17*(December), 1146–1156. https://doi.org/10.2119/molmed.2011.00169

Wynn, J. L., Scumpia, P. O., Winfield, R. D., Delano, M. J., Kelly-Scumpia, K., Barker, T., … Moldawer, L. L. (2008). Defective innate immunity predisposes murine neonates to poor sepsis outcome but is reversed by TLR agonists. *Blood*, *112*(5), 1750–1758. https://doi.org/10.1182/blood-2008-01-130500

Xie, Y., Kim, K. J., & Kim, K. S. (2004). Current concepts on Escherichia coli K1 translocation

of the blood-brain barrier. *FEMS Immunology and Medical Microbiology*, *42*(3), 271–279. https://doi.org/10.1016/j.femsim.2004.09.001

Yan, S. R., Qing, G., Byers, D. M., Stadnyk, A. W., Al-hertani, W., Bortolussi, R., & Mmun, I. N. I. (2004). Role of MyD88 in Diminished Tumor Necrosis Factor α Production by Newborn Mononuclear Cells in Response to Lipopolysaccharide, *72*(3), 1223–1229. https://doi.org/10.1128/IAI.72.3.1223

Zaghouani, H., Hoeman, C. M., & Adkins, B. (2009). Neonatal immunity : faulty T-helpers and the shortcomings of dendritic cells, (October), 585–591. https://doi.org/10.1016/j.it.2009.09.002

Zaleznik, D. F., Rench, M. A., Hillier, S., Krohn, M. A., Platt, R., Lee, M. T., … Baker, C. J. (1996). Invasive Disease Due to Group B Streptococcus in Pregnant Women and Neonates from Diverse Population Groups, (December), 276–281.

Zaman, K., Roy, E., Arifeen, S. E., Rahman, M., Raqib, R., Wilson, E., … Steinhoff, M. C. (2008). Effectiveness of maternal influenza immunization in mothers and infants. *The New England Journal of Medicine*, *359*(15), 1555–64. https://doi.org/10.1056/NEJMoa0708630

Zeitoun, A. a H., Gad, S. S., Attia, F. M., Abu Maziad, A. S., & Bell, E. F. (2010). Evaluation of neutrophilic CD64, interleukin 10 and procalcitonin as diagnostic markers of early- and late-onset neonatal sepsis. *Scandinavian Journal of Infectious Diseases*, *42*(4), 299–305. https://doi.org/10.3109/00365540903449832

Zhang, X., Deriaud, E., Jiao, X., Braun, D., Leclerc, C., & Lo-man, R. (2007). Type I interferons protect neonates from acute infl ammation through interleukin 10 – producing B cells, *204*(5), 1107–1118. https://doi.org/10.1084/jem.20062013

Zhu, J., Paul, W. E., Zhu, J., & Paul, W. E. (2014). CD4 T cells : fates , functions , and faults ASH 50th anniversary review CD4 T cells : fates , functions , and faults, 1557–1569. https://doi.org/10.1182/blood-2008-05-078154

Supplementary data

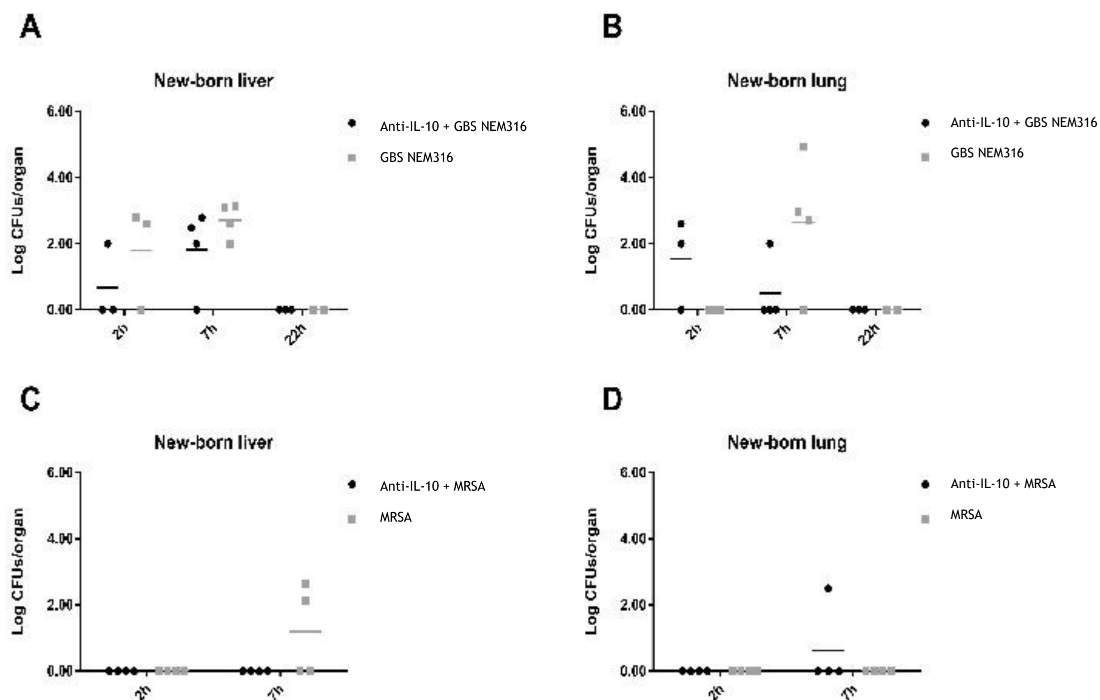


FIGURE 9 IL-10 neutralization reduces bacterial burden in new-born.

(A-D) Number of GBS and S. aureus CFU in liver and lung of neonatal WT (n=18) and anti-IL-10 mAb (n=17) treated animals at different timepoints post infection. New-born C57Bl/6 were treated with 75ug anti-IL-10 mAb or treated with 75ug of isotype control and 12h later were infected with $2x10^5$ GBS NEM316 CFU (A and B results represent data from two independent experiments) or $2,1x10^5$ *S. aureus* CFU (C and D). Results from individual mice are shown. The horizontal line represents the mean for each group.