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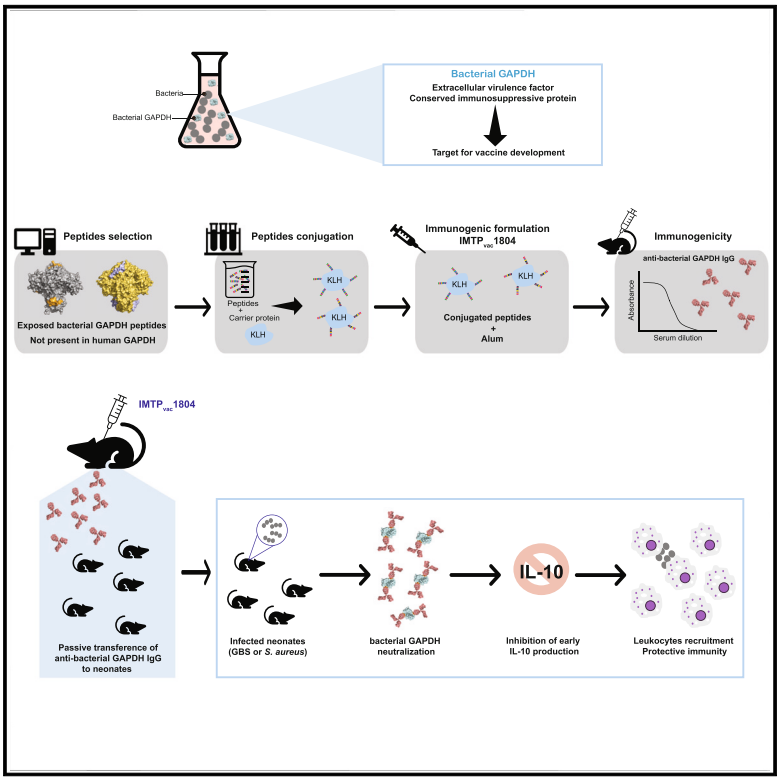
Maternal transfer of anti-GAPDH IgG prevents neonatal infections caused by *Staphylococcus aureus* and group B *Streptococcus*

Graphical abstract



Highlights

- *S. aureus* GAPDH induces IL-10 production in the host early upon infection
- GAPDH-induced IL-10 is directly associated with neonatal susceptibility to infection
- Neutralization of bGAPDH restores protective immunity towards GBS and *S. aureus*
- Proof of concept for a novel vaccine formulation that blocks bGAPDH is described

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In brief

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Maternal transfer of anti-GAPDH IgG prevents neonatal infections caused by *Staphylococcus aureus* and group B *Streptococcus*

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SUMMARY

Group B *Streptococcus* (GBS) and *Staphylococcus aureus* cause 200.000 neonatal deaths every year and no vaccine has been developed yet. Here, we described that extracellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *S. aureus* is an immunomodulatory protein. Antibody mediated neutralization of *S. aureus* extracellular GAPDH promotes a protective inflammatory response by inhibiting an early and abnormal production of IL-10 in infected neonatal mice. As an immunomodulatory role for extracellular GAPDH was already described for GBS, we selected peptides exposed on bacterial GAPDH from both bacteria but completely absent from human GAPDH. These peptides were chemically synthesized and conjugated to a carrier protein. Maternal vaccination with these conjugated peptides induced an increased survival of mouse pups from infection with GBS or *S. aureus*, when compared to controls. The addition of anti-bacterial GAPDH IgG into infected human cord-blood cells caused a significant reduction in bacterial replication, suggesting a putative efficacy for humans.

INTRODUCTION

Bacterial infections in the neonates are a concerning cause of mortality and morbidity. Approximately 30% of these deaths occur during the first day of life and around two-thirds within the first week.[1] Group B *Streptococcus* (GBS) and *Staphylococcus aureus* are two of the most common pathogens identified in neonatal infections.[2–6] The global implementation of intrapartum antibiotic prophylaxis has significantly reduced the incidence of GBS infections in the last decades;[7] nevertheless, a recent report indicates that the global toll for neonatal infections caused by GBS is far higher than previously recognized. Indeed, in 2020, GBS infections were associated with 518,000 preterm births, approximately 100,000 newborn deaths, and at least 46,000 stillbirths, worldwide. It was also estimated that 40,000 infants suffer from neurological deficits following GBS infections.[3]

Analysis of global, regional, and national mortality associated with bacterial pathogens across 204 countries estimated that in 2019 approximately 125,000 babies died in the neonatal period due to *S. aureus* infections.[8] Also, *S. aureus* is the most frequent pathogen isolated from neonatal intensive care units worldwide and the most common cause of late-onset sepsis (LOS) in preterm babies, particularly newborns with low- and very-low birthweight.[6,9–11]

Although antimicrobial resistance has a negative impact on the outcome of neonatal infections, the major obstacle in dealing with these infections is the rapid onset of the disease.[12,13] Both GBS and *S. aureus* can replicate very fast once inside the neonatal host and lead to severe disease within hours of infection, meaning that treatments are often administered too late,[14] with maternal vaccination being considered the best strategy to prevent disease and to significantly reduce the incidence of neonatal infections.[3,5,8]

Neonatal susceptibility to GBS infections is associated with the ability of this bacterium to produce and secrete glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[15] Extracellular bacterial GAPDH (bGAPDH) induces a rapid production of interleukin (IL)-10 that inhibits early events in the inflammatory response and impairs an efficient control of bacterial replication. Accordingly, protection against GBS neonatal infections in mice was achieved by antibody-mediated neutralization of bGAPDH.[15]

Herein, we explore the use of bGAPDH as a target antigen for the prevention of neonatal infections caused by two relevant bacterial pathogens. For that, we developed an immunogenic formulation ($IMTP_{vac}1804$) composed of peptides exposed on

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the surface of bGAPDH from GBS and *S. aureus* that are completely absent from the human homologue. Our formulation showed a robust immunogenic response in mice and, when used for maternal vaccination, it significantly increased survival of the offspring after lethal infection with GBS or *S. aureus*.

RESULTS

Extracellular GAPDH from *S. aureus* promotes susceptibility to infection

Maternal immunization with GBS GAPDH successfully protected the offspring against lethal infection with this bacterium,[15] showing that extracellular GAPDH is strongly associated with virulence. To evaluate if the same is true for *S. aureus*, we started by probing GAPDH in the culture supernatants of a methicillin-resistant strain of *S. aureus* (MRSA) by western blot (WB) analysis. Figure 1A depicts the presence of a 37 kDa band revealed by anti-GAPDH IgG. Mass spectrometry analysis confirmed the presence of GAPDH in culture supernatants of MRSA (the upper band was identified as an immunoglobulin binding protein) (Tables S1 and S2).

To analyze the role of bGAPDH in the neonatal susceptibility to MRSA, female mice were immunized with recombinant GAPDH (rGAPDH) from *S. aureus*. Pups born from GAPDH-immunized dams showed a significantly increase in the survival upon infection when compared with controls (Figure 1B). Passive immunization with anti-bGAPDH IgG also resulted in a significant increase in the survival rate of MRSA-infected pups, when compared with infected pups that received control IgG (Figure 1C). These results indicate that extracellular GAPDH from MRSA is indeed associated with neonatal susceptibility to infection.

As neonatal susceptibility to infection is often associated with the immaturity of the newborn immune system,[16–19] we evaluated the ability of infected pups to control bacterial replication upon anti-bGAPDH IgG administration. Mouse pups received anti-bGAPDH IgG (or control IgG) 24 h before infection with MRSA and liver, lungs, and blood were collected at 6 and 18 h after infection to quantify bacterial colony forming units (CFU). Antibody-mediated neutralization of bGAPDH promoted a faster bacterial clearance in all the organs analyzed (Figure 1D). Interestingly, and despite the immaturity of the newborn immune system, significant differences in organ colonization between controls and anti-bGAPDH IgG treated mice were observed in the liver and lungs even at the earliest time point analyzed (Figure 1D).

Neutralization of *S. aureus* GAPDH restores protective immunity

Having confirmed that extracellular GAPDH promotes susceptibility to MRSA infections by inhibiting the ability of the neonatal host to control bacterial replication, we wanted to assess if this is associated with an early production of IL-10, as it was already described for GBS infections.[15,20] For that, we collected blood, liver and lungs of MRSA-infected pups treated with anti-GAPDH IgG or control IgG, in order to analyze local gene expression [according to Livak et al.[21]] and to quantify serum cytokines.

A decrease in *il10* expression was observed at 6 h post infection in the liver of the anti-bGAPDH IgG treated pups and when compared with controls (Figure 2A). A tendency for a decreased *il10* expression was observed in the lungs of the anti-bGAPDH IgG group, although the results did not reach statistical significance when compared with controls (Figure 2B). Higher expression of *il10* in the organs of control pups is accompanied by higher bacterial counts in the same organs (Figure 1D), indicating that the production of this cytokine is indeed favoring bacterial dissemination.

A significant decrease in serum IL-10 concentration was also observed in the anti-bGAPDH IgG treated group when compared with controls (Figure 2C). Differences found between groups in the serum IL-10 concentration were only observed at 18 h post infection (Figure 2C), which is in accordance with the CFU counts depicted in Figure 1D, where differences in blood colonization are also only observed at this timepoint.

The decreased IL-10 production observed in pups treated with anti-bGAPDH seems to be associated with the ability of these mice to produce inflammatory molecules (Figures 2A–2C). As such, pups receiving anti-bGAPDH IgG have increased expression of *tnfa* at 6 h after infection and *cxcl2* and *icam1* at 18 h post-infection in the liver (Figure 2A) in comparison to controls. An increased expression of *icam1* in the lungs of anti-bGAPDH IgG is also observed at 6 h post-infection (Figure 2B). Increased serum levels of IL-6 and CXCL-1 can already be observed at 6 h post-infection (Figure 2C). Altogether, this reinforces the hypothesis that once bGAPDH is neutralized, mouse pups can trigger a protective inflammatory response toward MRSA.

In accordance with the observed increase in serum chemokines and organ-associated expression of adhesion molecules in mice upon bGAPDH neutralization (Figures 2A–2C), passive immunization with anti-bGAPDH IgG induced a significant increase in the number of inflammatory macrophages ($CD11b^+CD11c^+F4/80^+CXCR2^+Ly6C^+$ cells) in the lungs at 18 h after infection, when compared with the control group (Figures 2D–2F).

Contrary to what was previously described for GBS,[15] neutralization of GAPDH in MRSA infection did not result in significant differences from controls in the total number of neutrophils ($CD45^+CD11b^+F4/80^{neg}Ly6G^+$ cells) in infected organs (Figure S1).

$IMTP_{vac}1804$ is immunogenic and targets only bacterial GAPDH

Extracellular GAPDH was already described as a virulence-associated protein in different human pathogens.[22–26] Nevertheless, its homology with the human GAPDH (huGAPDH) has ruled out its use as a target antigen for human vaccines.[26,27] To overcome this, we developed a peptide-based formulation ($IMTP_{vac}1804$) composed of residues present on GAPDH of GBS and *S. aureus* but completely absent from huGAPDH. Peptide selection was performed using a three-step protocol. First, amino acid sequences of GAPDH from *S. aureus*, GBS and *Homo sapiens* were aligned and compared against each other. This allowed for the selection of peptides 10–20 amino acid long that were absent from the human protein. Second, all those peptides were mapped on the 3D structure models of the bGAPDH to choose those that, theoretically, are highly exposed to solvent, with higher probability of antibody recognition. Two peptides were selected

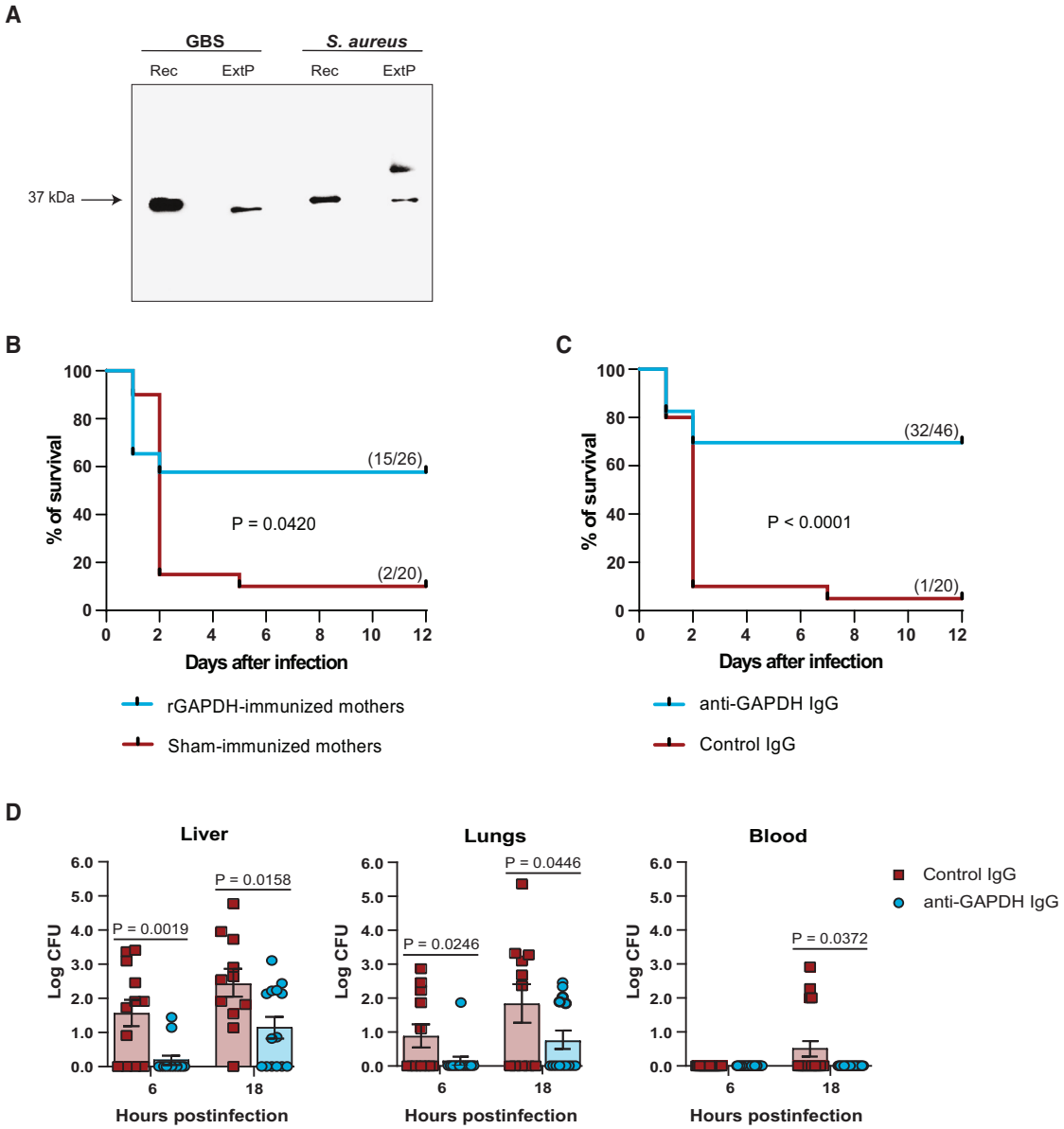

Figure 1. Antibody-mediated neutralization of extracellular bacterial GAPDH confers protection to the offspring against MRSA challenging infection

(A) Extracellular products (ExtP) from MRSA were separated by SDS-PAGE and analyzed by western blot using anti-GAPDH IgG purified from rGAPDH-immunized mice serum. Recombinant (Rec) GAPDH from *S. aureus*, GBS or GBS ExtP were used as positive controls.

(B) C57BL/6 females (6–8 weeks) were immunized sc with 20 μg of *S. aureus* rGAPDH in a saline suspension containing 0.125 mg of Alum as adjuvant, or with the adjuvant alone (sham-immunized). The animals received two administrations before mating, with a three-week interval between administrations. A boost administration was performed at gestational day 14. Neonates were sc infected 48 h after birth with 10^6 CFU of MRSA and survival was assessed for 12 days (C and D) Neonates were sc immunized (24 h after birth) with 150 μg of anti-GAPDH or control IgG and 24 h later, were infected sc with 10^6 CFU of MRSA. (B-C) In parentheses is shown the number of animals that survived versus the number of animals infected. Log rank test was used to determine differences between groups. *p* values are indicated in the graphs. (D) At indicated time points, bacterial load was assessed in the liver, lungs and blood. Results are presented as mean ± SEM. The differences between anti-GAPDH IgG and control IgG were analyzed using T-test (Mann-Whitney) and a $p \leq 0.05$ was considered significant. (B–D) Results show data from at least three independent experiments.

and synthesized: peptide #1 (EVKEGGFEVNGKFIKVSA) targets an exposed region on GBS GAPDH while peptide #2 (DVTVEQ VNEAMKNASNESF) addresses *S. aureus* GAPDH (Figures 3A–3C). Each peptide was individually conjugated with keyhole limpet hemocyanin (KLH). The choice of KLH as a carrier protein was based on its well-reported ability to enhance hapten immunogenicity and to induce strong T cell-mediated humoral responses.[28–30] Aluminum hydroxide (Alum) was used as adjuvant.

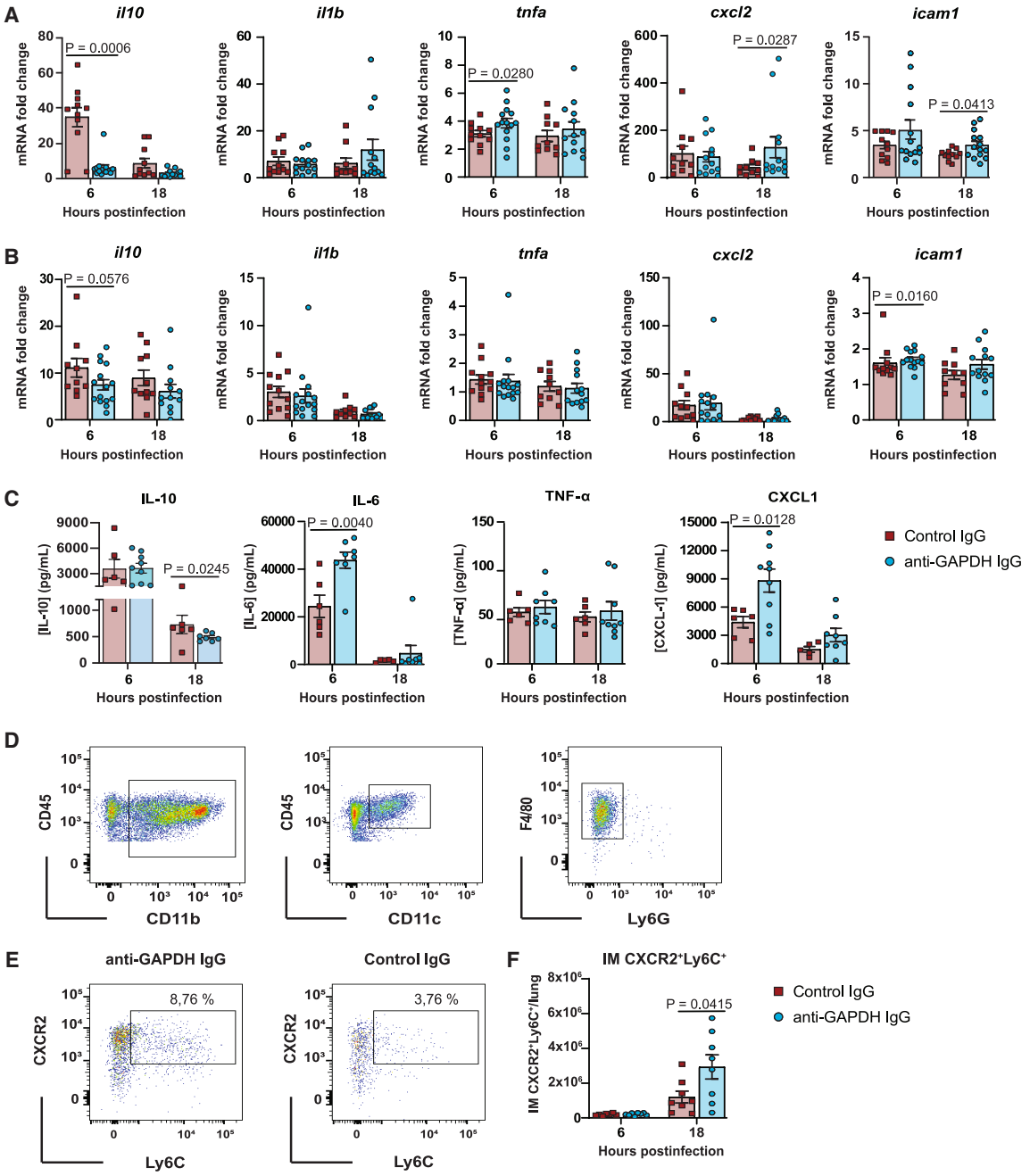

Figure 2. Antibody-mediated neutralization of *S. aureus* GAPDH inhibits early IL-10 production and promotes inflammatory response

C57BL/6 neonatal mice were sc immunized (24 h after birth) with 150 μg of anti-GAPDH IgG or control IgG, and 24 h later were infected sc with 10^6 CFU of MRSA. (A–C) At the indicated time points after infection, liver, lung and blood were collected. (A-B) mRNA expression of the indicated genes in the (A) liver and (B) lung. The fold change in gene expression was obtained through the calculation of $2^{-\Delta\Delta CT}$ and relative to β-actin mRNA. (C) Serum cytokines were quantified by ELISA. (D–F) Lung inflammatory macrophages (IM) were analyzed by flow cytometry. (D) Gating strategy for the analysis of $CD45^+CD11b^+$ cells that correspond to the following phenotype: $CD11c^+F4/80^+Ly6G^{neg}CXCR2^+Ly6C^+$. (E) Representative example of the frequency of activated IM in the lung of infected mice treated with anti-GAPDH IgG or with control IgG, at 18 h post infection. (F) Total numbers of IM are presented as mean ± SEM. The differences between anti-GAPDH IgG and control IgG were analyzed using T-test (Mann-Whitney) and a $p \leq 0.05$ was considered significant.

Finally, $IMTP_{vac}1804$ was tested *in vivo* to assess the immunogenicity against GBS and *S. aureus* GAPDH. Adult female mice received two administrations of $IMTP_{vac}1804$ with a 3-week interval between administrations. As shown in Figure 3D, peptide immunization induced a significant increase in the serum IgG titers toward bGAPDH and, as expected, anti-bGAPDH IgG induced by $IMTP_{vac}1804$ does not show any reactivity toward huGAPDH (Figure 3D). The cross-reactivity toward huGAPDH was analyzed

A

```
GAPDH_Mouse_(P16858)      --MVKVGVNGFGRIGRLVTRAAICSGKVEIVAINDPFIDLNYMVYMFQYDSTHGKFNGTV     58
GAPDH_Human_(P04406)      MGKVKVGVNGFGRIGRLVTRAAFNSGKVDIVAINDPFIDLNYMVYMFQYDSTHGKFHGTV     60
GAPDH_GBS_(Q9ALW2)        -MVVKVGINGFGRIGRLAFRRIQNVEGVEVTRIND-LTDPNMLAHLLKYDTTQGRFDGTV     58
GAPDH_S._aureus_(P0A038)  -MAVKVAINGFGRIGRLAFRRIQEVEGLEVVAVND-LTDDDMLAHLLKYDTMQGRFTGEV     58
                              ***.:*********. *        :::. :** : * : :.::::**: :*:* * *

GAPDH_Mouse_(P16858)      KAENGKLVINGKPITIFQERDPTNIKWGEAGAEYVVESTGVFTTMEKAGAHL-KGGAKRV     117
GAPDH_Human_(P04406)      KAENGKLVINGNPITIFQERDPSKIKWGDAGAEYVVESTGVFTTMEKAGAHL-QGGAKRV     119
GAPDH_GBS_(Q9ALW2)        EVKEGGFEVNGQFVKVSAEREPANIDWATDGVEIVLEATGFFASKEKAEQHIHENGAKKV     118
GAPDH_S._aureus_(P0A038)  EVVDGGFRVNGKEVKSFSEPDASKLPWKDLNIDVVLECTGFYTDKDKAQAHI-EAGAKKV     117
                          :. :* : :**: :.   * : ::: *   . : *:*.**.::  :**  *: : ***:*

GAPDH_Mouse_(P16858)      IISAPSA-DAPMFVMGVNHEKYDNSLKIVSNASCTTNCLAPLAKVIHDNFGIVEGLMTTV     176
GAPDH_Human_(P04406)      IISAPSA-DAPMFVMGVNHEKYDNSLKIISNASCTTNCLAPLAKVIHDNFGIVEGLMTTV     178
GAPDH_GBS_(Q9ALW2)        VITAPGGNDVKTVVFNTNHDILDGTETVISGASCTTNCLAPMAKALQDNFGVKQGLMTTI     178
GAPDH_S._aureus_(P0A038)  LISAPATGDLKTIVFNTNHQELDGSETVVSGASCTTNSLAPVAKVLNDDFGLVEGLMTTI     177
                          :*:**.  *   .*:..**:  *.: .::*.******.***:**.::*:**: :*****:

GAPDH_Mouse_(P16858)      HAITATQKTVDGPSGK-LWRDGRGAAQNIIPASTGAAKAVGKVIPELNGKLTGMAFRVPT     235
GAPDH_Human_(P04406)      HAITATQKTVDGPSGK-LWRDGRGALQNIIPASTGAAKAVGKVIPELNGKLTGMAFRVPT     237
GAPDH_GBS_(Q9ALW2)        HAYTGDQMILDGPHRGGDLRRARAGAANIVPNSTGAAKAIGLVIPELNGKLDGAAQRVPV     238
GAPDH_S._aureus_(P0A038)  HAYTGDQNTQDAPHRKGDKRRARAAAENIIPNSTGAAKAIGKVIPEIDGKLDGGAQRVPV     237
                          ** *. *   *.*      * .*..  **:* *******:* ****::*** * * ***.

GAPDH_Mouse_(P16858)      PNVSVVDLTCRLEKPAKYDDIKKVVKQASEGPLKGILGYTEDQVVSCDFNSNSHSSTFDA     295
GAPDH_Human_(P04406)      ANVSVVDLTCRLEKPAKYDDIKKVVKQASEGPLKGILGYTEHQVVSSDFNSDTHSSTFDA     297
GAPDH_GBS_(Q9ALW2)        PTGSVTELVATLE-KDVT---VEEVNAAMKAAANDSYGYTEDPIVSSDIVGISYGSLFDA     294
GAPDH_S._aureus_(P0A038)  ATGSLTELTVVLEKQDVT---VEQVNEAMKNASNESFGYTEDEIVSSDVVGMTYGSLFDA     294
                            . *:.:*.  **        : *: * :   :    ****. :**.*. . ::.* ***

GAPDH_Mouse_(P16858)      GAGIA---LNDNFVKLISWYDNEYGYSNRVVDLMAYMASKE- 333
GAPDH_Human_(P04406)      GAGIA---LNDHFVKLISWYDNEFGYSNRVVDLMAHMASKE- 335
GAPDH_GBS_(Q9ALW2)        TQTKVQTVDGNQLVKVVSWYDNEMSYTSQLVRTLEYFAKIAK 336
GAPDH_S._aureus_(P0A038)  TQTRVMSVGDRQLVKVAAWYDNEMSYTAQLVRTLAYLAELSK 336
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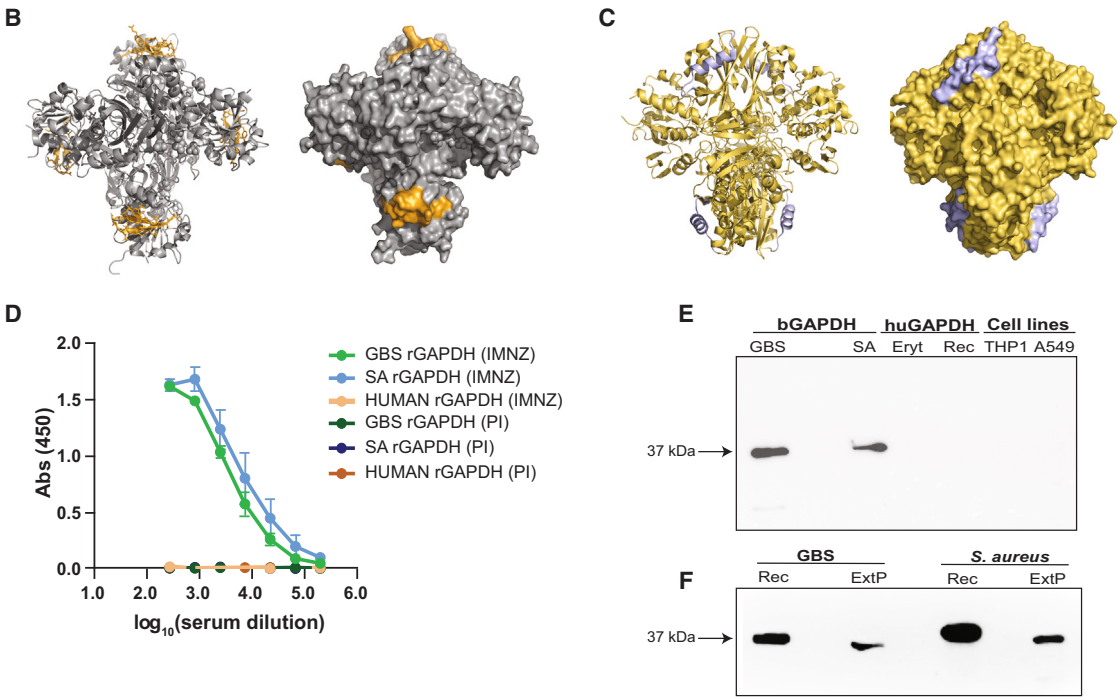

Figure 3. IMTP$_{vac}$1804 peptides are present at the surface of bacterial GAPDH and absent in the human homologue

(A) Alignment of protein sequences of GAPDH from human (Uniprot ID: P04406), GBS (Uniprot ID: Q9ALW2) and *S. aureus* (Uniprot ID: P0A038), obtained using Clustal Omega at EMBL-EBI. The IMTP$_{vac}$1804 peptides are highlighted in bold and underlined. An "*" (asterisk) indicates positions which have a single, fully conserved residue; a ":" (colon) indicates conservation between groups of residues with strongly similar properties, and a "." (period) indicates conservation between groups of residues with weakly similar properties.

(B and C) 3D representation of GBS (PDB: 5Y37) and *S. aureus* (PDB: 3LC2) GAPDH, respectively. Peptide 1 is represented in yellow and peptide 2 in purple.

(D) Reactivity of mouse anti-bGAPDH IgG (purified from IMTP$_{vac}$1804 immunized mice serum) against GBS, *S. aureus* or huGAPDH from pre-immune (PI) and immune sera (IMNZ), collected 1 week after the last immunization. Absorbance was determined at 450 nm. At least, four independent experiments were performed. Results are presented as mean ± SEM.

(legend continued on next page)

by ELISA and WB, demonstrating that no linear or structural epitopes of huGAPDH are being targeted by IMTP$_{vac}$1804 (Figures 3D and 3E). Also, anti-bGAPDH IgG purified from the animals immunized with the IMTP$_{vac}$1804 recognizes bGAPDH in the culture supernatants of GBS and *S. aureus* (Figure 3F).

The similarity and identity of GAPDH across different strains of GBS and *S. aureus* was evaluated (Figure S2), as well as the sequence conservation of IMTP$_{vac}$1804 peptides amongst the different strains for which bGAPDH coding sequence has been verified. The region corresponding to each peptide is highly conserved between all the serotypes of GBS and *S. aureus* (Figures S3A and S3B, respectively), indicating the probability of IMTP$_{vac}$1804 effectiveness against most, if not all, the serotypes of these bacteria.

Maternal vaccination with IMTP$_{vac}$1804 protects mouse pups from *S. aureus* and GBS infections

The development of a single maternal vaccine targeting bGAPDH could represent a major achievement in preventing infections caused by two of the most prevalent pathogens in neonatal infections.

As shown in Figures 4A and 4B, maternal immunization with IMTP$_{vac}$1804 significantly increased the survival of pups upon challenging infection with each of the bacterial pathogens. Like what was observed with rGAPDH immunization (Figures 1B and 1C), significant differences in the survival of pups born from IMTP$_{vac}$1804-immunized females versus controls are already observed in the first 48 h after infection (Figures 4A and 4B). Protection induced by IMTP$_{vac}$1804 immunization is associated with a significant increase in the serum titers of anti-bGAPDH IgG (Figures 4C and 4D). From Figures 4C and 4D it is possible to observe that in the pups born from IMTP$_{vac}$1804-immunized mothers there is an increase in the anti-bGAPDH IgG serum titers from day 1 (before infection) to day 14 (corresponding to 12 days after infection), most probably reflecting gut translocation of antibodies from the mother's milk.

IMTP$_{vac}$1804 immunization does not induce alterations in the gut microbiome

To evaluate any possible effect of IMTP$_{vac}$1804 immunization in the gut microbiome composition, fecal samples were collected at three different time points from IMTP$_{vac}$1804-or sham-immunized female mice: *i)* before immunization, *ii*) 2 days and *iii)* 30 days after the last immunization.

Principal coordinate analysis (PCoA) results did not support the separation of samples from the IMTP$_{vac}$1804-immunized group in collection times "Before Immunization" and "2 days after immunization" ([ANOSIM] R: 0.12239; $p < 0.012$), "Before Immunization" and "30 days after immunization" [ANOSIM] R: 0.30721; $p < 0.001$) and between "2 days after immunization" and "30 days after immunization" ([ANOSIM] R: 0.04561; $p < 0.104$).

Considering that no significant differences were detected at the different time points within groups, all samples within each group were considered for a global PCoA analysis of IMTP$_{vac}$1804-immunized versus sham-immunized mice. Statistical analysis of group distances indicated no significant differences ([ANOSIM] R: 0.01377; $p < 0.091$) between both groups (Figure 5A).

Operational Taxonomic Unit (OTU) distribution at the family level was very similar for all the time points analyzed within each group (IMTP$_{vac}$1804-and Sham-immunized) and no statistical differences were observed between IMTP$_{vac}$1804-and sham-immunized mice (Figures 5B and 5C). A group of mice treated with meropenem was used as positive control. Meropenem is a broad-spectrum carbapenem and currently is the second most commonly used antibiotic in neonatal intensive care units.[31] As expected, meropenem treatment induced a significant disruption of gut microbiome composition (Figure S4), in clear contrast with what was observed in IMTP$_{vac}$1804-immunized mice (Figure 5).

The efficacy of IMTP$_{vac}$1804 in preventing neonatal infections is dependent on being administrated to the mothers and, as such, any chronic or permanent effect on microbiome would be observed only in vaccinated mothers. Nevertheless, the vertical transfer of anti-GAPDH IgG from mothers to the fetus may transiently cause a disruption on the gut microbiome composition of the offspring. In order to analyze any possible effect of GAPDH neutralization on the establishment of gut microbiome in the early life, mice pups received 200 μg of anti-GAPDH IgG 24 h after birth. Controls were left untreated. Feces were collected at day 6 and day 23 after weaning, which corresponds to day 26 and day 49 after birth. No significant differences were found between immunized and non-treated mice (Figure S5), indicating once again that GAPDH neutralization does not interfere with gut microbiome composition. No discernible behavioral alterations or clinical symptoms were observed between sham- or IMTP$_{vac}$1804-immunized dams. No differences were observed between the number of pups per litter between groups as well (Figure S6), which supports the lack of an evident teratogenicity induced by IMTP$_{vac}$1804 immunization.

Anti-bGAPDH IgG from IMTP$_{vac}$1804-immunized animals promote bacterial clearance in human cord-blood

Targeting a common immunosuppressive protein instead of serotype-specific cell-wall-associated molecules, allows IMTP$_{vac}$1804 to protect neonatal mice from infections caused by *S. aureus* and GBS. However, translation of data from the animal model into humans is a critical step in drug development, and vaccine efficacy in the animal model does not always correlate with efficacy in humans.[32]

Here, we used an *ex vivo* model of bacteremia to evaluate the ability of anti-bGAPDH IgG (purified from the serum of goat immunized with IMTP$_{vac}$1804-immunized) to control bacterial growth in human cord-blood. The cord-blood used in this study was collected from healthy babies who have no detectable IgG

(E) Recombinant proteins from GBS, *S. aureus* and human GAPDH, human erythrocytes (Eryt) GAPDH and proteins from human cell lines (THP1 and A549) were separated by SDS-PAGE. Analysis was done by western blot using anti-bGAPDH IgG purified from IMTP$_{vac}$1804-immunized mice.
(F) Western blot analysis of blotted extracellular products (ExtP) of GBS and *S. aureus*. Recombinant (Rec) proteins were used as positive controls. Anti-bGAPDH IgG of pooled sera collected from IMTP$_{vac}$1804-immunized mice were used as developing Ab.

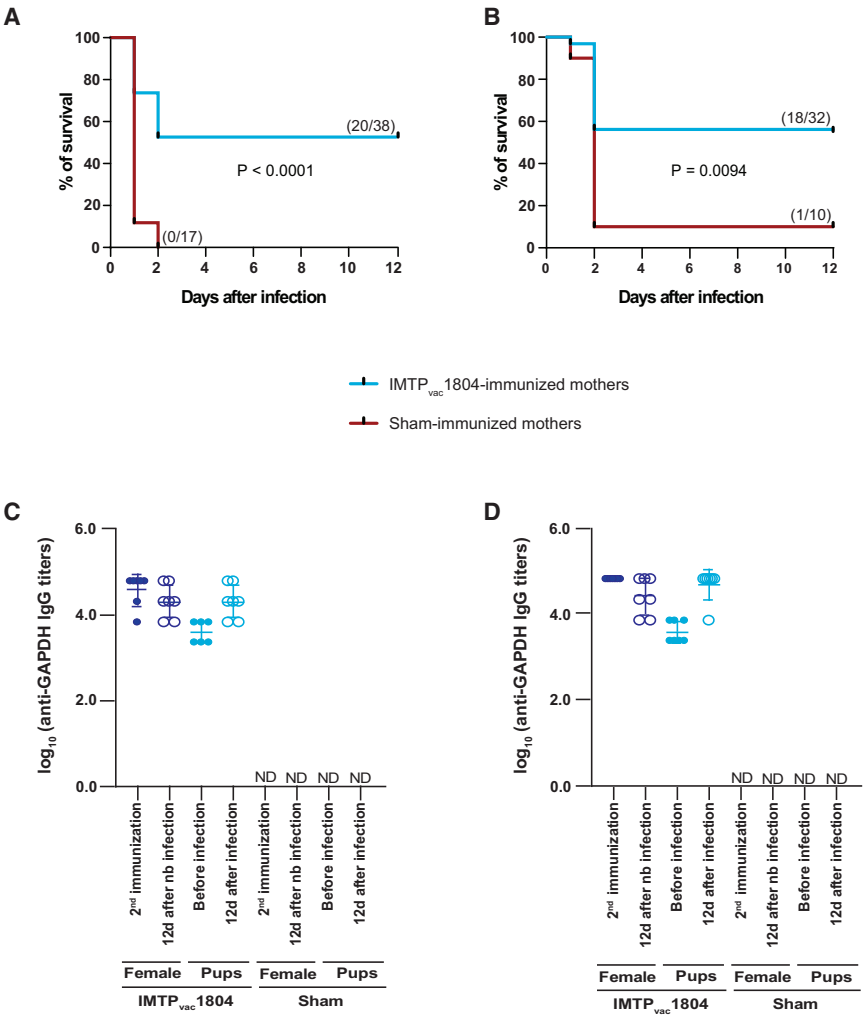

Figure 4. Maternal immunization with IMTP$_{vac}$1804 protects neonatal mice against GBS and *S. aureus* infection

C57BL/6 females (6–8 weeks) were sc immunized with IMTP$_{vac}$1804 or with the adjuvant alone (sham-immunized).

(A and B) The animals received two administrations before mating, with a three-week interval between administrations. A boost immunization was administered at gestational day 14. Neonates were infected sc 48 h after birth with (A) 4×10^5 CFU of GBS, or with (B) 10^6 CFU of MRSA. At least three independent experiments were performed. In parentheses is shown the number of animals that survived vs. the number of animals infected. Log rank test was used to determine differences between groups. *p* values are indicated in the graphs.

(C and D) Serum anti-bGAPDH IgG titers of females and neonates determined by ELISA. Blood was collected from IMTP$_{vac}$1804-or sham-immunized females 1 week after 2nd dose (2nd immunization) and 12 days post infection of the newborns (12 days after nb infection). Blood was collected from the offspring before infection (Before infection) or 12 days after infection of the newborn (12 days after infection). ELISA plates were coated with rGAPDH from (C) GBS or (D) *S. aureus*. IgG titers were calculated as the first value of serum dilution where OD 450 nm $\leq$ 0.1. Titers below 90 were considered non-detectable (ND). Results are presented as mean ± SEM.

titers for bGAPDH (data not shown) and upon mother's informed consent. Cord-blood was incubated with GBS or MRSA for 3 h in the presence of anti-bGAPDH IgG, or anti-KLH IgG as control.

As observed in Figures 6A and 6B, GBS and MRSA survived or increased in CFU/mL when incubated for 3 h in cord-blood in the presence of control IgG. However, the presence of anti-bGAPDH IgG significantly reduced the bacterial numbers in cord-blood infected with GBS (Figure 6A) or MRSA (Figure 6B). This result indicates that, as observed in animals, neutralization of bGAPDH restores the ability of human cord-blood cells to control bacterial replication.

DISCUSSION

In this study, we present the neutralization of extracellular bGAPDH as a strategy for the prevention of neonatal infections caused by GBS and *S. aureus*.

IMTP$_{vac}$1804 targets bGAPDH, an extracellular immunosuppressive protein conserved in these two bacteria. As previously reported for GBS,[15] we describe here that extracellular GAPDH from *S. aureus* is also contributing to an early production of IL-10 in infected neonates. This early production of IL-10 induced by *S. aureus* GAPDH inhibits critical steps of the inflammatory process, namely the expression and production of inflammatory mediators and the activation of inflammatory macrophages in the lungs of infected mice. Although neutralization of bGAPDH has the same overall protective effect in mice infected with MRSA or GBS, we can see distinct patterns regarding cell recruitment kinetics. While in GBS neonatal infections the neutralization of bGAPDH is associated with an increased recruitment of neutrophils into infected organs,[15] in MRSA infections we observe a more marked effect on the activation of lung inflammatory macrophages. This probably suggests intrinsic mechanisms for the effective control of each of the pathogens. Indeed, severity of GBS infections has been associated with an insufficient neutrophil response,[33–37] while activation of lung inflammatory macrophages has been described as a critical step in the control of *S. aureus* infections.[38–40] However, this does not exclude an important role of neutrophils in the overall response to *S. aureus* infection.

The differences in the amount of CFU observed in the organs analyzed does not seem to be solely explained by the differences in the expression of IL-10 in the same organs or at the same time point. This can be explained by the endocrinal role of IL-10, meaning that the site and time of production does not limit the systemic action of this cytokine. Moreover, we cannot rule out the action of

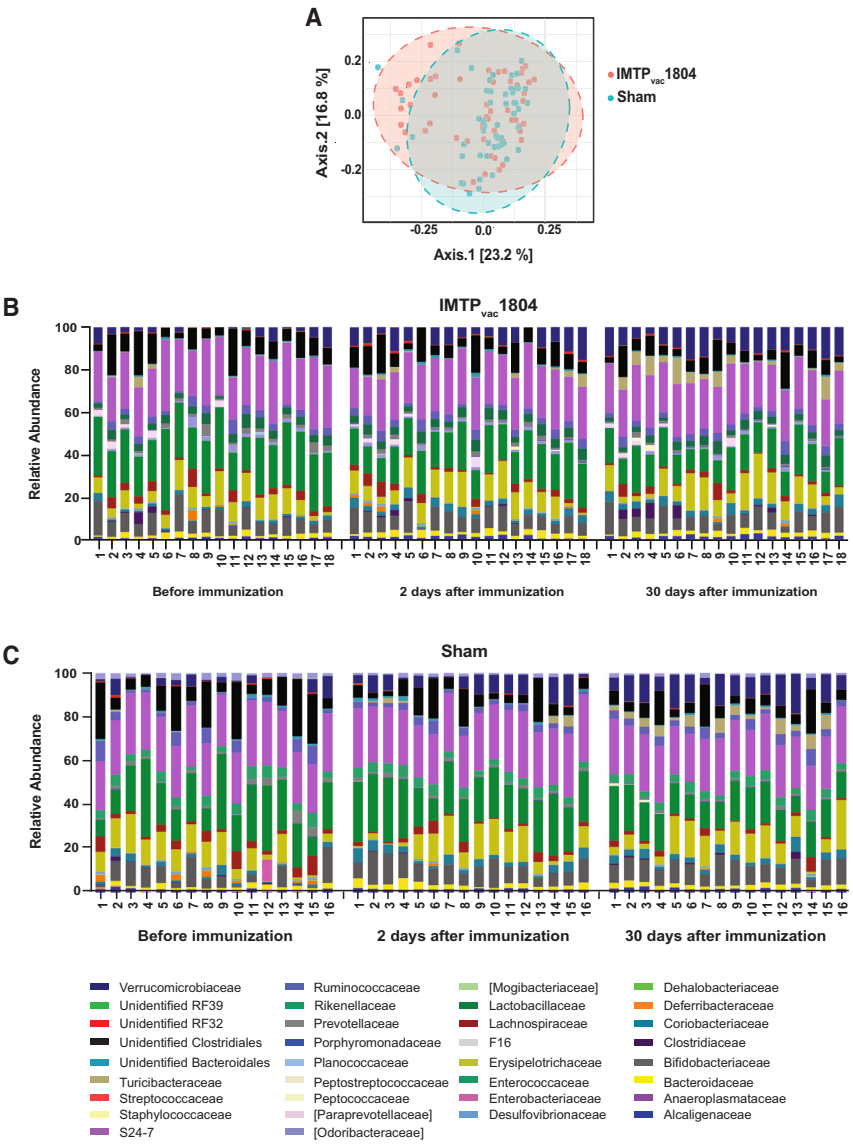

Figure 5. IMTP$_{vac}$1804 does not interfere with host microbiome

C57BL/6 females were immunized two times sc with IMTP$_{vac}$1804 or the adjuvant alone, with a three-week interval between administrations. Feces were collected before immunization, and two and thirty days after the last administration. At least, sixteen animals were used in each condition. (A) PCoA of IMTP$_{vac}$1804 versus sham-immunized females. Each dot represents a sample. Statistical analysis of group distance was performed using the ANOSIM test. Family microbial relative abundances in (B) IMTP$_{vac}$1804 and (C) sham-immunized group.

other anti-inflammatory molecules (such as lipoxins) that may contribute for the GAPDH-mediated immunosuppression.

Redundancy has an important role in biological systems and, as such, our immune system is endowed with redundant cellular and molecular mechanisms acting to protect us from infection. On the other hand, there are immunological checkpoints that are non-redundant and prevent excessive or uncontrolled responses. The production of IL-10 is a non-redundant immunological checkpoint that limits excess of inflammation that could induce permanent tissue damage. Thus, under a physiological response, IL-10 production is crucial to allow the termination of inflammation when the infectious agent is cleared.[41]

Excretion of GAPDH by GBS and *S. aureus* causes the host to produce a premature and abnormal quantity of IL-10. This way, GBS and *S. aureus* can modulate the host's immune system in their favor by limiting inflammation from the very beginning of infection. Interestingly, extracellular bGAPDH seems to address a central and non-redundant immunological checkpoint, making it more difficult for the host to compensate the immunosuppression induced by this virulence mechanism. Susceptibility of neonates to infection is usually regarded as a consequence of an immature immune system.[19] However, once bGAPDH is neutralized, neonatal mice can control infection caused by GBS or *S. aureus*. Also, neonates do have a higher propensity to produce IL-10 as part of a biological necessity to tolerate *neo* antigens.[42–44] The secretion of bGAPDH by two of the most common neonatal pathogens, seems to reflect an exquisite adaptation of these bacteria to the neonatal host.

Several attempts have been made to develop maternal vaccines to prevent neonatal disease caused by GBS.[45] This includes the vaccine candidates from GSK (no longer in pipeline) and Pfizer, targeting the capsular polysaccharides (CPS),[46,47] or the vaccine from MinervaX based on the N domains of the alpha-like proteins of GBS.[48] To the extent of our knowledge, there has not been in clinical development any maternal vaccine to prevent neonatal infections caused by *S. aureus*.

These vaccines have one common feature: they target surface antigens to promote opsonophagocytic killing. Nevertheless, the ability of these bacteria to secrete GAPDH may present a suitable explanation for the reason why it has been so difficult to develop effective vaccines against GBS or *S. aureus*. The data presented herein suggests that without the neutralization of the immunosuppressive activity of bGAPDH, host immunity against these bacterial pathogens will be inhibited even in the presence of anti-CPS IgG.

The demonstration of efficacy of bacterial vaccines in human trials is a difficult task due to the high number of patients that need to be enrolled to demonstrate a statistical relevant protection. This is mainly due to the serotype specificity of traditional vaccine candidates.[45] The need for large clinical trials can be

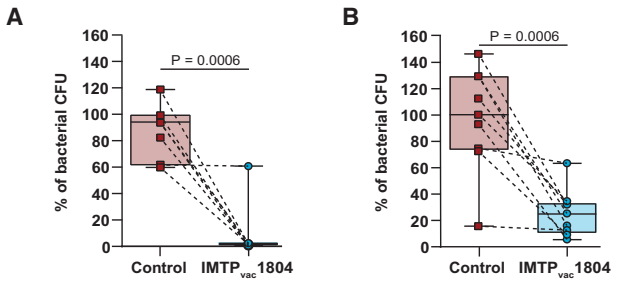

Figure 6. IMTP$_{vac}$1804-elicited anti-bGAPDH IgG can control bacterial replication in human cord blood

Total fresh human cord blood was diluted 1:2 in RPMI medium supplemented with 1% HEPES and 50 μM 2-Mercaptoethanol. Diluted blood was incubated with 200 μg of anti-bGAPDH IgG (purified from goats immunized with IMTP$_{vac}$1804), or with goat anti-KLH IgG and infected for 3 h with 10^7 CFU of (A) GBS or (B) MRSA. Percentage of bacterial CFU in each indicated condition relative to bacterial CFU in conditions with no IgG for GBS and MRSA (100%) are indicated in the graphs. Results are presented as box-plot and at least seven independent experiments were conducted. The dashed line in the box-plot representation corresponds to the same cord blood sample treated with anti-bGAPDH IgG (purified from the sera of goat immunized with IMTP$_{vac}$1804) or anti-KLH IgG (Control). The differences between groups were analyzed using a T-test (Mann-Whitney) and a $p \leq 0.05$ was considered significant.

avoided by targeting an antigen that is conserved in different bacteria and amongst all the serotypes of that bacteria. This will be a clear advantage of using a formulation that targets bacterial GAPDH.

The rationale for targeting bGAPDH in human vaccines is not entirely new. Different authors have performed non-clinical studies testing the efficacy of microbial GAPDH as a vaccine candidate.[22–26] Nevertheless, based on the available information, these attempts were abandoned due to the relative similarity of bacterial and human GAPDH.[26,27] The recognition of protective B- and T cell epitopes in *S. aureus* GAPDH was reported in different studies by Cui and colleagues,[49,50] however, these epitopes are also located in a region of the protein with significant homology with the human protein.

Ferreira and colleagues described punctual and reversible alterations in gut microbiome composition in mice pups born from mothers immunized with whole rGAPDH from GBS.[51] However, no information was provided on how rGAPDH was produced and it was not ruled out a possible contamination with endotoxin that could lead to the production of antibodies against bacterial lipopolysaccharide – and thus toward gut bacteria. This again argues in favor of using a peptide-based vaccine instead of whole bGAPDH.

Using a multi-step approach based on bioinformatic tools and *in vivo* immunogenicity assays, we were able to select peptides exposed on bGAPDH that, when conjugated to a carrier protein, have a strong immunogenicity and do not induce any cross-reactivity toward huGAPDH. Also, IMTP$_{vac}$1804 does not generate any alteration in gut microbiome composition.

The work presented herein opens the possibility for a safe neutralization of bacterial GAPDH. Maternal vaccination endows the offspring of vaccinated mothers to combat these bacterial infections once maternal IgG starts to be transferred, which in humans occurs around the third trimester of gestation. Future work will allow us to establish how soon during gestation babies could be protected from *in utero* ascending bacterial infections.

The efficacy and safety profile of IMTP$_{vac}$1804 shown here are the groundwork for the development of what we consider a new generation of vaccines. For that reason, future experiments must be conducted to optimize our IMTP$_{vac}$1804 formulation. The use of different carrier proteins or the inclusion of other peptides exposed on bGAPDH are an option to consider. The development of a formulation composed of GAPDH peptides from other human pathogenic bacteria will also be evaluated.

If successful, this approach based on host immunoregulation will significantly reduce neonatal mortality and considerably decrease the health care costs associated with these bacterial infections, while contributing to a significant reduction in antibiotic administration worldwide.

Limitations of the study

The main goal of this study is to show that neutralization of bacterial GAPDH is important to prevent neonatal infections caused by two different bacteria. This can be achieved by a single vaccine composed of peptides exposed on bacterial GAPDH of GBS and *S. aureus* and completely absent from human GAPDH. There are however limitations to this study. The mechanism by which human cord blood cells control bacteria replication is not elucidated. Although early IL-10 production was identified as the main mechanism of susceptibility to infection, the major source of this IL-10 production was not identified. Future work should also provide evidence on the use of other carrier proteins that may be more suitable to be used in human clinical trials.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be addressed to the lead contact, Pedro Madureira (pedro.madureira@immunethep.com)

Materials availability

During this work we generated *E. coli* expressing the recombinant form of *S. aureus*, GBS, and Human GAPDH. Further information can be found in the method details section (production and purification of rGAPDHs).

All unique/stable reagents generated in this study are available from the lead contact with a completed materials transfer agreement.

Data and code availability

- The data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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Real-time PCR analysis was performed at the i3S Cell culture and Genotyping platform, with the assistance of Paula Magalhães and Tânia Meireles.

AUTHOR CONTRIBUTIONS

F.L. and M.V. designed and performed all the experiments and analyzed the data presented in Figures 1, 2, 3, 4, 5, and 6. F.L. and M.V. contributed equally to this work. A.F. performed the experiment and analyzed the data presented in Figure 6, and the ELISA results regarding huGAPDH recognition. L.C. and C.N. contributed to performing the animal experiments. J.M., C.S., O.C., and M.D.C.A. are the medical doctors from CHUC responsible for collecting human cord blood samples. J.B.N. was responsible for the analysis of the gut microbiome composition in mice pups. P.C. and C.F. were responsible for recombinant protein production, antibody purification and obtention of bacterial extracellular products. P.C. conducted the protein structure analysis and peptide or protein sequence alignments. C.T. performed and analyzed the animal experiments and executed and analyzed the gene expression profile presented in Figure 2. M.V., F.L., C.T., and P.M. designed and discussed the experiments. P.M. wrote the paper, with contributions from all the authors. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

P.M. is the author of a patent related to this work (PCT/EP2015/063243).

All the rights on $IMTP_{vac}1804$ and on different forms to block GAPDH-mediated virulence mechanism are Immunethep property. F.L., M.V., A.F., L.C., C.N., J.B.N., P.C., C.F., C.T., and P.M. are Immunethep employees. All the other authors declare no financial or non-financial competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Fc-Block (TruStain FcX™, anti-mouse CD16/32)	Biolegend	Clone: 93; Cat #101320; RRID: AB_1574975
CD45-PerCP5/Cy5.5	Biolegend	Clone: I3/2.3; Cat # 147706; RRID: AB_2563538
CD11b-PE	Biolegend	Clone: M1/70; Cat #101208; RRID: AB_312791
Ly6G-AF488	Biolegend	Clone: 1A8; Cat #127626; RRID: AB_2561340
F4/80-AF647	Biolegend	Clone: BM8; Cat #123122; RRID: AB_893480
CD11c-BV421	Biolegend	Clone: N418; Cat #117330; RRID: AB_11219593
Ly6C-PE/Cy7	Biolegend	Clone: HK1.4; Cat #128018; RRID: AB_1732082
CXCR2-APC/Cy7	Biolegend	Clone: SA044G4; Cat #149313; RRID: AB_2734210
Mouse control IgG	InvivoMab	Cat #BE0083
Bacterial and virus strains		
S. aureus	Centro Hospitalar Universitário de Santo António	ST22, methicillin-resistant strain (MRSA)
GBS	Centro Hospitalar Universitário de Santo António	ST23, serotype III
E. coli BL21 Star™ (DE3)	ThermoFisher Scientific	Cat #C601003
Biological samples		
Human cord blood samples	Department of Obstetrics and Neonatology of Maternidade Bissaya Barreto from Coimbra Hospital and University Center (CHUC)	
Chemicals, peptides, and recombinant proteins		
Todd-Hewitt (TH) broth medium	Oxoid	Cat #CM0189B
RPMI	Gibco	Cat #61870010
Luria-Bertani (LB) Miller's broth medium	ThermoFisher Scientific	Cat #H26676
Kanamycin	NZYTech	Cat #MB02001
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	NZYTech	Cat #MB02602
NaCl	VWR	Cat #27800.360
Imidazole	Sigma-Aldrich	Cat #I202
Lysozyme	Sigma-Aldrich	Cat #62971
MgCl2	Merck	Cat #1.05833.0250
DNase I	Sigma Aldrich	Cat #DN25-100MG
PAGEBLUE	ThermoFisher Scientific	Cat #24620
Alum	Croda Pharma	Cat #AJV3012
KLH	Biosyn	Cat #KLH-NP
Peptide #1 (EVKEGGFEVNGKFIKVSA)	Ambiopharm	Cat #Api3454
Peptide #2 (DVTVEQVNEAMKNASNESF)	Ambiopharm	Cat #Api3450
Fetal-bovine serum	Gibco	Cat #10100
Penicillin/streptomycin	Gibco	Cat #15140
RNA later	ThermoFisher Scientific	Cat #AM7020
Turk's solution	Merck	Cat #1.09277.0100
Paraformaldehyde	Sigma-Aldrich	Cat #P6148
SuperScript VILO master mix	ThermoFisher Scientific	Cat #11754050
iQ™ SYBR® Green Supermix	BIO-RAD	Cat #1708884

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Tris	ThermoFisher Scientific	Cat #BP152
Triton X-100	VWR	Cat #M143
EDTA-free protease inhibitors	Roche	Cat #11873580001
HEPES	Sigma Aldrich	Cat #H0887
2-Mercaptoethanol	Gibco	Cat #31350
Critical commercial assays		
IL-10 Mouse Uncoated	R&D	Cat #DY417
IL-6 Mouse Uncoated	R&D	Cat #DY406
TNF-α Mouse Uncoated	R&D	Cat # DY410
CXCL-1 Mouse Uncoated	R&D	Cat #DY453
HEK-blue™ LPS detection KIT	Invivogen	Cat #rep-lps2
RNeasy Plus kit	Qiagen	Cat #74134
PowerMax® Soil DNA Isolation Kit	MoBio® Laboratories Inc	Cat #12988-10
KAPA HiFi HotStart PCR Kit	Roche	Cat #KR0369
SequalPrep 96-well plate kit	ThermoFisher Scientific	Cat #A1051001
Experimental models: Cell lines		
A549	ATCC	Cat #CRM-CCL-185
THP-1	ATCC	Cat #TIB-202
Experimental models: Organisms/strains		
C57BL/6	Charles River Laboratory	
CD1	Charles River Laboratory	
Goat	Eurogentec	
Oligonucleotides		
See Table S3 for oligonucleotide sequence and gene ID information		
Recombinant DNA		
pET28a plasmid	Novagen	Cat #69864-3
SA_GAPDH_pET28a	This work	
GBS_GAPDH_pET28a	This work	
Hum_GAPDH_pET28a	This work	
Software and algorithms		
Beacon Designer	Premier Biosoft	Version 7.9
Prism	GraphPad	Version 9.2.0
FlowJo	FlowJo, LLC	Version 10.8.1
Snapgene	Dotmatics	Version 7.0

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

C57BL/6 and CD-1 males and females six to eight-weeks old were purchased from Charles River Laboratory. The experiments were performed at the animal facilities of i3S – Instituto de Investigação e Inovação em Saúde da Universidade do Porto - and at the Centro de Neurociências e Biologia Celular da Universidade de Coimbra (CNC). Goat immunizations were performed at Eurogentec. All experiments were performed according to the European Convention for the protection of Vertebrate Animals used for Experiment and Other Scientific Purpose (2010/63/EU) and Portuguese Legislation (113/2013), and only after approval by the local ethics committee and Direção Geral da Alimentação e Veterinária (DGAV).

Experiments involving challenging infections were performed at the Animal Biosafety Level 2 (ABSL2) facility (according to the Portuguese Directive 102-A/2020), under negative pressure, 40 air changes/hours, temperature between 20-24 °C, 12 hours light/dark cycle and relative humidity of 55 % ± 10 %. Animals were kept in IVC polycarbonate type IIL cages, under negative pressure. Food (Mucedola Diet) and water (distilled) were autoclaved and provided *ad libitum*.

All procedures were planned to minimize animal suffering, following the institutional standard operating procedures. During the entire experiment, newborns were kept with their mother and nesting material was provided. Infected animals were monitored at least once a day.

The number of animals used in each experiment, as well as the number of independent experiments that were performed are indicated in the figure's legends. The animals were randomly allocated to each treatment group. In active immunization protocols the female was considered the experimental unit, while for passive immunization, the experimental unit was the newborn, since pups from the same litter received different treatments. Researchers were aware of the treatment administered to each animal. The number of animals used in each experiment was determined using the G*Power Software.[52]

Human cord-blood was obtained in the department of Obstetrics and Neonatology of Maternidade Bissaya Barreto from Coimbra Hospital and University Center (CHUC), under informed consent.

A549 and THP-1 human cell lines were obtained from ATCC and no further authentication was conducted. All cell lines tested negative for mycoplasma contamination.

METHOD DETAILS

Bacterial strains and growth conditions

S. aureus and GBS clinical isolates were kindly provided by the Microbiology Department of Centro Hospitalar Universitário de Santo António. For the challenging infections bacteria were grown until log phase in Todd-Hewitt (TH) broth medium (Oxoid, Hampshire, UK) with shaking, at 37°C. For the detection of native GAPDH, bacteria were grown in RPMI (Gibco, Paisley, UK) at 37°C.

S. aureus isolates correspond to a ST22 methicillin-resistant strain (MRSA), while GBS is a serotype III strain (ST23).

Production and purification of rGAPDHs

The expression of *S. aureus*, GBS and human rGAPDH was done in *E. coli* BL21 Star™ (DE3) strain (ThermoFisher Scientific, Carlsbad, California, USA), using pET28a plasmid (Novagen, Temecula, USA), coding for the GAPDH coding sequence (codon optimized for *E. coli* expression) followed by Leu-Glu (*Xho* I scar) and a C-terminal hexahistidine tag (SA_GAPDH_pET28a, GBS_GAPDH_pET28a and Hum_GAPDH_pET28a). Transformed *E. coli* was cultured on Luria-Bertani (LB) Miller's broth (ThermoFisher Scientific) medium, containing 50 μM kanamycin (NZYTech, Lisbon, Portugal) at 37°C with agitation, until OD_{600} reached 0.5. Then, temperature was decreased to 18°C and 45 min later, isopropyl β-D-1-thiogalactopyranoside (IPTG) (NZYTech) as added to a final concentration of 0.1 mM, and the expression left to occur at 18°C, overnight. After that, the pET28 derivative cells were harvested by centrifugation for 20 min at 6 000 x g and resuspended in binding buffer [20 mM phosphate buffer, pH 7.4, 500 mM NaCl (VWR, Radnor, Pennsylvania, US), 15 mM imidazole (Sigma-Aldrich, Saint Louis, Missouri, USA)]. Lysozyme (Sigma Aldrich) was then added prior to freezing at -20°C. The extract was thawed, and $MgCl_2$ (Merck, Darmstadt, Germany) and DNase I (Sigma Aldrich) were added for DNA digestion. The soluble fraction was isolated by ultracentrifugation (50 000 x g), filtered through a 0.22 μm syringe filter and applied to an HisTrap™ HP column (Cytiva, Upsala, Sweden) using an ÄKTA Pure 25 System (Cytiva). An imidazole stepwise elution gradient (50 mM, 150 mM, 300 mM and 500 mM) was used and rGAPDH eluted with 150 mM imidazole. A second purification was performed by size-exclusion chromatography (HiLoad Superdex 200 26/600 pg, Cytiva) using phosphate-buffered saline (PBS) solution as the mobile phase. An SDS-PAGE analysis was performed to confirm rGAPDH purification.

Mass spectrometry analysis

For the mass spectrometry analysis, bacterial culture supernatants were run in a 10% SDS-PAGE and the gel was left overnight to stain in PAGEBLUE (ThermoFisher Scientific). The gel bands of interest were sent for protein sequencing at the i3S Proteomics Scientific Platform.

$IMTP_{vac}1804$ formulation

$IMTP_{vac}1804$ is a conjugated peptide-based vaccine composed of two different peptides. Peptide #1 (EVKEGGFEVNGKFIKVSA) and Peptide #2 (DVTVEQVNEAMKNASNESF) were chemically synthesized and conjugated to clinical grade keyhole limpet hemocyanin (KLH, Biosyn, Carlsbad, California, USA) by Ambiopharm, under good manufacturing practices (GMP)-like conditions. Also, all the remainder components of the formulation were tested for the presence of endotoxins by the HEK-blue™ LPS detection KIT (Invivogen) and the ones tested positive were excluded.

A single $IMTP_{vac}1804$ formulation contains 100 μg of each peptide individually conjugated with KLH (1:1 ratio, w/w) and 125 μg of alum (Alhydrogel 2 %; Croda Pharma, Plainsboro, Nova Jersey, USA) as adjuvant, in 0.9 % NaCl (B.Braun, Melsungen, Germany) in a volume of 300 μL.

IgG purification

CD1 females were immunized intraperitoneally with three administrations of *S. aureus* rGAPDH, with a two-week interval between administrations. After the second administration, approximately 50 μL of blood was collected in heparinized tubes to determine the antibody titers. One week after the last administration, blood was collected by cardiac puncture. The serum from at least ten animals was pooled and stored until IgG purification.

Goat was immunized intramuscularly with four administrations of $IMTP_{vac}1804$ with a three-week interval, according to Eurogentec protocol. Titers were assessed one week after the third administration.

Antibody purification from the serum of GAPDH- or IMTP$_{vac}$1804-immunized animals was performed using ÄKTA Pure 25 System. Samples were applied to a Cyanogen Bromide (CNBr)-sepharose column (Cytiva) with immobilized rGAPDH. The eluted sample was desalted into binding buffer (20 mM phosphate, 300 mM NaCl, pH 7.4), using two tandem HiTrap Phast desalt 5 mL columns (Cytiva). For IgG purification, the sample was passed through an HiTrap Protein G HP 1 mL column (Cytiva) and eluted with elution buffer (100 mM Glycine-HCl, pH 2.7) into collection tubes containing neutralizing buffer (1 M Tris-HCl, pH 9). The buffer exchange to 0.9 % NaCl was done using the two tandem HiTrap Phast desalt columns described above.

Antibody quantification was done using NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific) by measuring absorbance at 280 nm.

Challenging infections

For active immunization studies, C57BL/6 females (6 - 8 weeks) were immunized subcutaneously (sc) with 20 μg of *S. aureus* rGAPDH or IMTP$_{vac}$1804 in a saline suspension containing 0.125 mg of Alum as adjuvant, or with the adjuvant alone (sham-immunized). The animals received two administrations before mating, with a three-week interval between administrations. An additional administration was performed at gestational day 14.

For passive immunization studies, C57BL/6 neonatal mice were sc immunized (24 hours after birth) with 150 μg of anti-GAPDH IgG or control IgG (InvivoMab, isotype control with unknown specificity).

Neonatal mice ($\leq$ 48 hours) were infected, subcutaneously (sc) with MRSA (10^6 CFU) or GBS (4×10^5), in a maximal volume of 40 μL. Survival curves were assessed for 12 days after infection.

For the quantification of organ CFU and the assessment of immunological response, newborns were anesthetized (isoflurane) and sacrificed by decapitation at the indicated time points. The liver, lungs and blood were aseptically collected. The organs used for CFU, cytometry and mRNA analysis were weighed after being divided. For CFU, the organs were homogenized in 500 μL of sterile PBS and plated in TH plates (the detection limit was 100 CFU/mL). For cytometry and mRNA analysis, a portion of each organ was stored in FACS buffer [2 % Fetal-bovine serum (FBS) (Gibco), 1 % penicillin/streptomycin (10 000 units/mL of penicillin and 10 mg/mL of streptomycin) (Gibco) and PBS)] or RNA later (ThermoFisher Scientific), respectively. Approximately 50 μL of blood per animal was collected in heparinized tubes, from which 10 μL were plated in TH plates.

Flow cytometry analysis

Samples were homogenized in FACS buffer and passed through a 40 μm nylon cell strain (VWR) to obtain a single-cell suspension. The total number of leukocytes per sample was determined by optical microscopy using Turk's solution (Merck). After that, samples were incubated for 5 min with Fc-Block (TruStain FcX™, anti-mouse CD16/32, Biolegend, San Diego, California, USA). Cells were then incubated for 25 min with a combination of different antibodies (Biolegend) that included, CD45-PerCP5/Cy5.5 (clone I3/2.3), CD11b-PE (clone M1/70), Ly6G-AF488 (clone 1A8), F4/80-AF647 (clone BM8), CD11c-BV421 (clone N418), Ly6C-PE/Cy7 (clone HK1.4) and CXCR2-APC/Cy7 (clone SA044G4).

Fixation was done with 1 % paraformaldehyde (Sigma-Aldrich) for 15 min. After one washing step, cells were kept overnight at 4°C. In the day after, samples were analyzed in the FACSCanto II (BD Bioscience, San Jose, California, USA) The results were analyzed using the FlowJo software version 10.8.1.

mRNA analysis

Total RNA was isolated from lungs and liver tissues that were stored at -80°C in RNAlater™ using the RNeasy Plus kit from Qiagen (Hilden, Germany). For each sample, the RNA quality was assessed in a Bio-Rad Experion automated electrophoresis station. The RNA was reverse transcribed with the SuperScript VILO master mix (ThermoFisher Scientific) following the manufacturer's instructions. Primer sequences (Table S3) were designed to specifically amplify cDNA using Beacon Designer 7.9 software. Real-time PCR analyses were performed on a BIO-RAD CFX384 Real Time System. PCR reactions were prepared in a final volume of 10 μL using 1 × iQ™ SYBR® Green Supermix (BIO-RAD, Hercules, California, USA), 125 nM of each primer and 1 μL of a 1:10 dilution of cDNA (synthesized from 0.5 - 1 μg of RNA). Thermal cycling conditions comprised an initial step at 95°C for 3 min, and 40 cycles of denaturation (95°C for 10 s), annealing (56°C for 30 s) and extension (72°C for 30 s). To confirm specificity of amplicons, a melt curve was performed at the end of the amplification going from 55°C to 95°C with 10 s increments of 0.5°C. The number of animals in each studied group was $9 \leq n \leq 15$ and samples were run in triplicate. Duplicates with Ct differences $\leq$ 0.5 were validated for analysis. The fold change gene relative expression was obtained through the calculation of $2^{-\Delta\Delta CT}$ based on the Livak method.[20] β-actin was used for data normalization and fold gene expression was determined in relation to the mean value of the non-manipulated (NM) animals' samples.

Serum cytokine quantification

After challenging infection, blood was collected in heparinized tubes and kept at 4°C until processing. The samples were centrifuged at 13 000 rpm, 10′, 4°C. Serum was collected, immediately frozen in liquid nitrogen and stored at -80°C. Cytokine and chemokine quantification was performed by ELISA (R&D, Minneapolis, Minnesota USA) according to the manufacturer's instructions. Sample dilution was optimized prior to analysis.

Gut microbiome analysis

C57BL/6 females were immunized (sc) two times, with a three-week interval, with IMTP$_{vac}$1804 or alum (sham-immunized). Meropenem was administered sc (1 mg/20 g of animal weight) during four consecutive days, as described elsewhere.[53] Fecal samples from adult mice were collected before immunization and two and thirty days after the second dose.

Samples were immediately snap-frozen in liquid nitrogen and stored at -80°C. The analysis was performed at Genoinseq (Biocant Park, Cantanhede, Portugal).

The different samples were kept on ice during DNA extraction. Metagenomic DNA was extracted with the PowerMax® Soil DNA Isolation Kit (MoBio® Laboratories Inc., Carlsbad, California, USA). The purified DNA preparations were quantified by UV-Vis (Nanodrop 2000c spectrophotometer, ThermoFisher Scientific) at 260 nm, concentrated in a centrifugal vacuum concentrator (SpeedVac SPD140 Vaccum Concentrator Kit, ThermoFisher Scientific) and stored at -20°C.

The samples of purified metagenomic DNA were prepared for Illumina Sequencing by 16S rRNA gene amplification of the bacterial community. The DNA was amplified for the hypervariable V3-V4 region with specific primers and further reamplified in a limited-cycle PCR reaction to add sequencing adapters and dual indexes. First PCR reactions were performed for each sample using KAPA HiFi HotStart PCR Kit (Roche, Basel, Switzerland) according to manufacturer suggestions, 0.3 μM of each PCR primer: forward primer Bakt_341F 5′–CCTACGGGNGGCWGCAG-3′ and reverse primer Bakt_805R 5′–GACTACHVGGGTATCTAATCC-3′ and 12.5 ng of template DNA in a total volume of 25 μL. The PCR conditions involved a 3 min denaturation at 95°C, followed by 30 cycles of 98°C for 20 s, 55°C for 30 s and 72°C for 30 s and a final extension at 72°C for 5 min. Second PCR reactions added indexes and sequencing adapters to both ends of the amplified target region according to manufacturer's recommendations. Negative PCR controls were included for all amplification procedures. PCR products were then one-step purified and normalized using SequalPrep 96-well plate kit (ThermoFisher Scientific), pooled and pair-end sequenced in the Illumina MiSeq® sequencer with the V3 chemistry at 2 x 300 bp (Illumina, San Diego, California, USA), according to manufacturer's instructions at Genoinseq (Cantanhede, Portugal).

Sequence data was processed at Genoinseq. Raw reads were extracted from Illumina MiSeq® System in FASTQ format and quality filtered with PRINSEQ version 0.20.4 to remove sequencing adapters, reads with less than 150 bases and trim bases with an average quality lower than Q25 in a window of 5 bases. The forward and reverse reads were merged by overlapping paired-end reads with AdapterRemoval version 2.1.5 using default parameters. The QIIME package version 1.8.0 was used for OTU generation, taxonomic identification, sample diversity and richness indices calculation. Sample IDs were assigned to the merged reads and converted to FASTA format. Chimeric merged reads were detected and removed using UCHIME against Greengenes database version 13.8. OTUs were selected at 97 % similarity threshold using the open reference strategy. Merged reads were pre-filtered by removing sequences with a similarity lower than 60 % against Greengenes database version 13.8 and the remaining merged reads were then clustered at 97 % similarity against the same database. Merged reads that did not cluster in the previous step were *de novo* clustered into OTUs at 97 % similarity. OTUs with less than two reads were removed from the OTU table. A representative sequence of each OTU was then selected for taxonomy assignment.

Beta-diversity was determined by the PCoA, based on the Bray-Curtis dissimilarity after normalization of the non-rarefied feature table with the trimmed mean of M-values (TMM), using MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/). Analysis of similarities (ANOSIM) was used to compare the microbial composition difference between groups.

For the analysis of gut microbiome composition in mice pups, neonates were immunized (sc) 24 hours after birth with 200 μg of anti-GAPDH IgG. Controls were left untreated. Feces were collected 26 and 49 days after birth. Samples were immediately snap-frozen in liquid nitrogen and stored at -80°C. The analysis was conducted by Microbiome Insights Inc. (Vancouver, BC, Canada). DNA was extracted using the PowerSoil DNA extraction kit (MoBio® Laboratories Inc., Carlsbad, California, USA). 16S rRNA gene V4 amplicons generated from mouse fecal samples were sequenced by the Illumina MiSeq. The generated Fastq files were quality-filtered and clustered into 97 % similarity OTUs using the mothur software package (http://mothur.org). High quality reads were classified using the Greengenes database version 13.8 reference taxonomy. OTUs were aggregated for taxonomy assignment.

Human GAPDH detection

A549 and THP-1 human cell lines were grown in DMEM (Gibco) or RPMI, respectively. Medium was supplemented with 10 % FBS, 1 x penicillin/streptomycin, at 37°C and 5 % CO_2. The pellet from each cell line was resuspended in 100 μL of lysis buffer [100 mM Tris (ThermoFisher Scientific), 150 mM NaCl, 1 % Triton X-100 (VWR), pH 7.4] containing complete EDTA-free protease inhibitors (Roche), for 30 min on ice. Samples were centrifuged for 30 min at 17 000 x g, 4°C, and supernatant was collected and stored at -80°C. Final protein concentration was determined by Lowry Protein Assay.

Human cell lines proteins, human erythrocytes GAPDH (Sigma-Aldrich) and recombinant GAPDH were separated by SDS-PAGE and analyzed by western blot using anti-bGAPDH IgG purified from IMTP$_{vac}$1804 immunized mice serum.

Ex-vivo model of bacteremia

Freshly collected cord-blood was diluted 1:2 in RPMI medium supplemented with 1 % HEPES (Sigma-Aldrich) and 50 μM 2-Mercaptoethanol (Gibco). Cells were incubated for 3 hours at 37°C, 5 % CO_2, with 10^7 CFU of GBS or MRSA, in medium containing 200 μg of goat anti-bGAPDH IgG (purified from the sera of mice immunized with IMTP$_{vac}$1804) or goat anti-KLH IgG

(control). At the end of the incubation period, serial dilutions of the cell culture were performed in sterile PBS and plated in TH for CFU analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad version 9.2.0. Survival curves significance was evaluated using the log-rank test. Outliers were determined considering mean ± 2 x SD. After that, normality distribution was assessed considering three tests (D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov). A nonparametric Mann-Whitney T-test was applied to assess differences between treatments. *P* values of less than 0.05 were considered statistically significant.