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RESEARCH PAPER

Streptococcus agalactiae glyceraldehyde-3-phosphate dehydrogenase (GAPDH) elicits multiple cytokines from human cells and has a minor effect on bacterial persistence in the murine female reproductive tract

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ABSTRACT

Streptococcus agalactiae glyceraldehyde 3-phosphate dehydrogenase (GAPDH), encoded by *gapC*, is a glycolytic enzyme that is associated with virulence and immune-mediated protection. However, the role of GAPDH in cellular cytokine responses to *S. agalactiae*, bacterial phagocytosis and colonization of the female reproductive tract, a central host niche, is unknown. We expressed and studied purified recombinant GAPDH (rGAPDH) of *S. agalactiae* in cytokine elicitation assays with human monocyte-derived macrophage, epithelial cell, and polymorphonuclear leukocyte (PMN) co-culture infection models. We also generated a *S. agalactiae* mutant that over-expresses GAPDH (*oe*GAPDH) from *gapC* using a constitutively active promoter, and analyzed the mutant in murine macrophage antibiotic protection assays and in virulence assays *in vivo*, using a colonization model that is based on experimental infection of the reproductive tract in female mice. Human cell co-cultures produced interleukin (IL)-1β, IL-6, macrophage inflammatory protein (MIP)-1, tumor necrosis factor (TNF)-α and IL-10 within 24 h of exposure to rGAPDH. PMNs were required for several of these cytokine responses. However, over-expression of GAPDH in *S. agalactiae* did not significantly affect measures of phagocytic uptake compared to an empty vector control. In contrast, *oe*GAPDH-*S. agalactiae* showed a small but statistically significant attenuation for persistence in the reproductive tract of female mice during the chronic phase of infection (10–28 days post-inoculation), relative to the vector control. We conclude that *S. agalactiae* GAPDH elicits production of multiple cytokines from human cells, and over-expression of GAPDH renders the bacterium more susceptible to host clearance in the female reproductive tract.

One-sentence summary: This study shows *Streptococcus agalactiae* glyceraldehyde 3-phosphate dehydrogenase, an enzyme that functions in glycolysis, gluconeogenesis and virulence, modifies phagocytosis outcomes, including cytokine synthesis, and affects bacterial persistence in the female reproductive tract.

Introduction

Streptococcus agalactiae, also known as Group B streptococcus (GBS), is a gram-positive commensal bacterium that inhabits the human gastrointestinal and genitourinary tracts [1–3]. The organism is part of the genital tract flora in ~25% of healthy women, and is a major cause of morbidity among neonates due to vertical transmission of the bacteria from colonized mothers to their infants. *S. agalactiae* can persist in the genital tract of carriers for months [4] and the condition of colonization can be modeled in mice with some similarities in pathophysiology [5]. *S. agalactiae* also causes acute disease in humans, and is an important

pathogen of animal and marine hosts [5]. The bacterium is associated with diverse disease presentations, including sepsis, meningitis and pneumonia [2], and causes skin and soft tissue infections, including urinary tract infections [6,7]. Despite substantial research aimed at developing a maternal vaccine for *S. agalactiae* to prevent neonatal disease in infants of vaccinated mothers, an effective vaccine has not yet been established [8].

Several surface-expressed *S. agalactiae* proteins promote adhesion to and invasion of human cells, and the binding of these proteins to components of the extracellular matrix can contribute to infection, as reviewed elsewhere [9]. Glyceraldehyde 3-phosphate dehydro

genase (GAPDH), encoded by the *gapC* gene, is a glycolytic enzyme of *S. agalactiae* but also exists in the form of a surface-expressed protein. GAPDH of *S. agalactiae* binds to human extracellular matrix proteins, and this effect has been linked with bacterial invasiveness *in vivo* [10]. In different *Streptococcus* spp., GAPDH is conserved [11,12] and, while it is typically studied as a glycolytic enzyme, it is expressed by numerous bacterial pathogens [13], and can contribute to immunomodulatory effects in addition to virulence [10,14]. Crystal structures of *S. agalactiae* GAPDH [15,16] reveal a unique surface of the protein, which has been proposed as potentially beneficial if targeted in vaccine design [17].

Several observations indicate that interactions between *S. agalactiae* GAPDH and the immune system are important in the context of disease pathogenesis. One study of immunized mice evaluated GAPDH as a vaccine antigen, and reported protective efficacy against systemic infection as a result of immunization [18]; this finding highlights the apparent immunogenicity of *S. agalactiae* GAPDH. However, immune-stimulatory effects of *S. agalactiae* GAPDH toward human cells are unknown. Another study reported inhibition of the production of IL-10 in mice immunized with *S. agalactiae* GAPDH, which appears to be necessary for host protection; this mechanism, mediated by maternal antibodies against *S. agalactiae* GAPDH, appears to confer immunity to offspring by promoting neutrophil recruitment [19]. Another study reported *S. agalactiae* recombinant GAPDH (rGAPDH) induces the production of IL-10 in sera of mice [14]. Thus, *S. agalactiae* GAPDH influences the production of IL-10, a key regulator of immunity [20], in mice. At the cellular level, GAPDH can be released from lysed *S. agalactiae* cells and induce apoptosis in murine macrophages [21]. However, the role of GAPDH in bacterial phagocytosis, and colonization of the female reproductive tract has not previously been reported.

In this study, we examined whether *S. agalactiae* GAPDH can stimulate cytokine production in human cells, and sought to examine its role in macrophage phagocytosis and bacterial colonization of the reproductive tract. We used human monocyte-derived macrophages (MDMs), epithelial cells, and primary polymorphonuclear leukocytes (PMNs) in co-culture models to determine the immune-stimulatory effects of *S. agalactiae* recombinant GAPDH (rGAPDH) toward cytokine profiles [22]. We also generated a mutant of *S. agalactiae* that over-expresses GAPDH (oeGAPDH) *in trans* from *gapC* and analyzed its phagocytosis and colonization of the reproductive tract of female mice.

Materials and methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Supplementary Table 1. *S. agalactiae* was routinely grown using liquid Todd-Hewitt broth (THB) (Thermo Fisher Scientific) cultures aerated by agitation at 200 rpm at 37°C, or on solid medium by supplementation with 1.5% bacteriological agar (Thermo Fisher Scientific). A mutant of *S. agalactiae* 874391 that over-expresses *gapC* was generated by introduction of pGU2753 in the WT strain to produce GU2852, as described below. Enumeration of *S. agalactiae* by colony counting used tryptone soya agar supplemented with 5% defibrinated horse blood (Thermo Fisher Scientific) and selective media, indicated below. *E. coli* DH5α or BL21(DE3)pLysS Rosetta strains were cultured using lysogeny broth (LB). Spectinomycin was used to select for pDL278 and derivatives in *S. agalactiae* and *E. coli* strains, and ampicillin for pET15b in *E. coli* (100 μg/mL] for both). The primers are listed in Supplementary Table 1.

rGAPDH protein expression, purification and generation of antisera

The coding sequence for *S. agalactiae* GAPDH was amplified from genomic DNA of *S. agalactiae* NEM316 and subcloned into pET-15b (Novagen) using *Nde*I and *Xho*I. rGAPDH was expressed in *E. coli* BL21(DE3)pLysS Rosetta (Invitrogen), as previously described [17]. Briefly, frozen bacterial cell pellets were lysed by freeze-thaw cycles in lysis buffer (25 mM HEPES pH 7.35, 0.1 M NaCl, 5 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine hydrochloride), and, after centrifugation (46,000 × *g*, 20 min 4°C), the cleared lysate was passed through a TALON column (Clontech Laboratories). Bound protein was eluted, concentrated and purified using a Superdex 200 HR 26/60 column (GE Healthcare Life Sciences) in 25 mM HEPES pH 7.35. SDS–PAGE was performed to assess any protein degradation, and limulus amebocyte lysate assays (Pierce) were performed after applying the samples to endotoxin removal columns (Sigma-Aldrich) to confirm absence of endotoxin. Aliquots of the protein were frozen at −80°C until use. An antiserum against *S. agalactiae* rGAPDH was generated in rabbits at the University of Alabama Proteomics Facility Core. Rabbits were administrated three doses of the protein (each 30 μg) in 0.5 ml of PBS i.p., with an intervening

period of three weeks between the doses. Sera were collected 30 days after the final dose, and pooled for use in immunofluorescence microscopy.

Culture of human MDMs, epithelial cells and PMNs

U937 monocytes (CRL-1593.2) and 5637 uroepithelial cells (HTB-9) were used similar to previously reported models of human cell co-culture [22–24]. Cell lines, purchased from ATCC, were confirmed as *Mycoplasma*-free regularly using a PCR Kit for *Mycoplasma* Detection (TaKaRa). MDMs were generated from monocytes as described elsewhere [25,26]. Primary human PMNs were purified from healthy donors by density gradient centrifugation using Polymorphprep (Axis-Shield, Rodelokka, Norway). PMNs were washed with ice-cold PBS supplemented with 2% human serum albumin, erythrocytes were removed by washing in lysis buffer, and cells were resuspended in culture media (RPMI 1640 medium with 2% human serum albumin and 10 mM HEPES; Life Technologies). Cells were routinely grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and other supplements, as described elsewhere [27,28]. Approval for this study was granted by the Griffith University Human Ethics Committee (GU Ref No: MSC/18/10/HREC).

Analysis of human cell co-culture cytokine responses to S. agalactiae rGAPDH

We co-cultured MDMs, epithelial cells, and PMNs in 96-well cell-culture plates in total volumes of 250 μl for assays. A dual-cell (DC) co-culture model combined 2×10^6 epithelial cells with 1×10^6 MDMs; a triple-cell (TC) model was used to examine the effect of PMNs in mixed cell populations, and comprised 1.8×10^6 epithelial cells, 6×10^5 MDMs, and 6×10^5 PMNs. These cell numbers were used to achieve a total cell number per well of 3×10^6 cells. Co-cultures were exposed to 5 μg of *S. agalactiae* rGAPDH or HEPES carrier (used for protein preparation) At 2 h, 8 h, and 24 h, supernatants were collected, clarified by centrifugation (500 x *g*, 10 min 4°C) and stored at −80°C. For multiplex assays, n = 6 biological replicates were tested for 27 biomarkers using BioPlex 27-plex kits (BioRad).

Generation of oeGAPDH-S. agalactiae

We constructed a system for over-expressing *gapC* of *S. agalactiae in trans* based on an approach described in a previous study [14]. Briefly, the coding sequence of *gapC* was fused to the promoter region of the kanamycin resistance gene *aphA-3* of *Staphylococcus aureus*,

separated by a *Kpn*I site and flanked by a 5′ *Eco*RI site and 3′ *Bam*HI site. This synthetic DNA assembly was generated in pUC57 (Genscript, USA) and the resultant 1496 bp *Eco*RI-*Bam*HI insert was excised by restriction digestion and subcloned into the *E. coli-Streptococcus* shuttle plasmid pDL278 [29] using T4 DNA Ligase to form pGU2753 (Supplementary Figure S1). The recombinant pGU2753 insert was mapped in its entirety using overlapping sanger sequencing reads (primers listed in Supplementary Table 1).

RNA extraction and qPCR transcriptional assays

Overnight THB cultures (1 mL) containing approximately 500 million bacteria were used for RNA extraction. The RNA was preserved by addition of 0.4 volumes of ice-cold 95% ethanol/5% phenol (vol/vol) and incubated on ice for 30 min. Cells were stored at −80°C until lysis at 37°C for 1 h in 100 μL TE buffer (RNase-free, Sigma-Aldrich) containing 100 U mutanolysin (Sigma-Aldrich) and 30 mg/mL lysozyme (Sigma-Aldrich). RNA was isolated using a Total RNA SV kit (Promega) and successful removal of trace DNA using Turbo DNA-free (Life Technologies) was confirmed by PCR. One thousand ng RNA was reverse-transcribed using Superscript IV according to the manufacturer's instructions (Life Technologies) and cDNA was diluted 1:50 in water prior to use in qPCR. Primers (Supplementary Table S1) were designed using Primer3 Plus [30,31] to quantify transcripts using Universal SYBR Green Supermix (Bio-Rad) using a Quantstudio 6 Flex (Applied Biosystems) system in accordance with MIQE guidelines [32]. Standard curves were generated using five-point serial dilutions of genomic DNA (5-fold) from WT *S. agalactiae* [33] and used to calculate primer efficiencies and relative transcript amounts. Relative expression ratios were calculated using CT values and primer efficiencies as described elsewhere [34]. Relative mRNA transcript amounts or expression ratios were normalized using *dnaN*, encoding DNA polymerase III β-subunit, from three independent biological replicates.

Immunofluorescence microscopy

Surface-expressed GAPDH in *oe*GAPDH-*S. agalactiae* was visualized using rabbit antisera against rGAPDH in immunofluorescence assays. Overnight cultures of bacteria grown in THB with Sp were washed three times in PBS (pH 7.4) (10,000 x *g*, 2 min; room temperature (RT)), then fixed with 4.0% paraformaldehyde (wt/vol) in PBS for 30 min at 37°C, prior to washing again in PBS (3x). The cells were labeled using rabbit anti-rGAPDH antisera as primary (1/500), and goat anti-

rabbit IgG Alexa-Fluor-488 as secondary (1/500; Thermo A11008) antibodies (each 30 min, 37°C). Each antibody labeling step was followed by three washes in PBS (pH 7.4), with final resuspension in 10 μL 0.2% n-propyl gallate (in 1:9 solution of PBS (pH 7.4): glycerol) (n-pg). These were diluted 1:20 in fresh n-pg and 7 μL were mounted under Zeiss High-Performance coverslips (no. $1^1/_2$ 18 mm x 18 mm) using VALAP [35], and stored at 4°C. Images were acquired using a Zeiss AxioImager.M2 microscope (*Carl Zeiss MicroImaging*) fitted with a Plan-Apochromat X63/1.40 objective lens and an AxioCam MRm Rev.3 camera. The Zen 2012 SP2 Imaging Software was used for image acquisition.

Macrophage phagocytosis assays

Phagocytosis of *oe*GAPDH-*S. agalactiae* and a control strain with empty vector was analyzed using J774A.1 macrophages in antibiotic protection assays, essentially as described elsewhere [25], with minor modifications. The antibiotic concentrations used to kill extracellular *S. agalactiae* were 250 U/mL penicillin, 250 U/mL streptomycin and 50 μg/mL gentamicin, which were used in combination [26]. Macrophages (1 x 10^5 cells) were seeded in 96-well tissue culture plates (Nunc, Rochester, New York, USA) and grown for 24 h at 37°C in 5% CO_2. Cultures of bacteria were grown overnight in THB Sp, washed three times in PBS (10,000 x *g*, 10 min), and resuspended in cRPMI without antibiotics. The target multiplicity of infection (MOI) was 50 bacteria per cell; this was confirmed in each assay by retrospective colony counts, noting equivalent CFU counts between strains required half-dilution of the *oe*GAPDH-*S. agalactiae* strain prior to inoculation of monolayers. To measure adhesion, monolayers were infected in antibiotic-free media for 1 h, then washed (5 times with PBS), and lysed by adding 50 μl of 0.1% Triton-X 100 and incubating for 10 min. Cell lysates were diluted in PBS to a volume of 200 μl and used for colony counts. For measuring uptake, infected monolayers were washed as above and, instead of adding Triton-X to lyse the cells, culture media with antibiotics were added to kill extracellular bacteria. The monolayers were then incubated at 37°C in 5% CO_2 for 2 h and washed and lysed as above to measure uptake, or incubated for 24–48 h and washed and lysed (for colony counts) to measure intracellular survival. The assays were performed with at least quadruplicate replicates, and were repeated six independent times. Data are shown as mean±SEM for independent experiments.

Mouse model of S. agalactiae colonization of the female reproductive tract

All protocols used for animal experiments were approved by the Griffith University Animal Ethics Committee (approval: MSC/03/12/AEC). Female 6-8-week-old C57BL/6 J mice were purchased from Animal Resource Center, Western Australia. The vaginal colonization protocols used in this study are reported elsewhere [36,37]. Mice received a single subcutaneous injection of 0.1 mg 17β-estradiol in castor oil at 24 h prior to bacterial inoculation to establish all mice in the estrus stage of the estrous cycle for inoculation (Day 0). Mice were inoculated with ~ 1 × 10^8 CFU of *S. agalactiae* (either *oe*GAPDH-*S. agalactiae* or control strain with empty vector) in 10 μL of PBS. At intervals following inoculation, the vaginal vault was sampled using cervico-vaginal swabs (Copan, Murrieta, CA). Cell suspensions prepared from the swabs were cultured on several types of media: 5% horse-blood tryptic soy agar (TSA, Oxoid, Adelaide, SA, Australia) for counts of total viable bacteria; 5% horse-blood Columbia agar (ColNAC; Oxoid) supplemented with 15 μg/mL nalidixic acid and 10 μg/mL colistin (Sigma-Aldrich, Castle Hill, NSW, Australia) for counts of gram-positive bacteria; for selection of *S. agalactiae*, we used CHROMID® Strepto B (STRB, bioMerieux, Marcy l'Etoile, France), as well as THA supplemented with 100 μg/mL spectinomycin to enumerate plasmid-carrying CFU. Cervicovaginal smears on glass slides stained with Diff-Quick were used to analyze the inflammatory infiltrate, as previously described [36,37]. Groups were 10–15 mice per treatment in each experiment, and experiments were repeated three independent times.

Statistical analysis

Cytokine data are presented as individual points for each biological replicate with means±SEM. Welch's Independent t test was used to compare mean cytokine levels (pg/mL) between groups in a single time point for data that were normally distributed. Data that were not normally distributed were compared using a Mann–Whitney U test. For multiple comparisons encompassing multiple time points, Kruskal-Wallis ANOVA was used followed by Dunn's post-hoc tests. Phagocytosis data were compared using independent samples t-test. For data derived from mouse experiments, colonization between the different treatment groups was compared using repeated measures two-way ANOVA of CFU data with a multiple comparisons posttest for individual time-points. We also analyzed these data using area under the curve (AUC) analysis, followed by a Mann-Whitney U-test. All statistical analyses were carried out using SPSS software (v26.0), and

GraphPad Prism software package 8.0. Statistical significance was accepted as $p < 0.05$.

Results

Cytokine responses of mixed human cell co-cultures to rGAPDH

Exposure of Triple Cell (TC) co-cultures of human epithelial cells, MDMs and PMNs to rGAPDH of *S. agalactiae* resulted in significantly increased production of several pro-inflammatory cytokines (Figure 1). The strongest responses were for IL-1β, IL-6, MIP-1α, MIP-1β and TNF-α, which were secreted in higher amounts in TC co-cultures exposed to rGAPDH compared to controls at one time point or more over the time course. Most responses were not apparent at 2 h but progressed to statistically significant at 8 h-24 h. Responses for other cytokines (*e.g.*, IL-12p70) were similar in rGAPDH and control groups, or the differences were relatively small (*e.g.*, IFN-γ) (Figure S2, and data not shown). PMNs contribute to immune responses of mice to rGAPDH [19], and so we analyzed the human cell co-culture responses according to the presence of PMNs. Comparing Dual Cell (DC) and TC co-culture models showed that the presence of PMNs contributed to the production of several pro-inflammatory cytokines (*e.g.*, IL-1β and IL-6) (Figure 1, compare 24 h rGAPDH groups in DC and TC co-cultures). Thus, *S. agalactiae* rGAPDH elicits several pro-inflammatory cytokines in human mixed cell co-cultures, and the presence of PMNs selectively amplifies some of these responses. The presence of PMNs in TC co-cultures exposed to rGAPDH did not alter the production of all pro-inflammatory cytokines, and the production of some cytokines, including MIP-1α, MIP-1β and TNF-α was lower in the rGAPDH-treated TC compared to DC group (Figure 1, compare DC and TC co-cultures, 2 h-8 h for TNF, 24 h for MIP-1α, MIP-1β).

In addition to pro-inflammatory cytokines, rGAPDH elicited the production of several regulatory and chemotactic cytokines in human mixed cell co-cultures (Figure 2). Among the responses detected in the DC model, only one, RANTES at 24 h was statistically significant (comparing rGAPDH to control). Comparing the rGAPDH and control groups in the TC model showed that the presence of PMNs led to significant production of IL-10 (minor but statistically significant increases at 2 h-8 h), IL-8 (8 h), G-CSF and GM-CSF (8 h-24 h) (Figure 2). The production of some regulatory and chemotactic cytokines was amplified by the presence of PMNs (*e.g.*, GM-CSF, G-CSF 24 h

comparing rGAPDH groups in DC and TC co-cultures) but the production of others, including IL-10 and RANTES was lower at 24 h in the rGAPDH-treated TC group compared to the DC group (Figure 2). Additionally, rGAPDH elicited minor but statistically significant increases in production of IL-1Ra and Eotaxin, as well as growth factors, such as platelet-derived growth factor (PDGF-bb) (Figure S2). For PDGF-bb, the presence of PMNs in combination with rGAPDH caused a reduction in the level of growth factor present in the controll mixed cell co-culture (Figure S2).

### Analysis of oeGAPDH-S. agalactiae and phagocytosis assays

We generated a system for over-expression of *gapC* in *S. agalactiae* 874391 by introducing pGU2753 to generate *oe*GAPDH-*S. agalactiae* (GU2852). We confirmed significantly elevated expression of the gene encoding GAPDH, *gapC*, using qRTPCR to show 4.0 ± 0.7-fold higher expression in the mutant vs WT (n = 3 replicates; $p < 0.05$). More abundant expression of GAPDH in the overexpression mutant vs the control (carrying empty vector) was detected using immunofluorescence microscopy with anti-GAPDH antisera (Figure S3) as well as using flow cytometry (Figure S4). Monolayers of murine macrophages were challenged with either *oe*GAPDH-*S. agalactiae* or WT *S. agalactiae* containing the empty vector backbone (pDL278) as a control strain (GU2672). Antibiotic protection assays were used to measure bacterial adhesion, uptake and intracellular survival of the strains. The numbers of bacteria recovered from macrophages did not differ between the *oe*GAPDH and control strain for adhesion, uptake or intracellular survival (Figure 3). Bacterial suspensions in these assays were plated in duplicate on nonselective and selective media containing spectinomycin to confirm approximately equivalent retention of plasmids in the two *S. agalactiae* strains. Thus, the over-expression of GAPDH in *S. agalactiae* 874391 does not perturb bacterial interactions with murine macrophages, as determined by quantitative culture techniques.

Female genital tract colonization

Inoculation of mice with either *oe*GAPDH-*S. agalactiae* or the control strain resulted in equivalent levels of colonization during the first 7 days post-inoculation, in terms of both bacterial loads and the proportions of mice in the different groups that exhibited culture-positive status for *S. agalactiae* and were consequently classified as colonized (Figure 4(a,b)). The proportions
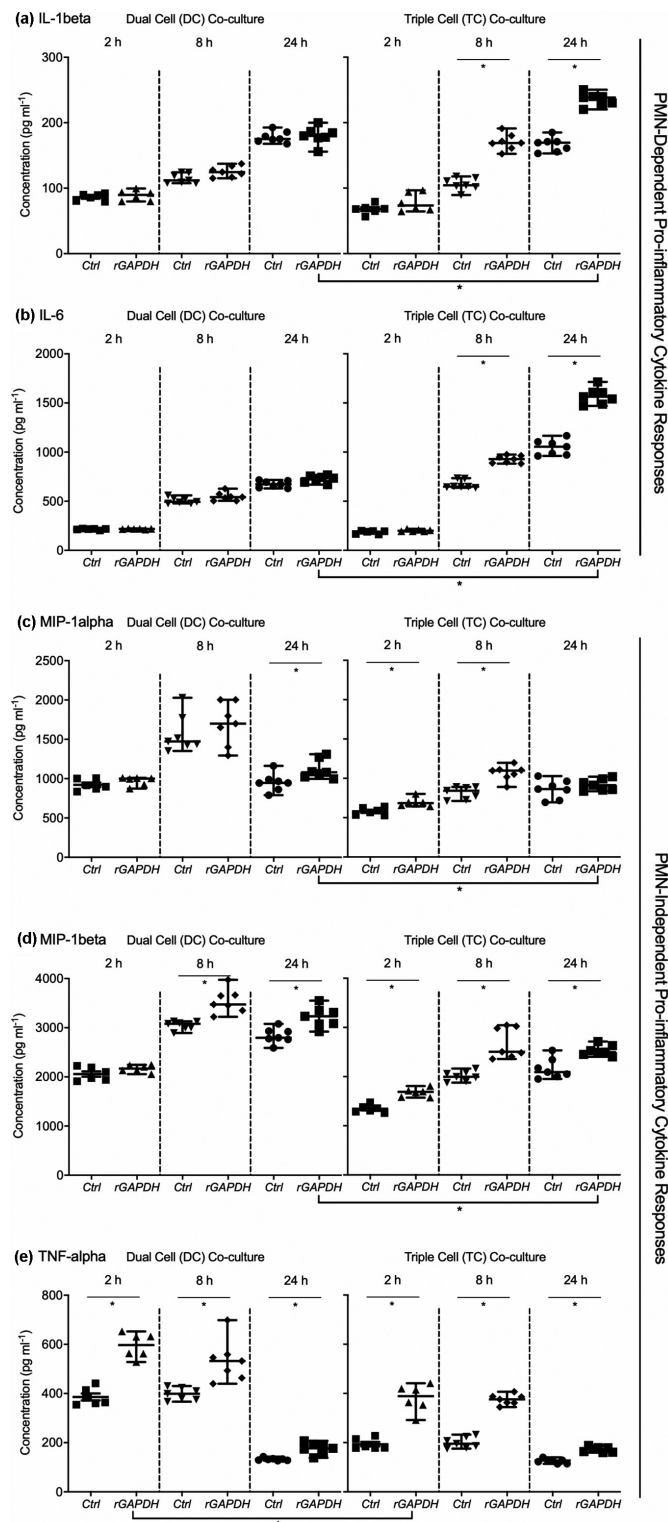
Figure 1. Pro-inflammatory cytokine responses of human cell co-cultures exposed to purified *S. agalactiae* rGAPDH. Dual-cell (DC) co-cultures comprised epithelial cells and MDMs; Triple-cell (TC) co-cultures also included PMNs. Cultures were exposed to 5 μg rGAPDH or carrier control (Ctrl) for 2 h, 8 h, or 24 h. Significant responses are indicated by asterisk notations from pairwise comparisons of treated and Ctrl groups at each time point. Responses for IL-1beta (a) and IL-6 (b) illustrate response patterns that were significantly affected by the presence of PMNs: *i.e.*, increased cytokine production in TC but not DC co-cultures. In contrast, significant responses for multiple other cytokines, including MIP-1alpha (c), MIP-1beta (d) and TNF (e) occurred independently of PMNs in DC co-cultures (responses that were at least equivalent to, or more significant compared to TC co-cultures). The presence of PMNs restricted some responses observed in DC co-cultures (*e.g.*, compare 24 h rGAPDH groups in DC and TC co-cultures). *$p < 0.05$.
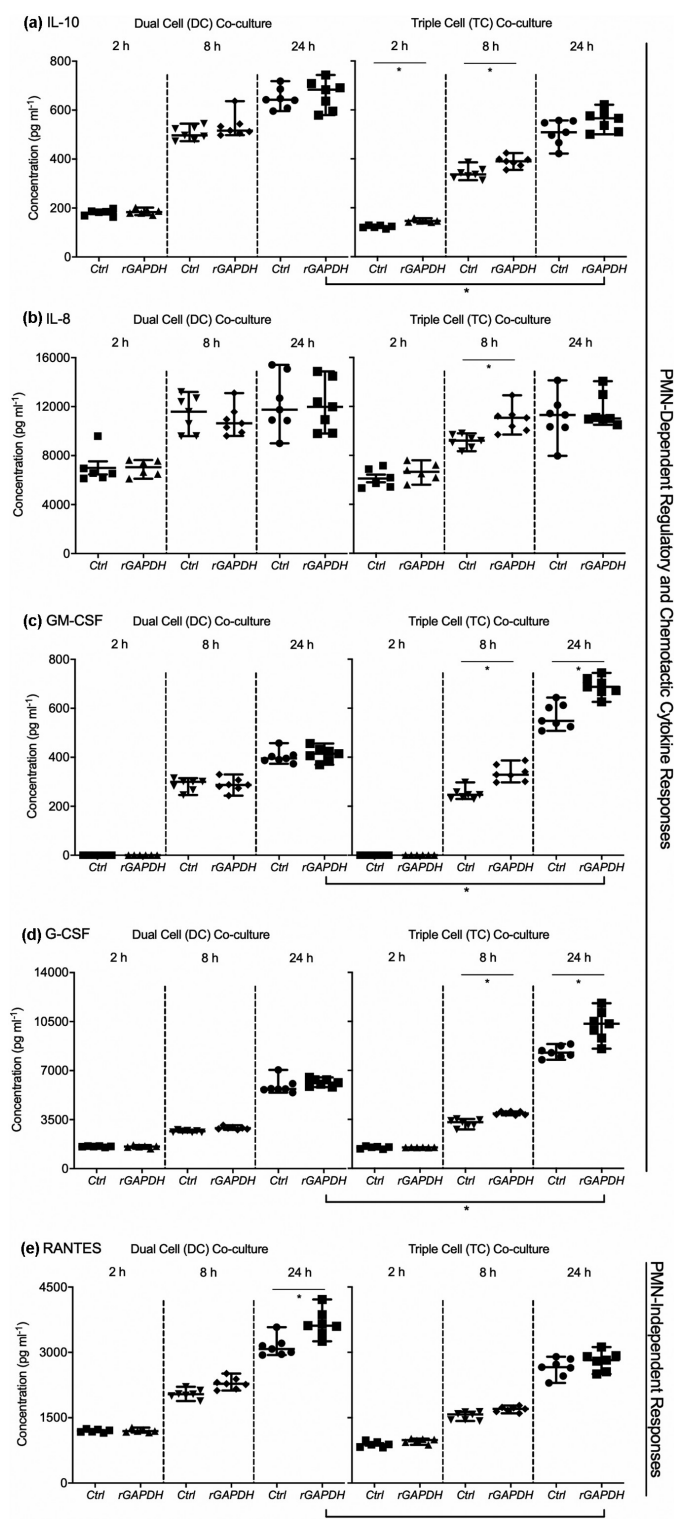
Figure 2. Regulatory and chemotactic cytokine responses of human cell co-cultures exposed to purified *S. agalactiae* rGAPDH. Dual-cell (DC) co-cultures and Triple-cell (TC) co-cultures were exposed to 5 µg rGAPDH or control (Ctrl) for 2 h, 8 h, or 24 h. The presence of PMNs significantly increased cytokine production in TC co-cultures for IL-10 (a), IL-6 (b), GM-CSF (c) and G-CSF (d). In contrast, a significant response for RANTES observed in DC co-cultures (e) was inhibited by the presence of PMNs (*i.e.*, compare 24 h rGAPDH group in DC and TC co-cultures). *$p < 0.05$.
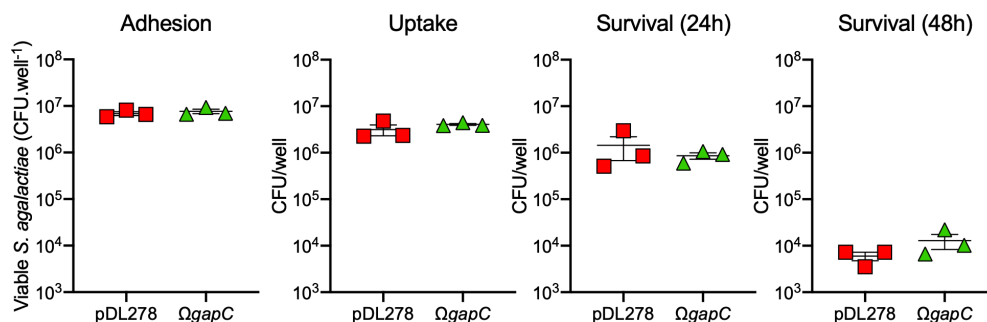
Figure 3. The effect of GAPDH over-expression in *S. agalactiae* (Ω*gapC*)on macrophage phagocytosis compared to control (pDL278 empty vector). Shown are measures of adhesion, uptake, and intracellular survival at 24 h and 48 h based on antibiotic protection assays in murine J774A.1 macrophages *in vitro* using a MOI of 50 bacteria per macrophage. Equivalent numbers of bacteria were recovered for both bacterial strains in each assay. Bars represent mean±SEM from three independent experiments.

of mice in each group that remained culture-positive for the entire duration of the experiment were also similar; beginning at day 10 post-inoculation, *S. agalactiae* was undetectable in 5 out of 40 mice (12.5%) for both groups; these proportions increased to 26/40 (65%) and 28/39 (72.5%) at day 28 for control and *oe*GAPDH-*S. agalactiae* groups, respectively. In other words, ~30-35% of mice remained colonized at day 28. An overall analysis for all culture-positive and culture-negative mice using a repeated measures two-way ANOVA of CFU data with a multiple comparisons posttest for individual time-points showed no significant difference between *oe*GAPDH-*S. agalactiae* compared to empty vector control strain based on THA-Spec or CHROMID (Figure 4(a,b)). At day 28, analyzing all culture-positive and culture-negative mice showed an average of 97% fewer *oe*GAPDH-*S. agalactiae* compared to the empty vector control strain based on mean values derived from THA-Spec (n = 39–40; means: $5.9 \pm 3.4 \times 10^4$ vs $2.5 \pm 1.7 \times 10^6$; medians: below LOD both, upper limits of 2.6×10^2 and 6.4×10^3, respectively; Figure 4a). Analysis of the subpopulation of mice that exhibited persistent colonization (including at day 28) using AUC analysis was used to gain insight into the cumulative CFU estimates between day 1 and day 28 for persistently culture-positive mice and demonstrated these mice had statistically significantly fewer *oe*GAPDH-*S. agalactiae* compared to the empty vector control ($p = 0.021$). The numbers of resident genital tract commensal flora in mice between the groups were similar overall, according to CFU estimates on TSA 5% horse blood for total bacteria (Figure 4c), and ColNAC (gram-positive bacteria; Figure 4d). Finally, the presence of either pGU2753 or pDL278 did not significantly affect the total numbers of resident genital tract commensal flora (Figure 4(c,d)). Cytological assessment of cervico-

vaginal cellular inflammatory infiltrates at days 10–17 in these mice showed no clear differences between the neutrophil or lymphocyte infiltrates elicited in response to oeGAPDH *S. agalactiae* versus the empty vector control strain (Figure S5).

In competitive mixed-infectionexperiments that used a 1:1 mixture of *oe*GAPDH-*S. agalactiae* and the WT, we noted a clear disadvantage of the *oe*GAPDH-*S. agalactiae* strain for chronic colonization compared to the WT (Figure S6). Collectively, these results establish that over-expression of GAPDH in *oe*GAPDH-*S. agalactiae* causes a small, but statistically significant attenuation for long-term persistence in the reproductive tract of female mice.

Discussion

The main findings of this study are (i) *S. agalactiae* rGAPDH triggers production of several pro-inflammatory, regulatory and chemotactic cytokines in human cells, (ii) increased expression of GAPDH-encoding *gapC* in *S. agalactiae* does not lead to significant changes in phagocytosis of the bacteria by murine macrophages, and (iii) *S. agalactiae* colonization of the female reproductive tract in mice is affected by over-expression of GAPDH, whereby increased expression results in a minor attenuation of bacteria for long-term persistence in the female reproductive tract in mice. Collectively, these findings provide new insight into the biology of GAPDH during host-pathogen interactions and how this glycolytic enzyme of the bacterium effects its virulence.

The elucidation of a diversity of pro-inflammatory, regulatory and chemotactic cytokines that *S. agalactiae* GAPDH elicits in human cells is useful to help understand the immunomodulatory role that this factor may
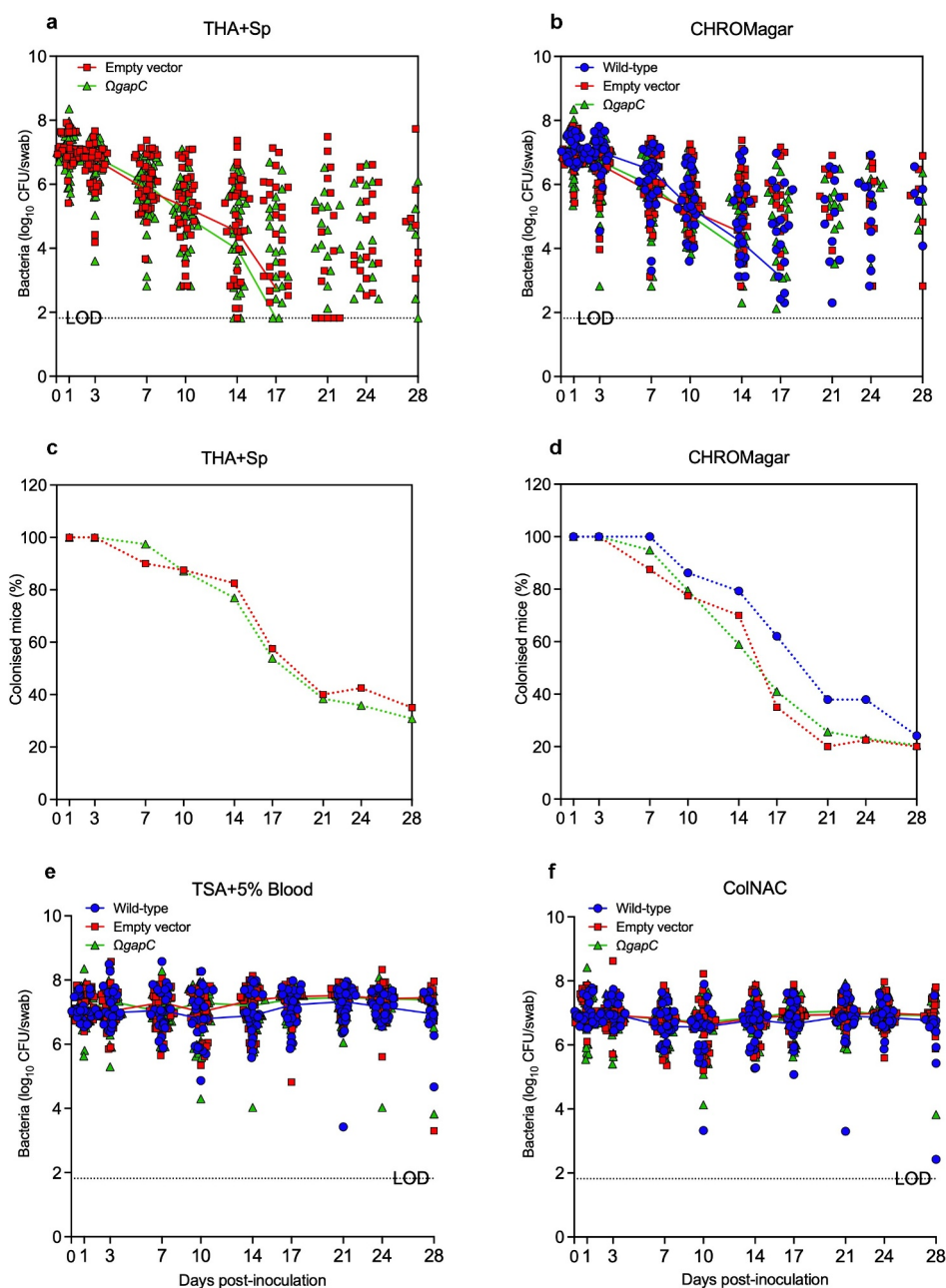
Figure 4. Persistence of *S. agalactiae* in the murine reproductive tract and effect of GAPDH. Mice were infected as per materials and methods, and samples were analyzed for numbers of *S. agalactiae* by quantitative colony counts on selective media as follows: (a) Sp-supplemented TH agar, and (b) CHROMID® Strepto B (CHROMagar). The percentage of mice in each group that exhibited culture-positive status are also shown for (c) Sp-supplemented, and (d) CHROMID® Strepto B (CHROMagar). The total numbers of resident genital tract commensal flora between the groups were determined using TSA 5% horse blood for (e) total bacteria, and (f) ColNAC for gram-positive bacteria. Data represent CFU/swab with each point representing an individual mouse, and are pooled from three independent experiments, each with 10–15 mice per group per experiment. Connecting lines are based on median values. The limit of detection (L.O.D) of the assay was 66 CFU/swab as indicated. Statistical significance was determined using repeated measures two-way ANOVA with a multiple comparison for individual time-point comparisons. We also performed an AUC analysis for the subpopulations of mice that exhibited persistent colonization for the duration of the 28-day assay, as described in the results.

play in the overall immune response to infection. In comparing the results of this study to prior studies on immunogenicity, it is notable that immunization of mice with *S. agalactiae* GAPDH led to increased production of IL-1β and IL-6 in the spleen and liver [18];

our findings of IL-1β and IL-6 production in mixed human cell co-cultures treated with rGAPDH from *S. agalactiae* are consistent with these results. The finding that *S. agalactiae* rGAPDH effects the production of immune regulatory IL-10 in human cells, which is

a master regulator of immunity [20] as well as IL-1Ra [38] that modulates inflammatory responses, expands the known immune regulatory actions of bacterial rGAPDH. Interestingly, more IL-10 was detected at 8 h in the TC model compared to the DC model, a response that paralleled lower TNF-a levels in the TC culture model at the early time points tested; perhaps such responses reflect a neutrophil-driven suppressive mechanism, given that IL-10 inhibits production of TNF-a [20], and neutrophils are significant producers of IL-10 in the context of infection [39]. Our interpretation of the other differences in cytokine responses between the DC and TC co-culture models is that these reflect the presence of neutrophils in the later. However, potential interrelationships among cytokine responses to rGAPDH, and the different cell types used in our co-culture models in this study remain hypothetical, so studies of the responses of single cell types in monocultures exposed to *S. agalactiae* rGAPDH are now warranted. Another interesting observation in this study is that for some cytokines, such as MIP-1a, the levels detected in DC culture were lower after 24 h compared 8 h. We cannot explain this finding but note that following release, cytokines and chemokines have relatively short half-lives [40]; turnover rates for different cytokines and related proteins can also differ substantially [41]. Determinants of protein half-life *in vitro* are multifactorial, encompassing post-transcriptional and post-translational factors [42,43]; determinants that may, conceivably, influence the levels of cytokines detected in our study.

In characterizing the responses of human cell cocultures to rGAPDH from *S. agalactiae* we recognize limitations of the current study, including the study design of using the carrier control and the identity of the individual cell-type(s) in the co-cultures that produced the cytokine responses. The carrier control used might contain trace proteins that may alter immune responses; another suitable control to account for protein purification contaminants would be a His-stop control based on pET-15b-*gapC*. For the identity of cytokine-producing cell types, our ability to assign precise roles for PMNs in mediating each cytokine response in the TC co-culture model following exposure to rGAPDH is restricted by our study design; also, differences in cell proportions between DC and TC cocultures necessitate care to avoid overinterpretation of the role of PMNs in amplifying or supressing cytokine responses in this model. Nonetheless, these models as applied achieved the goal of this part of the study in defining whether human cells can response to rGAPDH by producing cytokines. Moreover, the responses

detected in these models can be defined as being either dependent or independent of the presence of PMNs and support the central aim of determining whether human mixed cell co-cultures respond to *S. agalactiae* rGAPDH by producing cytokines. Co-culture models of bacterial infection can reveal complex, synergistic interactions between cells, including macrophages and epithelial cells [23]. Part of the rationale of using human bladder epithelial cells in the current study was to enable broad comparison with the findings of a previous study of cellular responses to whole *S. agalactiae* in human bladder epithelial cell cocultures [23]. The current study indicates that several cytokines produced in response to *S. agalactiae* rGAPDH, including IL-1β, IL-6 and IL-10 were shown to be induced by whole *S. agalactiae* [23]. It would be of interest to study the responses of epithelial cells derived from the reproductive tract to explore these responses in the context of *in vivo* vaginal cytokine responses in the murine model. Thus, studies could characterize cell-type contributions, including those of reproductive tract origin in the responses to *S. agalactiae* rGAPDH.

We used a study design that incorporated a mutant strain of *S. agalactiae* that over-expresses GAPDH to enable analysis of the effect of this over-expression on macrophage phagocytosis. The plasmid was designed to enable constitutive expression of GAPDH from *gapC* (Figure S7), based on a comparable system that demonstrated over-expression of GAPDH in *S. agalactiae* [14]. Assays of bacterial adhesion, uptake and intracellular survival demonstrated no significant difference in these measures compared to a control strain that carries the empty vector. In interpretating these findings, we note that several areas of the biology of *S. agalactiae* GAPDH were not examined in the context of host cellular responses and cellular interactions; for example, measures of cell death would be of interest given cytotoxic effects previously identified [21]. Furthermore, *S. agalactiae* GAPDH is a "sticky" protein [21] and the direct binding activities of rGAPDH to host cells would be of interest. In *Leishmania infantum*, GAPDH was shown to confer resistance against reactive oxygen species produced by host phagocytic cells [44]. While no studies have reported a role for in resistance to macrophage oxidative stress responses, there is considerable evidence that GAPDH contributes to virulence of *S. agalactiae in vitro*; it confers an ability to bind plasminogen and fibrinogen [12], and when secreted, exerts immuno-modulatory properties, including B and T cell activation [14]. Despite these multiple activities of GAPDH, the current study shows no role in

phagocytic uptake or survival of *S. agalactiae* inside macrophages.

Interestingly, despite no significant effect of GAPDH over-expression on phagocytic uptake and intracellular survival of *S. agalactiae in vitro* in this study, a small but statistically significant effect on the persistence of the bacteria *in vivo* was observed using a murine model of reproductive tract colonization, based on analysis of only culture-positive mice that exhibited colonization for the duration of the assay to 28-days, inclusive. In this model, *oe*GAPDH *S. agalactiae* was unable to persist as efficiently as a vector control strain in the reproductive tract of female mice, according to quantitative colony counts of viable bacteria. The differences in mean numbers of bacteria recovered from the mice were small comparing the two strains over the entire experimental time course of 28-days, but were statistically significant, according to an area-under-the-curve analysis. These findings were unexpected because GAPDH has been associated with increased virulence in mice [14]. Using an intraperitoneal challenge model with one million *S. agalactiae* cells, analysis of liver infection at day 5 showed a significant contribution of GAPDH to survival of the bacteria [14]. This is exciting, because while intraperitoneal challenge does not model a natural route of infection with *S. agalactiae* in humans, it offers proof of principle that GAPDH of the bacteria has a role in virulence. A finding of immune protection against *S. agalactiae* infection in neonatal mice based on a murine model of maternal vaccination with GAPDH [19] also supports a virulence-associated role for *S. agalactiae* GAPDH *in vivo*. The major differences in murine models examined in these previous studies and the current study are important to consider when interpreting findings about the effects of *S. agalactiae* GAPDH *in vivo*. For example, experimental infection of neonatal [19] or adult mice [14] using intraperitoneal administration of 1–5 million *S. agalactiae* cells is very different to the model of vaginal colonization used in this study; the colonization model is focused on bacterial persistence and host responses in the local niche of the reproductive tract [37], as a parallel to the carrier condition that occurs in adult women [45]; in contrast, measures of neonatal survival and viable bacteria in the liver in the few days immediately following disseminated infection are more reflective of acute, potentially life-threatening disease. Additionally, we note some loss of plasmid from *S. agalactiae* of both the GAPDH over-expression strain and the empty vector strain over time in our assays, accordingly to CFU counts on THA-Sp compared to CHROM agar. This likely reflects an absence of selection pressure *in vivo*; Sp treatment of mice (e.g., in drinking water) was not included in our study design because of likely effects on the microbiota that we sought to avoid. Notwithstanding experimental limitations and differences, the findings of this study indicate that GAPDH over-expression in *S. agalactiae* has a small but statistically significant effect on bacterial persistence in the reproductive tract of mice that remain chronically colonized. Distinct effects of GAPDH on bacterial persistence in the host might depend on the nature of infection and tissue context, which needs to examined in future studies.

The effects of GAPDH toward bacterial persistence during host-pathogen interactions *in vivo* are likely to be a function of known properties of the protein. On the one hand, GAPDH proteins are cytoplasmic glycolytic enzymes that, despite lacking identifiable secretion signals, are expressed at the surface of bacterial cells where they exhibit non-glycolytic functions, including adherence to host components [21]. In an infected host, surface-exposed GAPDH in bacteria would be accessible to interact with plasminogen, actin, and fibrinogen, and this might affect colonization; however, a role in binding of *S. agalactiae* GAPDH to cytoskeletal and extracellular matrix proteins has not yet been reported. GAPDH has been localized on the surface of multiple *S. agalactiae* serotypes [12], and the gene encoding it, *gapC*, is essential and conserved among strains. It induces production of IL-10 in the host [14,19], causes death of macrophages [21] and has B cell stimulatory effects independent of BCR specificity [14]. The current study establishes that *S. agalactiae* GAPDH induces multiple cytokine responses in human cells. Beyond these known properties, the way in which *S. agalactiae* GAPDH is detected by the immune system, and how the host responds to such exposure are not well understood.

In the current study, we also performed competitive index experiments using mice that received a mixed infection of equal numbers of WT and *oe*GAPDH-*S. agalactiae* to supplement the findings based on single-strain challenge experiments. These experiments showed a more dramatic disadvantage in the *oe*GAPDH strain compared to WT strain for chronic colonization; however, it is important to recognize the imperfect nature of this experimental design with potential for bias in the assay because the empty vector control strain is unable to be differentiated from the *oe*GAPDH strain in competition assay (both carry Sp-R markers).

In conclusion, *S. agalactiae* GAPDH elicits multiple pro-inflammatory, regulatory, and chemotactic cytokines from human mixed cell co-cultures. Over-expression of GAPDH renders *S. agalactiae* more susceptible to

clearance in the female reproductive tract of mice, but this is unrelated to the efficiency at which mouse macrophages phagocytose and kill the bacteria *in vitro*. This offers new insights into the effects of GAPDH in cell and cytokine responses at the host-pathogen interface.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability statement (DAS)

The datasets generated during and analysed in the current study are available from the corresponding author on reasonable request.

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