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Protein Kinase D Mediates Inflammatory Responses of Human Placental Macrophages to Group B *Streptococcus*

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Abstract

Problem: During pregnancy, Group B *Streptococcus* (GBS) can infect fetal membranes to cause chorioamnionitis, resulting in adverse pregnancy outcomes. Macrophages are the primary resident phagocyte in extraplacental membranes. Protein kinase D (PKD) was recently implicated in mediating pro-inflammatory macrophage responses to GBS outside of the reproductive system. This work aimed to characterize the human placental macrophage inflammatory response to GBS and address the extent to which PKD mediates such effects.

Method: Primary human placental macrophages were infected with GBS in the presence or absence of a specific, small-molecule PKD inhibitor, CRT 0066101. Macrophage phenotypes were characterized by evaluating gene expression, cytokine release, assembly of the NLRP3 inflammasome, and NF κ B activation.

Results: GBS evoked a strong inflammatory phenotype characterized by the release of inflammatory cytokines (TNF α , IL-1 β , IL-6 (P = 0.05), NLRP3 inflammasome assembly (P = 0.0005), and NF κ B activation (P = 0.05). Pharmacological inhibition of PKD suppressed these responses, newly implicating a role for PKD in mediating immune responses of primary human placental macrophages to GBS.

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Conclusion: PKD plays a critical role in mediating placental macrophage inflammatory activation in response to GBS infection.

Keywords

Placental Macrophage; Group B *Streptococcus*; Inflammation; Infection; Cytokine; Inflammasome

Introduction

Streptococcus agalactiae, also known as Group B *Streptococcus* (GBS), is a Gram-positive bacterium that colonizes the gastrointestinal and genitourinary mucosa of approximately 18% of pregnant women worldwide¹ and is a major cause of adverse pregnancy outcomes². Rectovaginal colonization is the major risk factor for invasive infection during pregnancy, including intrauterine infection (chorioamnionitis)³. Streptococcal chorioamnionitis, characterized by inflammation of the fetal membranes, can lead to preterm premature rupture of the membranes (PPROM), preterm labor, stillbirth and early-onset neonatal sepsis². Both PPRM and preterm labor are driven by host inflammatory responses to infection. Defining the mechanisms underpinning these responses within the fetal membranes is an important step towards developing better preventive and therapeutic strategies against perinatal GBS infection.

GBS has a strong capacity to adhere to, invade, and traverse intact fetal membranes^{4–6}. Chorioamnionitis caused by GBS results from bacteria ascending from the vagina, through the cervix, and into intact fetal membranes⁷. Once bacteria invade the membranes, a series of tissue paracrine signaling events occur that orchestrate host cellular responses to infection^{8,9}, which, ideally, eliminate the microbial threat. However, a robust inflammatory response can accelerate membrane weakening by reducing connective tissue integrity leading to membrane rupture, triggering preterm birth, and providing bacteria access to the fetus^{10–12}. The response to GBS infection in chorioamnionitis includes production of diverse proinflammatory cytokines such as IL-1 β , IL-6, and TNF- α ^{8,12–14}, each of which potentially plays a role in disease pathogenesis. For example, infection of the choriodecidua, the outermost maternally-derived layer of human fetal, was found to trigger the release of beta-defensin antimicrobial peptides from the amnion, the fetally-derived innermost layer of the extraplacental membranes, as a result of paracrine (IL-1 β) signaling⁸. Moreover, IL-1 β was shown to be responsible for both neutrophil recruitment in a mouse model GBS infection and weakening of tight junctions in the epithelial barrier^{15,16}. Macrophages are well known producers of IL-1 β , IL-6, TNF- α , and other mediators implicated in chorioamnionitis. We therefore focused on mechanisms of immune activation by human placental macrophages, which are found in both the placenta and extraplacental membranes, infected with GBS *ex vivo*.

A recent study reported a potential role for the protein kinase D (PKD) family of serine/threonine kinases in GBS-mediated immune activation¹⁷. Activation of the PKD1 isoform was demonstrated in mouse peritoneal macrophages as well as human and mouse macrophage cell lines infected with GBS¹⁷. Furthermore, PKD signaling was crucial for

orchestrating inflammatory responses to GBS by macrophages *in vitro* and by the host *in vivo*¹⁷. These results have not yet, to our knowledge, been evaluated in a primary human tissue macrophage or in a model of reproductive tract infection. Therefore, the hypothesis that GBS drives proinflammatory responses by human placental macrophages through a process that depends upon PKD activation was tested.

Materials and Methods

Bacterial strains and growth

GB00037 is a serotype V, multilocus sequence type ST-1, invasive strain of GBS isolated from the blood of a septic neonate with early onset disease^{18,19} and is available upon request. GBS was grown in 10 mL Todd-Hewitt broth at 37°C overnight (~16 h), centrifuged for 10min at 1500 rcf. Bacteria was then washed by resuspending in 2 mL PBS and centrifuging for 3 min at 8,609 rcf, and resuspended in RPMI 1640 (Gibco) prior to infection. Serial dilutions of GBS were plated onto Sheep's Blood agar plates to determine colony forming units (CFU). CFU values were used to determine multiplicity of infection (MOI, number of bacteria per macrophage).

Human subjects, tissue collection and placental macrophage isolation.

This study was reviewed and approved by the Vanderbilt University Institutional Review Board (protocol #140699). It involved using deidentified human cells and tissues for which specific consent was not required. Placental samples were provided by the Cooperative Human Tissue Network at Vanderbilt University, which is funded by the National Cancer Institute. Placental macrophages were isolated from villous placenta according to our previously published protocol^{20,21}. Briefly, tissue was washed three times in PBS, digested to a single cell suspension in 25% Percoll and loaded onto a 50% Percoll gradient to remove cellular debris. Macrophages were isolated from single cell suspensions using MACS Miltenyi CD14 microbeads. Purity of freshly isolated macrophages was ~90% on average, as assessed by percentage of viable, CD68+ cells, determined by flow cytometry.

Placental macrophage cell culture and infection

Placental macrophages were seeded at a density of 4×10^6 cells per well in a polystyrene, 6-well culture plate in RPMI 1640 (Gibco) supplemented with 10% charcoal-stripped, dextran treated heat inactivated FBS at 37°C with 5% carbon dioxide overnight (~16 h). Before treatment, cells were visually inspected to verify proper morphology. Cells were pretreated with 5 μ M PKD inhibitor CRT0066101 (CRT, Tocris Bioscience; Ellisville, MO) or vehicle for 1hr prior to infection, after which time the inhibitor was removed. Cells were then infected with GBS (MOI of 0.1:1 or 10:1) following CRT pretreatment for indicated time points at 37°C with 5% carbon dioxide. Cells and supernatants were collected for downstream analysis.

Reverse-transcriptase PCR

RNA was isolated from polarized macrophages using the Trizol method followed by RNA washing using the Qiagen® RNeasy plus mini kit (Bio-Rad). RNA quality and quantity were assessed using Nanodrop®. One microgram total RNA was used as template for cDNA

synthesis. cDNA was generated in the BIO-RAD® MJ Mini Personal Thermal Cycler using BIO-RAD® iScript™ Reverse Transcription supermix (Bio-Rad Laboratories, Hercules, CA) for RT-qPCR as outlined by the manufacturer. Following cDNA synthesis, samples were diluted 1:10 in ultrapure water. ThermoFisher Scientific Taqman® primers specific for M1 (CCR7, CD80 Cat# 4331182 and 331182 respectively) and M2 (CD163, CD209 Cat# 4331182 and 4331182 respectively) polarization transcripts were used in conjunction with BIO-RAD® iQ™ Supermix to carry out qPCR using the ThermoFisher Scientific StepOnePlus™ real-time PCR system (Waltham, MA). Twenty microliter reactions were generated comprised of 10 µL iQ™ Supermix, 4 µL H₂O, 5 µL cDNA, and 1 µL Taqman® primer. Reactions were placed in Applied Biosystems StepOnePlus™ real-time PCR system using the thermal cycling protocol as outlined by the manufacturer (3 min 95° for activation; 12 sec 95° denaturation step; 45 sec at 60° annealing; 40 cycles). Fold change values were calculated using the 2^{-ddCt} method where changes in gene expression were normalized to GAPDH as a housekeeping gene and relative to untreated cells as the calibration sample.

Pregnant mouse model of vaginal ascending GBS infection

GBS infection of pregnant mice and subsequent analyses were performed as previously described with some modifications^{22,23}. Briefly, C57BL6/J mice were purchased from Jackson Laboratories and mated in harem breeding strategies overnight. Pregnancy was confirmed the following day by the presence of a mucus plug to establish the embryonic day (E0.5). On embryonic day 13 (E13.5) dams were anesthetized using isoflurane chambers, and $\sim 1 \times 10^3$ GBS strain GB00037 colony forming units (CFU) in THB medium supplemented with 10% gelatin was introduced into the vagina (total inoculum volume 50 µL). Sham controls were inoculated with an equivalent volume of THB medium containing 10% gelatin. Animals were housed singly until embryonic day 15 (E15.5) at which time they were sacrificed by carbon dioxide euthanasia procedures and necropsy was performed to isolate reproductive tissues including the placenta and decidua for flow cytometry. Four to six animals were utilized for each experimental group derived from three separate experiments (sham treated uninfected animals or GBS-infected animals). All protocols were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center (M1700085).

Flow cytometry of mouse macrophages

Mouse placental and decidual macrophages were evaluated via flow cytometry as previously described with modification²³. Briefly, placenta and decidua were collected from each animal and pooled, weighed, minced with sterile scissors and digested in a solution containing 1 mg/mL collagenase, 1 mg/mL hyaluronidase, and 150 µg/mL DNase I with agitation for 1 h at 37°C. Samples were washed using RPMI 1640 +/+ medium (containing 1% antibiotic and 10% fetal bovine serum), centrifuged at 1500 RPM at 4°C for 10 min followed by 100 µm nylon mesh filtration to eliminate remaining particulates. Cells were then washed and centrifuged on a Percoll gradient as previously described followed RBC lysis buffer (eBiosciences). After two more washes with RPMI 1640 +/+, cells were counted, and one million cells were aliquoted into flow cytometry tubes. Cells were surface stained for 20 min at 4°C with anti-mouse CD45 (Biolegend; Pacific Blue, clone 30-F11),

anti-mouse CD11b (eBioscience; Alexa Fluor 700, clone M1/70), anti-mouse F4/80 (eBioscience; PE, clone BM8), and FcR blocking reagent (Miltenyi). Cells were then stained with a live/dead viability dye (Life Technologies; Aqua). Cells were resuspended in FACS buffer and immediately acquired on a BD LSR2 Flow Cytometer (BD Biosciences). Analysis was performed with BD FACS Diva software (BD Biosciences).

Gene expression array

RNA was isolated from macrophages using the Trizol method. RNA quality and quantity was assessed using a spectrophotometer (NanoDrop™). The Qiagen RT² Profiler™ PCR Array Human Inflammatory Response & Autoimmunity kit was used as outlined by the manufacturer to evaluate expression of 84 inflammation and immunity related genes. Briefly, genomic DNA was eliminated from the RNA isolation product followed by first strand cDNA synthesis. Twenty-five microliter cDNA samples were placed on 96-well PCR plates in the Applied Biosystems StepOnePlus™ real-time PCR system using the thermal cycling protocol as outlined by the manufacturer (10 min 95° for hot start activation; 15 sec 95° denaturation step; 1 min at 60° annealing; 40 cycles). Fold regulation was calculated.

Gene Expression Data Analysis

Using the GeneGlobe Data Analysis Center on the Qiagen® website, Ct values for samples were determined using the algorithm $2^{(-\text{Avg}(\Delta\text{Ct}))}$ and normalized to two housekeeping genes within the array, *B2M* and *RPLPO*. Resulting normalized Ct values were exported to Excel. Next, fold change and fold regulation were determined using the geometric mean of normalized Ct values for GBS-infected and uninfected samples. Normalized Ct values of GBS-infected samples were plotted against uninfected samples to graphically represent fold regulation. All statistical analysis was carried out on raw Ct values normalized to housekeeping genes.

Enzyme-linked immunosorbent assays (ELISAs)

Supernatants were collected and evaluated for cytokine release using R&D duo-set ELISA as instructed by the manufacturer. Supernatants were evaluated for TNFα (Cat# DY210–05), IL-1β (Cat# DY201–05), and IL-6 (Cat# DY206–05).

Immunofluorescence Staining for Quantification

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton/0.2% Bovine Serum Albumin in PBS, and probed using anti-phospho-NFκB p65 (Cat# 3033S, Cell Signaling Technology, Danvers, MA, United States) and anti- IκBα (L35A5) (Cat# 4814S, Cell Signaling Technology, Danvers, MA, United States) and ProLong™ Gold Antifade Mountant with DAPI (Life Technologies, Grand Island, NY). Alternatively, cells were stained for NLRP3/ASC using the Duolink® proximity ligation assay (PLA®) as described by the manufacturer. Images obtained using Nikon Eclipse microscope. For each sample, 2–5 fields were imaged per condition. Fields were chosen at random, excluding fields where aggregates of cells were observed (individual cells facilitated image analysis). Images were exported to ImageJ® for quantitation. Total field fluorescence intensity for each color (representative of a specific antibody-protein conjugate) was measured and normalized to

DAPI fluorescence. Normalization was calculated using the algorithm (protein fluorescence intensity)/(DAPI fluorescence intensity). DAPI represents the nucleus of the cell allowing for fluorescence to be normalized to the total number of cells per field. Phosphorylated NF κ B p65- I κ B α Index was calculated using DAPI-normalized data and the algorithm (phosphorylated NF κ B p65)/(I κ B α) for each condition. Uninfected controls were set to 1 and all treatments are expressed as fold change compared to uninfected

Magnetic bead array for inflammatory mediator quantification

Placental macrophages were cultured in 6-well plates using a density of 4×10^6 macrophages per well in 2mL RPMI 1640 supplemented with 10% charcoal-stripped, dextran treated, heat inactivated FBS at 37°C with 5% carbon dioxide. Following 1 hr pretreatment with CRT0066101 (CRT, Tocris Bioscience; Ellisville, MO) or vehicle and subsequent removal of CRT0066101, cells were infected with GBS (0.1 bacterium per macrophage) for 6 hours, and supernatants collected. A custom 15-plex Millipore MILLIPLEX[®] MAP Human Cytokine/Chemokine Magnetic Bead Panel - Immunology Multiplex Assay was run according to instructions provided by the manufacturer. Briefly, 25 μ L of supernatant or standard/quality control was loaded into a 96-well plate followed by addition of assay buffer, matrix solution, and magnetic beads and allowed to incubate overnight at 4°C. Wells were then washed and incubated for 1hr with detection antibodies followed by incubation with Streptavidin-Phycoerythrin for 30 min. Wells were then washed and the plate was analyzed using x-map technology on the Luminex[®] MagPix[®] machine. Results were graphed and analyzed using GraphPad Prism[®] (GraphPad Software, La Jolla, CA).

Western immunoblotting for PKD in human placental macrophages

Placental macrophages were cultured in 6-well plates using a density of 4×10^6 macrophages per well in 2mL RPMI 1640 supplemented with 10% charcoal-stripped, dextran treated, heat inactivated FBS at 37°C with 5% carbon dioxide. Macrophages were then infected with GBS (MOI 0.1:1) for 45min. Supernatant was aspirated, and cells were collected in cold PBS and centrifuged for 5min at 2380 RCF to form a pellet. Cell pellet was resuspended in cold lysis buffer (0.5% IGEPAL-CA360/ 250 mM NaCl/ 50 mM Tris HCl) and incubated at 4°C for 30 min with agitation. Cells were then centrifuged for 5 min at 13,000 x *g* at 4°C. Supernatant (lysate) was then transferred to fresh chilled tubes. Total protein concentration was quantified using DC Protein Assay (Bio-Rad). Twenty micrograms of protein was loaded into 10% Mini-PROTEAN TGX precast electrophoresis gels (Bio-Rad) for western. Membranes were probed for anti-phospho-PKD Ser744/748 (Cat# 2054S, Cell Signaling Technologies), anti-PKD1/PKC μ (D4J1N) (Cat# 90039, Cell Signaling Technologies), and anti- β -Actin (8H10D10) (Cat# 3700S, Cell Signaling Technology).

Lactate dehydrogenase cytotoxicity assay

Cytotoxicity was determined as previously described²⁴. Briefly, serum-free supernatants were collected and centrifuged to pellet cellular debris. Supernatants were analyzed using the Cytotoxicity Detection kit (Sigma-Aldrich) per the manufacturer's instructions. Results were expressed as fold change in cytotoxicity using medium without cells as the low control and cells treated with 2% Triton X-100 as the high control. Cytotoxicity was calculated

using the following equation: cytotoxicity = (experimental value – low control)/(high control – low control). Resulting values expressed as fold change compared to uninfected.

Membrane-based antibody array for detecting cytokines in placental macrophage supernatant

Cell supernatants were evaluated for cytokine expression following 1 hr CRT pretreatment and 6 hr GBS infection using the R&D Proteome Profiler™ Human XL Cytokine Array Kit as instructed by the manufacturer. Briefly, supernatants were incubated overnight on membranes spotted with specific antibodies. Resulting protein/antibody complexes were immobilized onto the membrane. Streptavidin-Horseradish Peroxidase and chemiluminescent detection reagents were then added and chemiluminescence was detected using LI-COR imager and software. HLIimage++ software by Western Vision Software was used to determine membrane densitometry values. Densitometry values were used to carry out statistical analysis in GraphPad Prism. Heatmapper® software (www.heatmapper.ca) was used to graphically represent values and obtain z-scores.

Membrane-based antibody array for detecting cytokines in THP-1 supernatant

THP-1 monocyte-like cells (ATCC TIB-202) were differentiated into macrophages as previously described²⁵. Briefly, THP-1 cells were incubated with 100nM phorbol 12-myristate 13-acetate (PMA; Sigma) in RPMI 1640 medium with 2% charcoal-stripped, dextran treated, heat inactivated FBS for 24 hrs. Cells were then pretreated with the PKD inhibitor CRT0066101 (CRT, Tocris Bioscience; Ellisville, MO) for 1 hr prior to infection, after which time the inhibitor was removed and fresh RPMI 1640 was added. Cells were then infected with log phase GBS using a multiplicity of infection (MOI) of 10:1 for 1 hr. Extracellular bacteria and media were aspirated and the cells were washed with PBS and RPMI 1640 supplemented with 2% FBS, 100µg/ml gentamicin (Gibco), and 5µg/ml penicillin G (Sigma). The cells were incubated in 100nM PMA at 37°C with 5% CO₂ for 6 hrs prior to collecting the culture media for cytokine profiling as previously described²⁶. The Abcam Human Cytokine Antibody Array Kit (ab133998), was performed according to the manufacturer's instructions with an Amersham Imager 600 (GE Life Sciences). Densitometry was performed using ImageJ to determine relative protein levels of each cytokine; densitometry values were corrected using internal positive controls for each array and the normalized values were graphed. Densitometry values from each infection condition were compared to the corresponding mock infection condition to calculate fold changes.

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism version 7.03 for Windows, (GraphPad Software, San Diego, California, United States). Changes in gene expression in human placental macrophages in response to GBS infection were evaluated via paired Student t-testing incorporating a false discovery rate (FDR) approach with the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli²⁷. Analysis of cytokine data released from placental macrophages when two conditions were compared relied on paired Student t-test, while data obtained when 3 or more conditions were assessed relied on one-way ANOVA with Bonferroni multiple comparison test. Membrane-based antibody array data were evaluated by two-way ANOVA and Bonferroni multiple comparison test. A two-

way ANOVA and Bonferroni multiple comparison test was used to analyze differences in NLRP3-ASC complex fluorescence. Statistical significance of mean fluorescence intensity of cells stained for phosphorylated p65 and I κ B α was determined using two-way ANOVA and Bonferroni multiple comparison test.

Results

GBS induces proinflammatory activation of placental macrophages in the gravid uterus

Bacteria such as GBS generally activate macrophages towards an inflammatory phenotype, but little has been done to characterize the response of macrophages present at the maternal-fetal interface. Therefore, primary human placental macrophages were used to evaluate how GBS infection impacts expression of genes involved in inflammation. In the absence of infection or other stimuli, evidence suggests that placental macrophages exist in a relatively anti-inflammatory (M2-like), tolerogenic activation state^{28–32}. The data confirmed that human placental macrophages more highly expressed the M2 markers CD163 and CD209 than the M1 markers CCR7 and CD80 (Supporting Information Fig. 1). Similarly, mouse placental macrophages more highly expressed the M2-like marker CD206 than the M1 marker CD11c^{33,34} (Supporting Information Fig. 2) in the absence of infection. Human placental macrophages were infected with an invasive clinical isolate of GBS using a low multiple of infection (MOI; 1 bacterium for every 10 macrophages). An RNA array evaluating the expression of 84 inflammation-related genes in response to infection was performed and revealed a strong shift upward in the expression of proinflammatory genes (Fig. 1A). Several cytokine genes associated with chorioamnionitis, including *IL-6*, *TNF α* , and *IL-10* were upregulated, though neither *IL-1 β* nor *IL-8* were among them. Moreover, the most highly upregulated transcripts were *SELE* (E-Selectin) and *CCL4* (MIP-1 beta). Transcripts for chemokines previously associated with labor such as *CXCL10*, *CCL19*, and *CCL21* were also upregulated³⁵. GBS infection also upregulated NF κ B and NOS expression. Transcripts for *CCL2* (*MCP-1*) were reduced during infection, but the trend was not statistically significant. A complete dataset from the mRNA arrays is provided (Supplemental Table 1). In mice, at 48 hr post vaginal inoculation with GBS, we primarily observed bacterial invasion of the decidual tissue and to a lesser extent the junctional zone of the mouse placenta²³. Macrophage populations observed in the decidua indeed revealed a shift from a balanced M1/M2 population to a more M1-dominant population, whereas no such proinflammatory shift was observed in macrophages isolated from the adjacent placenta (Supporting Information Fig. 2).

Because gene transcription does not always correlate with protein expression, the extent to which GBS infection impacted inflammatory protein production by human placental macrophages was assessed, using an antibody-conjugated bead array. In response to GBS infection, placental macrophages released significantly higher amounts of inflammatory cytokines such as TNF- α , IL-1 β , MIP-1 α (CCL3) and IL-6 compared to uninfected cells (Fig. 1B). As in the mRNA array, IL-10 release was also increased in response to GBS infection. Interestingly, CCL2 (MCP-1) release was significantly suppressed in response to GBS (mimicking the trend in mRNA suppression) and no changes in IL-8 were seen (also similar to the mRNA data).

GBS induces inflammatory cytokine release in a PKD-dependent manner

A recently published study conducted in mouse macrophages and the human THP-1 macrophage-like cell line reported that GBS infection induced proinflammatory responses through a PKD-dependent mechanism¹⁷. Because PKD localization, activation, and function are often context dependent^{36,37}, whether PKD played a role in placental macrophage inflammatory responses to GBS was assessed. PKD1 expression and GBS-induced PKD1 activation, indicated by phosphorylation, was confirmed in placental macrophages via Western immunoblot (Supporting Information Fig. 3). To assess the role of PKD in GBS-mediated cytokine release, placental macrophages were infected with GBS in the presence or absence of the highly specific, small molecule PKD inhibitor, CRT^{38,39}. Five micromolar CRT was used to inhibit PKD activation as previously reported¹⁷ and no cytotoxicity was observed using this concentration for 1 hr pretreatment in human placental macrophages as determined by lactate dehydrogenase (LDH) release (Supporting Information Fig. 5). A 100-panel cytokine membrane-based antibody array was used to evaluate cytokine release in response to infection *in vitro* (Fig. 2A). Accompanying values from the dataset are included (Supplement Table 2). GBS infection significantly induced 8 proinflammatory mediators (8% of the total) and each of the 8 mediators appeared to be upregulated in a PKD-dependent manner (Fig. 2B). None of the inflammatory mediators were significantly downregulated. Supporting previous findings and confirming the magnetic bead array results above, proinflammatory cytokines TNF α , IL-1 β , and IL-6 were induced in a PKD-dependent manner compared with control (uninfected) samples (Fig. 2C).

GBS induces inflammasome activation in a PKD-dependent manner

GBS was previously shown to activate the macrophage inflammasome after phagocytosis by virtue of bacterial RNA leaking from the phagolysosome to activate cytoplasmic NLRP3^{40,41}. This activation was reported to be dependent on expression of the GBS toxin β -hemolysin. A recent *in vivo* study suggests macrophages require PKD to activate the NLRP3 inflammasome⁴². With this in mind, it was noted that GBS infection of placental macrophages led to measurable increases in extracellular IL-1 β without increasing mRNA transcripts for pro-IL-1 β (above), suggesting that PKD-dependent NLRP3-dependent activation and cleavage of pro-IL-1 β might explain the increased release of IL-1 β found in cell supernatants. Thus, human placental macrophages were infected with GBS and evaluated for assembly of the NLRP3/ASC complex using a proximity ligation assay⁴³. GBS activated the inflammasome, as indicated by increased NLRP3-ASC complex fluorescence, and this was completely blocked by CRT (Fig. 3A, 3B).

GBS-induced NF κ B activation is PKD-dependent.

NF κ B is a known regulator of several physiological responses, particularly those related to inflammatory responses to stimuli. Under oxidative stress and cholinergic signaling, PKD is able to mediate NF κ B activation^{44,45}. Inhibitors of PKD such as CRT were also shown to attenuate NF κ B activation and inflammatory responses in pancreatitis rat models⁴⁶. Placental macrophages were infected with GBS in the presence or absence of CRT to evaluate modulation of NF κ B activation, as estimated by the ratio of phosphorylated NF κ B p65 to I κ B α , since the former will increase as NF κ B is activated and the latter will be

degraded⁴⁷. Indeed, in response to GBS infection, NF κ B activation was significantly upregulated in PMs and this was blocked by pharmacological PKD inhibition (Fig. 4).

Discussion

Group B *Streptococcus* is an important perinatal pathogen. Worldwide, it is estimated that GBS causes as many as 3.5 million preterm births and nearly 57,000 stillbirths per year, many as a result of intrauterine infection during pregnancy (chorioamnionitis)². Despite the clinical importance of GBS during pregnancy, many fundamental questions remain regarding the regulation of innate immune responses to GBS.

Macrophages are the major resident phagocyte in uninfected fetal membranes^{48–50}. Placental macrophages, also known as Hofbauer cells, are fetally-derived phagocytes that reside within the chorioamnion⁵¹; they are presumably engaged in host defense but are also important for governing maternal-fetal immune tolerance and membrane remodeling/rupture during labor. Chorioamnionitis leads to a robust inflammatory response that can ultimately lead to preterm labor, preterm premature rupture of membranes, and infection of the fetus, which can result in stillbirth or neonatal sepsis². Because macrophages are important sentinels of innate immunity and produce inflammatory mediators that have potent paracrine actions on neighboring cells, we sought to further define responses of human placental macrophages to GBS in this context. Our results implicate PKD-dependent signaling in orchestrating the human placental macrophage response to infection in this human macrophage population.

Toll-like receptors (TLRs) are among the most well characterized pathogen recognition receptors known to recognize GBS. Several GBS components such as RNA and DNA are recognized by TLRs^{52–60}. Much of the work characterizing TLR-dependent activation suggests that GBS-induced inflammatory responses signal through the adaptor protein MyD88^{53,61–63}. There is a gap in knowledge regarding the contribution of intermediate scaffolding proteins activated downstream of MyD88 following TLR ligation. The serine/threonine kinase PKD was identified as being a critical mediator of MyD88-dependent inflammatory activation⁶⁴.

PKD is involved in a multitude of cellular activities including vesicular trafficking, transcription factor and signaling modulation, inflammatory activation, and cellular detoxification^{17,37,42,65–67}. While there are three isoforms of PKD (PKD1, PKD2, PKD3), PKD1 is the most well characterized and was found to be activated by all TLR ligands except TLR3^{17,64}. PKD1 was also found to be activated by GBS to induce inflammatory responses in THP-1 and mouse macrophages¹⁷. PKD contains an N-terminal regulatory domain which functions in localization and interaction, a catalytic domain containing phosphorylation sites for activation, and a C-terminal extension. PKD1 activation is conferred through phosphorylation at serine residues Ser⁷⁴⁴ and Ser⁷⁴⁸ located within the catalytic domain, and Ser⁹¹⁶ located in the C-terminal extension^{68,69}.

PKD also plays a critical role in inflammasome formation and activation. In its inactivated, unphosphorylated state, PKD recruits NLRP3 to Golgi membranes, and upon

phosphorylation, frees NLRP3 to form active inflammasome complexes⁴². While GBS has been shown to activate the NLRP3 inflammasome^{40,41}, to our knowledge there are no reports of PKD being involved in GBS-mediated NLRP3 activation. This putative link between GBS infection and PKD-mediated inflammasome activation is new. Considering PKD's role in inflammatory activation, it is no surprise that PKD activation has been linked to NF κ B signaling. Inhibiting PKD with pharmacological inhibitors resulted in an inhibition of NF κ B signaling; consistent with this, the PKD gene (*PRKDI*) promoter contains binding sites for NF κ B^{44–46,70}. Moreover, GBS infection was shown to downregulate the expression of I κ B α in THP-1 macrophages and mouse spleen; I κ B α downregulation was reversed using the potent PKC/PKD1 inhibitor Gö6976¹⁷. Our findings in placental macrophages suggest that PKD is critical for GBS infection-induced NLRP3 inflammasome formation, inflammatory cytokine release, and mediating NF κ B signaling events, all of which were abrogated upon PKD inhibition by CRT.

Our gene expression array revealed a multitude of upregulated transcripts in response to GBS infection. Interestingly, *SELE* (E Selectin) and *CCL4* (MIP-1 β) were the most highly induced transcripts in placental macrophages by GBS infection, suggesting that cellular adhesion and leukocyte recruitment may be key functions of placental macrophages in response to bacterial threats. Of particular importance amongst cytokines observed were the inflammatory cytokines TNF α , IL-1 β , and IL-6 because of their established association with chorioamnionitis and adverse pregnancy outcomes. Surprisingly, IL-1 β transcripts were not significantly upregulated, yet we observed significant increases in IL-1 β protein release, suggesting that GBS infection induces post-transcriptional modification of IL-1 β . None of the 84 inflammatory transcripts evaluated were suppressed in response to infection, highlighting GBS' ability to induce an inflammatory phenotype in placental macrophages.

Of 100 inflammatory mediators observed in placental macrophage cell supernatant, eight were significantly upregulated in response to GBS, each of which was sensitive to PKD inhibitor treatment. No cytokines were significantly downregulated in response to GBS. We also infected the human THP-1 macrophage-like cell line with GBS and observed increased release of many inflammatory mediators including several that were observed to be up-regulated in placental macrophages (Supporting Information Fig. 4)¹⁷. Many, though not all, of these mediators appeared to depend on PKD activation. These results suggest that our findings are generalizable beyond placental macrophages. Interestingly, cytokines and chemokines we identified to be upregulated by GBS are also key cytokines in chorioamnionitis pathogenesis and preterm birth^{71–73}. Unlike THP-1 or RAW 264.7 macrophages, placental macrophages did not induce IL-8 or IL-12 release, highlighting a similar yet unique response to GBS infection in human placental macrophages. It is interesting to speculate that the absence of IL-8 and IL-12 induction by GBS might serve as a mechanism to prevent neutrophil infiltration or activation of uterine natural killer cells in fetal membranes during infection, but this will need to be explored in future investigations.

Strain differences between GBS clinical isolates may contribute to different phenotypic outcomes in macrophages. While no significant upregulation of IL-8 or IL-12 was observed using GB00037 (type V strain), a previous study observed upregulation of IL-8 and IL-12 in THP-1 and RAW 264.7 cells infected with a type 1a strain of GBS¹⁷. We previously

reported varying capacities for phagocytosis across different GBS strains⁷⁴. While our work did not explore differences in macrophage inflammatory activation across GBS strains, future experiments examining strain specific contributions to macrophage immune activation would better address this.

Our results indicate that GBS induces PKD-dependent NLRP3 inflammasome formation as indicated by the assembly of NLRP3 together with ASC. The exact mechanism involved in mediating GBS-induced NLRP3 activation remains to be defined, but previous reports suggest that DNA and RNA from GBS might prompt NLRP3 activation^{40,41,56}. Moreover, PKD was previously implicated in the phosphorylation and release of bound NLRP3 from Golgi membranes, allowing free NLRP3 to form active inflammasome complexes⁴². Inhibition of PKD caused NLRP3 to be retained at Golgi membranes inhibiting NLRP3 inflammasome assembly. While our studies did not explore NLRP3 localization, our results in response to GBS infection accord with the observation that inhibiting PKD also inhibits NLRP3-ASC assembly, suggesting that GBS could potentially be inciting PKD to release Golgi-bound NLRP3 to form active NLRP3-ASC complexes. Our IL-1 β cytokine release data support the PKD-dependent activation of the inflammasome. IL-1 β is of particular interest in models of chorioamnionitis because of its role in weakening tight junctions in the placental barrier and its ability to stimulate the release of the antimicrobial peptide human beta defensin into the amniotic cavity^{16,75}. This human beta defensin was shown to be able to directly kill GBS⁸. The ability of IL-1 β to induce human beta defensin release in the amniotic cavity even when GBS has not crossed the physical barriers of the gestational membranes highlights a critical role for IL-1 β in paracrine mediated signaling resulting in bacterial clearance. Our work identifies placental macrophages as being one of the sources of IL-1 β release in gestational tissues.

GBS infection was also found to upregulate phosphorylated NF κ B p65 and reduce the amount of detectable I κ B α in a PKD-dependent manner in human placental macrophages. These findings solidify PKD as a key regulator in inducing inflammatory responses in human placental macrophages. The extent to which NF κ B p65 activation directly effects inflammatory gene transcription is yet to be determined, but given the potent and promiscuous effect of NF κ B p65 on inflammatory gene activation, we suspect that NF κ B p65 may potentially be a key mediator of PKD-mediated inflammation.

Limitations of this work include the lack of *in vivo* data to support a role for PKD in mediating the natural course of perinatal GBS infection. Future studies will be needed to determine such effects. In addition, our *in vitro* studies utilized GBS without opsonizing the bacterium with either complement or immunoglobulin. It is possible that opsonization would change our findings. Because GBS possesses a polysaccharide capsule that prevents opsonization^{76–78}, we were interested in first defining the nature of cellular responses to unopsonized bacteria. Our work does not address the contribution of strain specificity and/or virulence factors to placental macrophage inflammatory activation. Future experiments will need to utilize varying strains of GBS to evaluate differences in macrophage activation amongst clinical strains. The use of a small molecule PKD inhibitor, despite its reported high specificity³⁹, is a limitation that will require future studies in macrophage-specific

PKD-null cells or animals to confirm what the pharmacological studies here and elsewhere suggest about the importance of PKD to macrophage responses to GBS infection.

In summary, our work demonstrates that GBS induces proinflammatory activation in human placental macrophages in a PKD-dependent manner. This activation is characterized by the upregulation of inflammatory genes, the release of inflammatory cytokines, NLRP3 activation, and NF κ B activation (Fig 5). These findings highlight a potentially important contribution of PKD in mediating inflammation in human placental macrophages, making PKD regulation a possible target for helping to prevent or treat GBS infections during pregnancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A **B**

Figure 1. GBS induces inflammatory profile in maternal-fetal macrophages.

Placental macrophages were infected with GBS (MOI 0.1:1) for 6 hrs. Isolated mRNA was used to analyze gene expression via PCR array. (A) Scatter plot showing changes in fold regulation for 84 genes associated with inflammation comparing infected cells to uninfected cells. Gene expression was normalized to the housekeeping genes *B2M* and *RPLPO*, $n=4$. Paired t -test used for statistical analysis. Discovery determined using the Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, no statistical significance was detected. (B) Supernatants were collected and evaluated for cytokine release using an antibody-conjugated bead array, $n=10$. Error bars represent SEM. Paired t -test used for statistical analysis. (** $P < 0.01$, and **** $P < 0.0001$).

Figure 2. GBS induces proinflammatory cytokine release in PKD-dependent manner. Human placental macrophages were infected with GBS (MOI 0.1:1) for 6 hr. in the presence or absence of the PKD inhibitor CRT. Supernatants were collected and evaluated for cytokine expression. (A) Membrane-based antibody array displaying cytokine densitometry values expressed as heatmap (n=3). (B) Selected cytokines from antibody array that were significantly upregulated in response to GBS infection. (C) TNF α , IL-6, and IL-1 β ELISA (n = 5). Error bars represent SEM. Two-way ANOVA and Bonferroni's multiple comparison test used for statistical analysis. (* P 0.05, ** P 0.01, and **** P 0.0001)

Figure 3. GBS induces PKD-dependent activation of NLRP3 inflammasome.

Human placental macrophages were infected with GBS (1 bacterium per human cell) or treated with PKD-1 inhibitor CRT for 6h. Cells were then stained for NLRP3/ASC (red) using DuoLink and DAPI (nuclei, blue). **(A)** Images taken at 40X. Images in neighboring panel represent zoomed in yellow boxes highlighted in panel A. (n=3) **(B)** Quantitation of NLRP3-ASC complex fluorescence. Error bars represent SEM. Two-way ANOVA and Bonferroni's multiple comparison test used for statistical analysis (* p < 0.05, ** p < 0.01, and *** p < 0.001)

Figure 4. Activation of NFκB signaling pathway in response to GBS infection.

Human placental macrophages were infected with GBS (MOI 0.1:1) and evaluated for NFκB signaling. Mean fluorescence intensity of cells stained for p-P65 and IκBa at 6h was measured and expressed as relative index (n=1 with 4 replicates). Error bars represent SEM. Two-way ANOVA and Bonferroni's multiple comparison test used for statistical analysis (* P 0.05).

Figure 5. GBS Mediated Macrophage Activation in Gestational Membranes.

Schematic summarizing GBS activation of PKD in placental macrophages leading to downstream upregulation of inflammatory genes, NLRP3 inflammasome activation, and inflammatory cytokine release.