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Decidual stromal cell-derived PGE₂ regulates macrophage responses to microbial threat

Lisa M. Rogers, BS^{*}, Anjali P. Anders, MD[†], Ryan S. Doster, MD^{*}, Elizabeth A. Gill, BS[‡], Juan S. Gnecco, PhD^{§,¶}, Jacob M. Holley, MSC^{*}, Tara M. Randis, MD, MS^{#,**}, Adam J. Ratner, MD, MPH^{#,**}, Jennifer A. Gaddy, PhD^{*,||}, Kevin Osteen, PhD^{§,¶,||}, and Dr. David M. Aronoff, MD^{*,§,¶}

^{*}Division of Infectious Diseases, Department of Medicine, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN

[§]Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN

[†]Division of Newborn Medicine, Department of Pediatrics, Washington University School of Medicine, St. Louis, MO

[‡]The University of the South, Sewanee, TN

[¶]Department of Obstetrics and Gynecology, Vanderbilt University Medical Center, Nashville, TN

^{||}Tennessee Valley Healthcare Systems, Department of Veteran Affairs, Nashville, TN

[#]Department of Pediatrics, New York University School of Medicine, New York, NY

^{**}Department of Microbiology, New York University School of Medicine, New York, NY

Abstract

Problem: Bacterial chorioamnionitis causes adverse pregnancy outcomes yet host-microbial interactions are not well characterized within gestational membranes. The decidua, the outermost region of the membranes, is a potential point of entry for bacteria ascending from the vagina to cause chorioamnionitis. We sought to determine whether paracrine communication between decidual stromal cells and macrophages shaped immune responses to microbial sensing.

Method of Study: Decidual cell-macrophage interactions were modeled *in vitro* utilizing decidualized, telomerase-immortalized human endometrial stromal cells (dTHESCs) and phorbol ester-differentiated THP-1 macrophage-like cells. The production of inflammatory mediators in response to LPS was monitored by ELISA for both cell types, while phagocytosis of bacterial pathogens (*E. coli* and Group B *Streptococcus* (GBS)) was measured in THP-1 cells or primary human placental macrophages. Diclofenac, a nonselective cyclooxygenase inhibitor, and prostaglandin E₂ (PGE₂) were utilized to interrogate prostaglandins as decidual cell-derived paracrine immunomodulators. A mouse model of ascending chorioamnionitis caused by GBS was

utilized to assess the co-localization of bacteria and macrophages *in vivo* and assess PGE₂ production.

Results: In response to LPS, dTHESC and THP-1 co-culture demonstrated enhancement of most inflammatory mediators, but a potent suppression of macrophage TNF- α generation was observed. This appeared to reflect a paracrine-mediated effect of decidual cell-derived PGE₂. In mice with GBS chorioamnionitis, macrophages accumulated at sites of bacterial invasion with increased PGE₂ in amniotic fluid, suggesting such paracrine effects might hold relevance *in vivo*.

Conclusion: These data suggest key roles for decidual stromal cells in modulating tissue responses to microbial threat through release of PGE₂.

Keywords

microfluidics; pregnancy; prostaglandins; fetal membranes; chorioamnionitis; infection

INTRODUCTION

Chorioamnionitis is a major cause of preterm birth (PTB) ¹, preterm premature rupture of membranes (PPROM) ², stillbirth ³, and neonatal sepsis ^{4, 5}. Bacterial chorioamnionitis provokes inflammatory responses that trigger early labor ⁶ and contribute to a fetal inflammatory response syndrome ¹. An estimated 25–50% of all PTBs are infection-related ^{7, 8}, and this contribution increases to more than 90% of PTBs occurring very early in gestation ^{9, 10}. In surviving children, chorioamnionitis is associated with long-term health sequelae ^{1, 11}. Unfortunately, these infections are often clinically silent until an adverse event occurs ¹².

The gestational membranes, also referred to as fetal or extraplacental membranes, are composed of three layers, including the fetally-derived amnion and chorion and the maternally-derived decidua. The decidual plane is the outermost layer of the membrane and thus the initial component to sense microbes ascending from the lower reproductive tract (vagina and cervix). The decidua is composed mainly of decidual stromal cells, along with macrophages and lymphocytes ^{13, 14}. Data suggest that decidual stromal cells and macrophages exhibit reciprocal paracrine communication ¹⁵ although this has not been explored in the context of infection. Decidual stromal cells are considered non-immune cells, but express functional pattern recognition receptors which are responsive to pathogen-associated molecular patterns, including lipopolysaccharide (LPS) ^{16–19}.

Prostaglandins (PGs) are ubiquitous lipid mediators derived from cyclooxygenase (COX)-dependent metabolism of arachidonic acid, an omega-6 polyunsaturated fatty acid found in cell membrane phospholipids ²⁰. PGs are involved in many aspects of parturition, including maternal immune tolerance to the fetus ^{21–24} and labor ^{25–28}. One particular PG, PGE₂, has potent immunomodulatory actions on both innate and adaptive immunity ^{20, 29–33}, and has been implicated as a decidual stromal cell-derived paracrine mediator of lymphocyte and natural killer cell function ^{22–24}. The extent to which PGE₂ or other PGs explain paracrine immunoregulation between decidual stromal cells and macrophages is not well defined.

In the current study, we assessed the nature of paracrine immunoregulation of macrophage-decidual stromal cell interactions, identifying an apparent role for decidual cell-derived PGE₂ in modulating macrophage responses to microbial threat.

MATERIALS AND METHODS

Note on Methods

A complete Materials and Methods section is provided as Supporting Information.

Bacterial strains and growth conditions

A spontaneous streptomycin resistant mutant generated from Group B *Streptococcus* (GBS) strain NCTC 1084 (1169-NT1; serotype V, provided by V. Nizet), the *E. coli* strain UTI89 / UPEC (provided by M. Mulvey) or the GBS strain 37 (GB37; serotype V, sequence type ST-1, provided by S. Manning) were used as indicated.

Mouse chorioamnionitis model

Animal experiments were performed in accordance with the Animal Welfare Act, U.S. federal law, and NIH guidelines. All experiments were carried out under a protocol approved by either Vanderbilt University Institutional Animal Care and Use Committee (IACUC; M/14/034), a body that has been accredited by the Association of Assessment and Accreditation of Laboratory Animal Care (AAALAC), or Columbia University IACUC (AC-AAAE3951, AC-AAAD1332). GBS infection of pregnant mice and subsequent analyses were performed as previously published³⁴ with minor modifications³⁵.

Mouse tissue immunohistochemistry

Pregnant animals were sacrificed on day E17.5 (96 h infection) and the entire fetal-placental unit (most proximal to cervix, right side) was removed, separated and fixed in 4% paraformaldehyde and paraffin-embedded. NYU Experimental Pathology Immunohistochemistry Core Laboratory performed macrophage staining and imaging. A subset of pregnant animals was sacrificed on day E15.5 (48 h infection) immunohistochemistry staining was performed by the Vanderbilt Translational Pathology Shared Resource.

Human gestational membrane culture and placental macrophage isolation

Human gestational membranes were excised from placental tissues from women who delivered healthy, full term infants by cesarean section without labor. De-identified tissue samples were provided by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute. All tissues were collected in accordance with Vanderbilt University Institutional Review Board (approval #131607) and Declaration of Helsinki. Gestational membranes were processed into 12-mm punch biopsies as published³⁶ or mounted on transwell devices as we have previously described^{37, 38}. Placental macrophages were isolated and cultured according to our previously published protocol³⁹.

Culture and decidualization of telomerase-immortalized human endometrial stromal cells

Telomerase-immortalized human endometrial stromal cells, THESC (American Type Culture Collection (ATCC) CRL-4003; Manassas, VA) can be decidualized into cells mimicking primary decidual stromal cells⁴⁰. Decidualized THESC cells (dTHESCs) were generated as originally published⁴⁰ using 0.5 mM 8-Bromo-cAMP (Tocris Bioscience; Minneapolis, MN), 1 μ M medroxyprogesterone acetate (MPA; Sigma), and 10 nM estradiol (E2; 17 β -estradiol-acetate; Sigma). The stromal cells were assessed for decidualization by measurement of human insulin-like growth factor binding protein-1 (IGFBP-1) and prolactin by ELISA (Alpha Diagnostic International; San Antonio, TX).

THP-1 macrophage culture and differentiation

The human monocytic leukemia-derived cell line, THP-1 cells (ATCC #TIB202), was maintained in RPMI 1640 (Invitrogen), 10% charcoal-stripped FBS, and 1% antibiotic/antimycotic solution (referred to as RPMI^{+/+}) in T-75 flasks until use in experiments. Phorbol myristate acetate (PMA, 5 ng/mL; Sigma) was used to differentiate the nonadherent monocytes into macrophage-like cells on the day prior to co-culture with dTHESCs (day 7).

THP-1 and dTHESC co-culture experiments

Inflammatory responses of individual cell cultures were performed in parallel with the co-culture of dTHESCs and PMA-differentiated THP-1 cells. Using a ratio of 1 THP-1 cell to 10 dTHESCs, THP-1 cells (1×10^5 /well) or dTHESCs (1×10^6 /well) were plated alone and in co-culture (1×10^6 dTHESCs + 1×10^5 THP-1 cells) in DMEM/F12^{+/+} media. Briefly, after THESC decidualization on day 8, PMA-differentiated THP-1 cells were added to the dTHESC wells for co-culture (or cells were plated individually as described above). Each of the cell groups (THP-1 alone, dTHESC alone, and co-culture) were rested 1 h before being treated with or without 1 μ g/mL LPS (ultrapure lipopolysaccharide from *E. coli* K12 strain; InvivoGen, San Diego, CA) in 1 mL total volume. These conditions were maintained for 24 h.

Organ-on-chip microfluidic device cell culture

Similar experimental analyses were performed using single-chamber organ-on-chip devices. The microfluidic organ-on-a-chip devices were assembled using our previously described protocol⁴¹.

Conditioned media (CM) experiments

THESCs were plated and decidualized as described above. On day 8 the media was changed and rested until day 10 (48 h), when supernatants were harvested and stored at -20°C . We refer to these supernatants as CM from untreated dTHESCs. In some instances, harvested dTHESC CM was subjected to heat-inactivation by treating for 10 min at 85°C . Gestational membrane 12-mm tissue punches were rested for 4 h in DMEM^{+/+} after excision from the placenta before being cultured in fresh DMEM^{+/+} for 24 h to generate CM, which was stored at -20°C until later use in macrophage culture experiments. THP-1 cells were PMA-treated as before and 1×10^5 cells were plated into individual wells of a 96-well plate (Corning). Cells were allowed 1 h for adherence before CM from untreated dTHESCs (heat-

inactivated or not) or gestational membrane CM were added to the THP-1 cells (200 μ L per well). Cells were pretreated in CM for 1 h prior to being treated with or without 1 μ g/mL LPS for 24 h.

Exogenous recombinant human cytokine treatment of macrophages

THP-1 cells were plated at 1×10^5 cells per well in a 96-well plate (200 μ L) and allowed to adhere for 1 h prior to the addition of 0.1 – 200 μ g/mL exogenous rhMIC-1, rhTGF- β 1, rhIL-10, rhVEGF, or rhG-CSF (each from R&D Systems) for 1 h before THP-1 cells were exposed or not exposed to 1 μ g/mL LPS for 24 h. Supernatants were collected for ELISA analysis.

Exogenous PGE₂ treatment of macrophages

THP-1 cells were plated at 1×10^5 cells per well in a 96-well plate (200 μ L) and allowed to adhere for 1 h prior to the addition of exogenous 1 μ M PGE₂ (Cayman Chemical, Ann Arbor, MI). Pretreatment with PGE₂ lasted for 15 m before the THP-1 cells were exposed or not exposed to 1 μ g/mL LPS or GBS (GB37) at a multiple of infection (MOI) of 10:1 for 24 h. For GBS treatment, macrophages were treated in the absence of antibiotic with live GBS for 1 h before 0.5% antibiotic/antimycotic was added for 24 h. Supernatants were collected for ELISA analysis.

Exogenous diclofenac treatment of dTHESC cells

A nonselective COX inhibitor, diclofenac (Cayman Chemical), was used to block PGE₂ production in dTHESCs *in vitro* to generate CM supernatants for later THP-1 experimentation. Briefly, a 6-well dish containing 1×10^6 dTHESCs in 1 mL was treated for 1 h with 3 μ g/mL diclofenac or solvent control (0.1% ethanol) to prime the stromal cells before DMEM/F12^{+/+} was changed and diclofenac was added again for 48 h with fresh media. CM supernatants were harvested, frozen, and assayed for the presence of PGE₂ by ELISA. Following dTHESC CM supernatant generation, THP-1 cells were PMA treated and plated at 1×10^5 per well in a 96-well plate (200 μ L) and rested for 1 h prior to pretreatment with diclofenac or solvent control-containing CM supernatant. After 1 h with CM supernatants, THP-1 cells were treated or not with 1 μ g/mL LPS for 24 h. Supernatants were collected and stored at -20°C for ELISA analysis.

Enzyme-linked immunosorbent assays

Enzyme-Linked Immunosorbent Assay (ELISA) analysis was performed according to manufacturers' instructions. Heat maps were generated from mean data using Morpheus software (Broad Institute, Cambridge, MA).

Macrophage phagocytosis assays

The ability of either THP-1 cells or human placental macrophages to internalize fluorescently-labeled, unopsonized bacteria was determined using fluorescein isothiocyanate (FITC)-labeled GBS (prepared as described⁴²) or commercially available FITC-conjugated *E. coli* Bioparticles, (Thermo Scientific)⁴³. Phagocytic activity was quantified as described⁴³.

Statistical analysis

For all statistical analyses, an α value of <0.05 was considered statistically significant ($P < 0.05$). 1-way repeated-measures ANOVA followed by Tukey's post-test was performed for experiments comparing more than two experimental groups. Paired Student *t*-tests were utilized to assess statistical significance for experiments with two groups. A *synergistic* response for inflammatory mediators measured in co-culture of THP-1 cells and dTHESC cells was defined when a statistically significantly greater numerical response in co-culture was observed, compared to the mathematical sum of the individual cultures. A suppressive response was defined as a statistically significantly lower numerical response measured in co-culture compared to the sum of the individual cultures. An *additive* response was defined as a statistically insignificant change seen in the co-culture when compared to the sum of the individual cultures.

RESULTS

Co-culture alters the inflammatory response of dTHESCs and THP-1 cells to LPS

Chorioamnionitis has been associated with increased recruitment of monocytes and macrophages into human gestational membranes, where they interact with decidual stromal cells ⁴⁴. 1×10^5 THP-1 macrophage-like cells were cultured either alone or together with 1×10^6 decidualized THESC cells (dTHESCs) (ratio of 10 dTHESC cells to 1 THP-1 cell) and then exposed to 1 $\mu\text{g/mL}$ of LPS for 24 h. Decidualization of THESC cells was confirmed by enhanced secretion of prolactin and insulin-like growth factor binding protein 1 (IGFBP-1) by day 6 of culture (data not shown). From culture supernatants, a panel of protein inflammatory mediators was quantitated by ELISA. Mediators selected for measurement included those implicated in chorioamnionitis and/or known to modulate macrophage production of TNF- α . Results are summarized in a heat map (Figure 1A) or presented individually in Figure 1B (for TNF- α) and Supplemental Figure 1 (all other mediators). The dTHESC cells generated significantly greater CCL2 in response to LPS stimulation versus control. The THP-1 macrophage-like cells produced significantly more TNF- α in response to LPS than unstimulated cells. The production of IL-1 β , CCL5, IL-10, macrophage inhibitory cytokine (MIC)-1, granulocyte colony stimulating factor (G-CSF), and transforming growth factor beta 1 (TGF- β 1) in response to LPS was not significantly altered in either cell type alone. VEGF production was below the limit of detection (LOD; 30 ng/mL) for each cell type tested. Co-cultured cells demonstrated a synergistic increase in the production of both CCL2 and G-CSF in response to LPS compared to the sum of the individual cell cultures. Co-culture led to additive LPS-stimulated responses for IL-10, IL-1 β , MIC-1, TGF- β 1 and CCL5, which were not statistically different than the mathematical sum from the individual cell types exposed to LPS. The dTHESC/THP-1 co-culture resulted in a significant *suppression* of the usual TNF- α production in response to LPS (Figure 1B).

Direct cell-cell contact is not required for dTHESC suppression of LPS-induced TNF- α production by THP-1 cells

Because THP-1 cells, and not dTHESC cells, generated measurable and significant increases in TNF- α in response to LPS, we tested the hypothesis that dTHESC cells inhibited LPS-

induced TNF- α production by THP-1 cells through the release of soluble, paracrine mediators. Supporting this, conditioned media (CM) obtained from dTHESC cells caused significant suppression of TNF- α production by LPS-stimulated THP-1 cells (Figure 1C).

Micro-scaling dTHESC-THP-1 cell co-culture in an organ-on-chip device recapitulates paracrine regulation of LPS-induced TNF- α production

Traditional cell culture uses high media-to-cell volume ratios, which potentially dilutes mediators and masks relevant physiological effects⁴⁵. We applied a microfluidic organotypic model⁴⁵ to assess the responses of dTHESC and THP-1 cells to LPS and define the extent to which such a model recapitulates traditional cell culture findings (Figure 2A). Individual cell and co-culture experiments performed in single-chamber organ-on-chip devices illustrated a statistically significant increase in TNF- α production by THP-1 cells after a 24 h exposure to LPS and suppression of the TNF- α response when co-cultured with dTHESC cells (Figure 2B), confirming the results from traditional plate-based culture experiments. Confluent cells within the organ-on-chip device were stained and assessed by immunofluorescence, which confirmed the presence of THP-1 cells by Cell Tracker Green staining and dTHESCs by anti-vimentin staining (Figure 2C). Cell viability was not reduced in the device (assessed using NucBlue® Live ReadyProbes® Reagent (Hoechst 33342) (data not shown).

dTHESC-derived prostaglandins impair macrophage TNF- α production

Protein mediators can suppress LPS-induced TNF- α expression by macrophages, including IL-10⁴⁶, TGF- β 1⁴⁷, G-CSF⁴⁸ and the TGF- β family member MIC-1⁴⁹. However, recombinant, human preparations of these mediators did not suppress THP-1 expression of TNF- α in response to LPS (Supplemental Figure 2) in our model system. Heating dTHESC CM to protein-denaturing conditions (10m, 85°C²¹) did not eliminate the paracrine inhibitory effect of the CM on LPS-induced TNF- α production by the THP-1 cells (Figure 3A). Prostaglandins (PGs) are heat-resistant lipid mediators. PGE₂ is well-known to impair macrophage expression of TNF- α in response to LPS through cAMP-dependent signaling⁵⁰. Decidual cells synthesize PGE₂^{24, 51}. We confirmed that dTHESC cells produced PGE₂, which was not altered by the heat treatment (Figure 3B) and found that exogenous PGE₂ significantly impaired the ability of either LPS-treated or GBS-exposed THP-1 cells to generate TNF- α (Figure 3C and D). Treatment of dTHESC cells with the cyclooxygenase inhibitor diclofenac significantly prevented PGE₂ synthesis (Figure 3E), and CM from diclofenac-treated dTHESC cells, but not from cells treated with the ethanol vehicle control, lost the capacity to limit macrophage TNF- α following LPS (Figure 3F).

Decidual cell-derived prostaglandins impair macrophage phagocytosis of *Streptococcus agalactiae* and *E. coli*

Prostaglandins such as PGE₂ are known to inhibit macrophage (and THP-1 macrophage-like cell) phagocytosis of bacterial pathogens^{43, 52}. Conditioned cell supernatant obtained from dTHESC cells significantly impaired the unopsonized ingestion of GBS and *E. coli* by THP-1 cells, actions that were prevented through the pharmacological inhibition of PG production using diclofenac (Figure 4A–B). We confirmed that exogenously-added PGE₂ could significantly impair macrophage ingestion of unopsonized GBS and *E. coli* using

either THP-1 cells or placental macrophages (Figure 4C–F). We also tested the ability of human placental macrophages to phagocytose *E. coli* in response to dTHESC conditioned media treated or not treated with diclofenac and observed similar suppression as we did with THP-1 cells (Figure 4G).

PGE₂ is produced by human gestational membranes, and this effect is increased by infection *ex vivo*

Chorioamnionitis in humans is associated with increased PGE₂ in the amniotic fluid ⁵³ and animal models show similar increases in amniotic fluid following bacterial infection, as demonstrated in rabbits ⁵⁴ and nonhuman primates ⁵⁵. *Ex vivo*, we utilized human gestational membrane 12-mm punch biopsies to assess the capacity of full thickness membrane tissues to produce PGE₂ basally and following exposure to live GBS. The average PGE₂ concentration in CM from uninfected membrane punches was 3643 ± 1791 pg/ml (mean ± SEM) and was increased significantly after infection with either of two strains of GBS *ex vivo* (Figures 5A and 5B). Exposure of THP-1 cells to uninfected CM from gestational membrane punches resulted in a significant attenuation of LPS-induced TNF-α production (Figure 5C). Using human gestational membrane tissues organized into transwell constructs to provide directionality to infection, we found that GBS was able to invade into full-thickness membrane tissues and that macrophages appeared to accumulate in areas of infection, based on CD68 immunohistochemistry, suggesting that migration of these cells towards the microbial invaders occurred *in situ* after infection (Figure 5D).

Murine model of ascending chorioamnionitis results in GBS gestational tissue invasion, PGE₂ secretion and macrophage recruitment *in vivo*

Using an ascending mouse model of bacterial chorioamnionitis, we interrogated macrophage influx and bacterial dissemination into the gestational tissues. Histopathologic evaluation of gestational tissues identified yolk sac membrane and placental invasion by GBS at both 48 h (Figure 6A–B, bottom panels) and 96 h (Figure 6D–E), as well as an influx of F4/80+ macrophages into the placental tissues at both time-points (Figure 6A–B, top panels; Figure 6D–E), which was not observed in sham-infected controls. Amniotic fluid PGE₂ accumulation was significantly increased following GBS infection at 48 h (Figure 6C).

DISCUSSION

Bacterial chorioamnionitis, an inflammatory condition of the gestational membranes, is an important cause of adverse pregnancy outcomes. A common paradigm holds that chorioamnionitis is usually caused by ascending bacterial infection from the vagina ⁵⁶. The decidua is the outermost layer of the gestational membranes and potentially the first to contact microbes during ascending infection ¹⁹. Defining early events in the pathogenesis of chorioamnionitis, such as when bacteria first contact the decidua, might reveal actionable targets for disease prevention or therapy.

The decidua is composed of non-immune and immune cells, including decidual stromal cells, macrophages and lymphocytes ^{13, 57}. Though not considered immune cells *per se*, decidual stromal cells express pattern recognition receptors (PRRs) including toll-like

receptors and nucleotide-binding oligomerization domain receptors¹⁹. Previous studies have shown that these PRRs are functional and responsive to pathogen associated molecular patterns (PAMPs) including LPS, leading to a pro-inflammatory cascade (reviewed in¹⁹). That decidual stromal cells and macrophages can communicate through paracrine mediators has been observed¹⁵ but not explored in the context of the host response to bacterial infection.

The present study newly documents the capacity of dTHESC cells to regulate the response of THP-1 macrophage-like cells to LPS and intact bacteria, through the elaboration of prostaglandins, such as PGE₂. We found that decidualized stromal cells (dTHESCs) significantly increased their expression of CCL2 in response to LPS, which was consistent with previously published findings of other investigators^{58–61}. The dTHESC cells did not demonstrate significantly increased production of CCL5, IL-10, TGF-β1, MIC-1, G-CSF, or IL-1β following LPS exposure. Prior studies have reported mixed results in TNF-α production in response to LPS by decidual stromal cells^{58, 62, 63}, and VEGF was below the limit of detection in our system. In our studies, PMA-differentiated THP-1 macrophage-like cells produced significant increases in TNF-α in response to LPS. Interestingly, dTHESC-THP-1 co-culture demonstrated a synergistic increase in production in CCL2 and G-CSF but an antagonistic (suppressive) response in the production of TNF-α.

The ability of the dTHESC-THP-1 co-culture to impair TNF-α production in response to LPS was replicated in a microfluidic organ-on-chip model of the decidua. These data also support the use of microfluidic devices to model cell-cell interactions⁴⁵. Importantly, we used conditioned cell supernatants to establish that the immune modulating factor(s) derived from dTHESC cells could suppress TNF-α production by THP-1 cells in response to LPS in a paracrine fashion and independent of cell-cell contact. Furthermore, we also found that supernatants from human gestational membranes cultured *ex vivo* could also blunt the same TNF-α response to LPS by the THP-1 macrophages, suggesting that such events could occur *in situ*.

The capacity for decidual stromal cells to suppress macrophage TNF-α is consistent with an existing body of literature suggesting that macrophage activation is actively suppressed within the decidua basalis^{64–66}. This immunomodulatory action of decidual stromal cells has been implicated in promoting maternal immune tolerance against the semi-allogeneic fetus^{64, 65}. The mechanisms whereby decidual stromal cells modulate macrophage immune activation remain unclear. Unidentified decidual extracellular matrix components have been implicated^{64, 65} and the present studies suggest that soluble lipid mediators might be involved. Paracrine factors known to suppress macrophage TNF-α expression include PGE₂, TGF-β, IL-10, G-CSF and MIC-1^{49, 67–69}. Our studies, using either heat to denature proteins or applying recombinant human preparations of TGF-β, IL-10, G-CSF, VEGF and MIC-1 failed to support a role for these mediators in suppressing TNF-α. We therefore concentrated our efforts on lipid mediators as possible candidates.

Prostaglandin E₂ is an oxygenated metabolite of the cell membrane constituent arachidonic acid and is synthesized by the sequential metabolism of arachidonic acid by cyclooxygenase (COX) and PGE synthase (PGES) enzymes. Its expression is greatly augmented at sites of

infection and inflammation³³. Our group and others have shown that PGE₂ is a potent negative regulator of innate immunity^{43, 52, 70–82}. Bacteria are among the most potent inducers of PGE₂ production^{83, 84}. Once produced, PGE₂ signals in an autocrine or paracrine manner via four unique G protein-coupled E prostanoïd (EP) receptors, termed EP1, EP2, EP3, and EP4⁸⁵. In simple terms, the EP1 receptor signals via increasing intracellular calcium, EP2 and EP4 receptors activate adenylate cyclase to increase intracellular cyclic adenosine monophosphate (cAMP) levels, while the EP3 receptor can signal via both calcium and suppression of cAMP production. This is important because most immunomodulatory actions of PGE₂ result from EP2 and/or EP4-dependent cAMP signaling cascades^{29, 30, 43, 52, 80, 86–88}. Inhibiting PGE₂ synthesis or signaling has been shown to improve innate immunity against diverse pathogens, particularly with regards to phagocyte function^{43, 71, 72, 80, 89–92}. In addition, studies suggest a beneficial effect of inhibiting PGE₂ synthesis during severe infections (reviewed in⁷⁹). Targeting PGE₂ synthesis or signaling is a host-centered approach for enhancing immune defenses and such tactics are increasingly important in the modern age of antibiotic resistance.

We and others have accrued data specifically demonstrating a suppressive impact of PGE₂ on female reproductive tract host defense^{70, 81, 93–95}. Aronoff and colleagues reported that the intrauterine application of the PGE₂ analogue misoprostol impaired innate immune defenses and increased mortality in a rat model of *Clostridium sordellii*, a cause of postpartum and post-abortion endometritis⁹⁴. Misoprostol was a potent suppressor of macrophage anti-bacterial actions against this pathogen⁹⁴. Rogers, *et al.* confirmed similar actions of native PGE₂ on macrophage phagocytosis, intracellular killing, and cytokine responses to infection with both *C. sordellii*⁵² and the postpartum sepsis agent Group A *Streptococcus*⁸¹. Mason and colleagues also found that mice treated with intrauterine PGE₂ (to mimic the elevated PGE₂ levels of pregnancy) were highly vulnerable to a lethal Group A *Streptococcus* endometritis, and mice genetically lacking the microsomal PGE synthase-1 enzyme (who could not generate normal levels of PGE₂) were protected⁸¹. In addition, mice lacking the EP2 receptor for PGE₂, or treated with an EP4 receptor antagonist, were protected from lethal infection⁸¹. A study by Slama, *et al.* revealed that a stable PGE₂ analogue delivered into the maternal cervix postpartum in cows increased the incidence of puerperal endometritis⁹³. Lastly, systemically-increased PGE₂ facilitated the establishment of chlamydial uterine infections in mice⁷⁰. Thus, we speculate that high PGE₂ levels in the female reproductive tract during pregnancy increase susceptibility to infection, and it is known that decidual cells synthesize PGE₂^{23, 24, 51}.

In the current study, we confirm that PGE₂ accumulates in dTHESC conditioned media supernatants, and that heat-inactivation of these conditioned media supernatants results in a suppression of LPS-induced TNF- α similar to that which we saw when we used non-heat-treated conditioned media. Heat-treatment itself did not affect PGE₂ levels in the conditioned media, confirming PGE₂ is in fact a heat-tolerant lipid. We show that exogenously added PGE₂ is able to suppress both LPS- and GBS-induced TNF- α production by THP-1 macrophages, suggesting it might play a paracrine role in the dTHESC conditioned media supernatant suppression of LPS-induced TNF- α that we saw. Treatment of dTHESC cells with the cyclooxygenase inhibitor diclofenac significantly prevented PGE₂ synthesis, and conditioned media supernatant from diclofenac-treated dTHESC cells, but not

from cells treated with the ethanol vehicle control, lost the capacity to limit macrophage TNF- α following LPS stimulation. Prostaglandins such as PGE₂ are known to inhibit macrophage phagocytosis of bacterial pathogens^{43, 52}. In our hands, conditioned media supernatant obtained from dTHESC cells significantly impaired the unopsonized ingestion of GBS and *E. coli* bioparticles by THP-1 cells and human primary placental macrophages, actions that were prevented through the pharmacological inhibition of PG production using diclofenac. We confirmed that exogenous PGE₂ impaired macrophage ingestion of GBS and *E. coli* bioparticles using both THP-1 cells and placental macrophages.

Chorioamnionitis in humans is associated with increased PGE₂ in the amniotic fluid⁹⁶ and animal models show similar increases following bacterial infection, as demonstrated in rabbits⁵⁴ and nonhuman primates^{55, 97}. We utilized an *ex vivo* punch biopsy system of human gestational membranes to assess the capacities of the choriodecidua and the amnion to produce PGE₂ following exposure to GBS. Uninfected conditioned media obtained from human gestational membrane punch biopsies cultured *ex vivo* significantly impaired LPS-induced TNF- α production by THP-1 cells, and microbial (GBS) stimulation of the choriodecidual surface stimulated significant PGE₂ generation. GBS-infected gestational membranes show GBS invasion of the tissue and increased CD68+ macrophage staining at the site of infection. Using a murine model of ascending bacterial chorioamnionitis, we show that GBS ascends and invades gestational membrane and placental tissues after 48 and 96 hours of infection. F4/80+ macrophages traffic to areas of GBS infection, and amniotic fluid PGE₂ accumulation is significantly increased.

Our studies and model systems in general have several limitations. We employed cell lines as opposed to primary cells. While dTHESCs and PMA-differentiated THP-1 cells are standard models for primary DSCs and macrophages, respectively, they are immortal cells which likely differ from primary cells in ways that are not fully characterized. Another potential limitation is that we studied only two cell types, while in normal gestational membranes more than two cell types are present. Future models could incorporate both immune and non-immune cells in addition to decidualized stromal cells and macrophages. Furthermore, the ratios of decidualized stromal cells to macrophages we used might not reflect the *in vivo* situation. Finally, we recognize that LPS is a single PAMP, not an intact pathogen. Future studies will benefit from the introduction of living microbes into models of the human decidua or gestational membrane.

These results newly identify significant paracrine interactions between decidual stromal cells and macrophages in response to PAMP-based and microbial immune stimulation. The factors and molecular mechanisms governing these effects, in part, are due to the heat-stable lipid PGE₂. Future studies aimed at identifying other possible paracrine mediators with potent immunomodulatory actions in the decidua will help shed new light on the nature of maternal-fetal immune tolerance and host susceptibility to chorioamnionitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Co-culture alters the inflammatory response of dTHESCs and THP-1 cells to lipopolysaccharide (LPS) driven by dTHESC conditioned media (CM) paracrine signaling. Macrophages, stromal cells, direct co-cultures, or dTHESC conditioned media cultures were stimulated with 1 μ g/mL LPS for 24 h and supernatants were collected for ELISA analysis. **A.** Heat map of inflammatory mediator analysis results from 6-well plates containing 1×10^5 THP-1 cells, 1×10^6 dTHESCs, or co-culture combined in 1 mL media. **B.** TNF- α production (pg/mL; n=13) from 6-well plates containing 1×10^5 THP-1 cells, 1×10^6 dTHESCs, or co-culture combined in 1 mL media. **C.** TNF- α production (pg/mL; n=6) from 96-well plates containing 1×10^5 THP-1 cells using 200 μ L dTHESC CM. STATISTICS: **(B-C)** 1-way RM ANOVA with Tukey's post-test; mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$.

Figure 2: TNF- α profile of THP-1 cells, decidualized THESCs, and co-culture cells cultured in single-chamber chip devices.

2×10^6 /mL THP-1 cells, 5×10^6 /mL dTHESCs, or co-culture cells were loaded into chip devices and rested for 1 h before being stimulated with $1 \mu\text{g}/\text{mL}$ LPS for 24 h. **A.**

Photographic image and schematic of the device. **B.** TNF- α analysis by ELISA (pg/mL; $n=5$). **C.** Representative fluorescent images of cells within the chip devices (10 $\times$; blue represents NucBlue Live (Hoechst 33342), green represents CellTracker Green, and red represents anti-Vimentin).

STATISTICS: **(B)** 1-way RM ANOVA with Tukey's post-test; mean $\pm$ SEM; *** $p < 0.001$.

Figure 3: The heat-tolerant immunomodulatory lipid PGE₂ plays a role in the LPS-induced suppression of TNF-α seen in coculture cells in a paracrine manner.

dTHESC conditioned media (CM) was heat-inactivated at 85°C for 10 m before being placed onto 1×10^5 THP-1 cells in a 96-well plate for 1 h (200 μL). Cells were then stimulated with 1 μg/mL LPS for 24 h before supernatants were harvested for TNF-α ELISA analysis. **A.** Heat-inactivated dTHESC CM suppresses LPS-induced TNF-α (pg/mL; n=3). **B.** Validation that PGE₂ remained in the dTHESC CM after heat-inactivation (pg/mL; n=3). **C.** 1×10^5 THP-1 cells in a 96-well plate were pretreated for 15 m with 1 μM exogenous PGE₂ before being stimulated with 1 μg/mL LPS for 24 h. PGE₂ appears to be a potent suppressor of LPS-induced TNF-α (pg/mL; n=4). **D.** 1×10^5 THP-1 cells in a 96-well plate were pretreated for 15 m with 1 μM exogenous PGE₂ before being stimulated with live GBS at a multiple of infection (MOI) of 10:1 for 24 h. PGE₂ appears to be a potent suppressor of GBS-induced TNF-α (pg/mL; n=7). **E.** Treatment of 1×10^6 dTHESCs in a 6-well plate with the cyclooxygenase inhibitor diclofenac significantly prevented PGE₂ synthesis (pg/mL; n=3), and **F.** CM from diclofenac-treated dTHESC cells lost the capacity to limit macrophage TNF-α following 24 h LPS stimulation (pg/mL; n=3) (1×10^5 THP-1 cells in a 96-well plate with 200 μL). STATISTICS: (A, C, D, E, F) 1-way ANOVA followed by Tukey's post-test or (B) paired student *t*-test; mean ± SEM for all graphs; **p*<0.05, ***p*<0.01, and ****p*<0.001.

Figure 4: Prostaglandins from conditioned dTHESC medium and exogenous PGE₂ impair macrophage phagocytosis of *Streptococcus agalactiae* (GBS) and *E. coli*.

2×10^5 THP-1 cells in a 384-well plate were treated for 30 m with dTHESC-generated CM (3 μg/mL diclofenac or 0.1% solvent control) before being stimulated with **A.** heat-killed FITC-GBS at MOI 150:1 (n=3), or **B.** FITC-*E.coli* bioparticles at MOI 50:1 (n=3) for 3 h. **C.** 2×10^5 THP-1 cells or **D.** placental macrophages were treated with 1 μM exogenous PGE₂ for 15 m before being stimulated with heat-killed FITC-GBS at MOI 150:1 for 3 h. **E.** 2×10^5 THP-1 cells or **F.** placental macrophages were treated with 1 μM exogenous PGE₂ for 15 m before being stimulated with FITC-*E.coli* bioparticles at MOI 50:1 for 3 h. **G.** 2×10^5 placental macrophages were treated for 30 m with dTHESC-generated CM (3 μg/mL diclofenac or 0.1% solvent control) before being stimulated with FITC-*E.coli* bioparticles at MOI 50:1 (n=3) for 3 h. Phagocytosis was quantified by fluorescence. Data are presented as Phagocytic Activity (% of untreated control). STATISTICS: (**A, B, G**) 1-way ANOVA

followed by Tukey's post-test and (C-F) paired student *t*-test; mean $\pm$ SEM for all graphs;
p*<0.05, *p*<0.01, and ****p*<0.001.

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Figure 5: *Ex vivo* model of chorioamnionitis results in GBS membrane invasion, PGE₂ secretion and macrophage recruitment.

Using a 12-mm punch biopsy culture system of human gestational membranes, conditioned media (CM) supernatant was generated for use on THP-1 cells. **A.** 24 h GBS-infected gestational membrane biopsies secrete PGE₂ *ex vivo* in response to 1×10^6 CFU GBS (GB37) or **B.** GB1084 (fold change compared to control; $n=7-8$). **C.** 24 h uninfected CM obtained from the gestational membrane punch biopsies significantly reduced TNF-α production by 1×10^5 THP-1 cells in a 96-well plate after 1 h pretreatment and 24 h stimulation with 1 μg/mL LPS (pg/mL; $n=4$). **D.** Human gestational membranes were organized into transwell constructs and infected with GBS (1×10^6 CFU GB37) on the choriodecidual surface for 48 h before tissue processing and immunohistochemistry analysis. Tissues were stained with anti-CD68 for macrophages (top panels) or anti-GBS (bottom panels). Tissue analysis shows invasion of human membrane tissues *in vitro* by GBS and the presence of macrophages within the choriodecidual (CD) at the site of infection.

AE = amniotic epithelium. STATISTICS: (A, B) paired student t-test; mean $\pm$ SEM; * $p<0.05$ and ** $p<0.01$; (C) 1-way ANOVA with Tukey's post-test; mean $\pm$ SEM; ** $p<0.01$.

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Figure 6: Mouse model of ascending chorioamnionitis results in GBS gestational tissue invasion, PGE₂ production and macrophage recruitment.

Pregnant mice at day E13.5 were intravaginally inoculated with 1×10^3 CFU GBS (GB37) and sacrificed on E15.5 (48 h after infection). Placental tissues were dissected and evaluated by immunohistochemistry for GBS invasion and the presence of F4/80+ macrophages.

Tissue analysis demonstrates that after 48 h of ascending infection, GBS invades into (A) gestational membrane (B) and placental tissues (bottom panels). F4/80+ macrophages traffic to the site of tissue infections (top panels of A and B). C. Amniotic fluid was isolated from control- and GBS-infected mice and assayed for PGE₂ following 48 h GBS (GB37) vaginal infection (pg/mL; n=3). Bacterial infection results in a statistically significant increase in amniotic fluid PGE₂. D. Pregnant mice at day E13.5 were intravaginally inoculated with 1×10^7 CFU GBS (GB1084) and sacrificed on E17.5 (96 h after infection). Placental tissues were dissected and evaluated by immunohistochemistry for GBS invasion and the presence of F4/80+ macrophages. Immunohistochemistry from D. sham and E. GBS-infected mouse

gestational tissues demonstrate that GBS invades placental tissues and F4/80+ macrophages influx into infected tissues. STATISTICS: (C) paired student t-test; mean $\pm$ SEM; ** $p<0.01$.

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