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Peptidoglycan Induces Necrosis and Regulates Cytokine Production in Murine Trophoblast Stem Cells

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Abstract

Problem—Intrauterine bacterial infection during pregnancy may lead to adverse outcome. The objective of this study was to assess whether peptidoglycan (PGN) derived from Gram-positive bacteria induces trophoblast stem (TS) cell death or alters TS cell cytokine production.

Method of study—TLR transcript expression was assessed by RT-PCR. Protein expression was determined by confocal microscopy or flow cytometry. 7-Aminoactinomycin D (7-AAD) staining was used to assess TS cell death. Morphological features of cell death were evaluated by transmission electron microscopy. The presence of cleaved caspase-3 and HMGB1 protein was examined by western blot. Cytokine levels in cell supernatants were determined using a mouse cytokine 23-plex panel.

Results—TLR2 and TLR4 protein was expressed from the 1-cell through the blastocyst stage of murine embryo development. Murine TS cells expressed TLR2 and TLR6 but not TLR1 or TLR4 RNA. Only TLR2 protein was detected at the plasma membrane of TS cells. PGN induced TS cell death by a caspase-3 independent mechanism. The cell death pathway induced by PGN was morphologically consistent with necrosis. Finally, PGN induced HMGB1 release and increased MIP-1 β secretion while inhibiting the constitutive release of RANTES.

Conclusion—PGN-induced TS cell necrosis and the subsequent release of HMGB1 and MIP-1 β may regulate an infection-induced inflammatory response at the maternal-fetal interface and thus may play a role in the pathogenesis of infection-associated pregnancy complications.

Keywords

TLR; trophoblast stem cell; necrosis; proinflammatory cytokines

Introduction

Intrauterine bacterial and viral infections are clinically associated with specific pregnancy complications including intrauterine growth restriction (IUGR), preeclampsia, and preterm birth¹. It is postulated that the detrimental effects of infection on pregnancy are due to the

activation of the innate immune system which leads to excessive inflammation and apoptosis at the maternal-fetal interface and thus poor pregnancy outcome ²⁻⁵.

Toll-like receptors (TLRs) are innate immune receptors, which detect foreign microbes through the recognition of conserved pathogen-associated molecular patterns or PAMPs ⁶. PAMPs are widely expressed among microbes but are not present in host cells thus TLR recognition of PAMPs is one mechanism by which the host detects non-self ⁷. In addition to exogenous ligands TLRs also recognize endogenous ligands, which may be released by an inflammatory response or by damaged tissue thus they are referred to as danger signals ⁸. TLR activation triggers inflammation, which is critical for protection against bacterial and viral infections ⁸.

TLRs are expressed at the maternal-fetal interface on both maternal immune cells and gestational tissues including trophoblast cells of the placenta ⁹. TLR expression among trophoblasts differs by lineage and gestational age ¹⁰. Importantly, TLRs have been implicated in inflammation-associated pregnancy complications. The role of TLRs in preterm birth has become increasingly well-defined ¹¹⁻¹⁸. In addition, it is postulated that TLRs play a role in the pathogenesis of pre-eclampsia ^{19, 20}.

Previous studies demonstrated that TLR activation induces direct effects on trophoblast cells ⁹. Ligation of TLR2 with PGN induced apoptosis in human first trimester trophoblast cells by a TLR1 and TLR2 dependent mechanism ^{21, 22}. The presence of TLR6 attenuated this response ²¹. In contrast, activation of TLR4 with LPS did not induce apoptosis rather it resulted in increased cytokine production by trophoblasts ^{22, 23}. Trophoblast cells were also shown to respond to the TLR3 ligand poly (I:C) resulting in the secretion of antiviral factors and the upregulation of chemokine production ^{23, 24}. Importantly, TLR activation on trophoblasts regulates immune cell migration through the production of chemokines ²³. Thus a growing body of evidence suggests that trophoblast cells are a pregnancy-specific component of innate immunity ²⁵. Indeed, the placenta may play a pivotal role in controlling bacterial and viral infections at the maternal-fetal interface.

Although TLR responses in human first trimester trophoblasts are becoming increasingly well defined little is known regarding the ability of trophoblast progenitor cells specifically trophoblast stem cells to detect and respond to pathogens at the maternal-fetal interface. As TLR signaling may contribute to pregnancy pathologies associated with placental dysfunction we sought to determine whether bacterial products adversely affect the cells involved in the earliest stages of placentation. The objective of this study was to begin to characterize the ability of early embryos and TS cells to sense bacterial products. In addition, we wished to examine the ability of TS cells to respond to PGN, a TLR2 ligand. In this study we report that TLR2 and TLR4 protein are expressed from the 1-cell through the blastocyst stage of murine preimplantation embryo development. Murine TS cells expressed TLR2 and TLR6 but not TLR1 or TLR4 RNA. Interestingly, only TLR2 protein was detected at the plasma membrane of TS cells. PGN induced TS cell death in a manner that was consistent with necrosis. In addition, PGN induced HMGB1 release and increased MIP-1 β secretion while inhibiting the constitutive release of RANTES. The ability of PGN to regulate the release of proinflammatory molecules from TS cells has the potential to modulate an infection-induced inflammatory response by altering the composition of the immune cell populations or their activation status at the maternal-fetal interface. These alterations may ultimately contribute to infection-associated pregnancy complications. Therefore, bacterial infections may adversely affect placentation at its earliest stages by inducing TS cell necrosis and by regulating the release of proinflammatory molecules.

Materials and methods

Embryo recovery

Embryos were recovered as previously described²⁶. Briefly, three-week old female mice (B6SJLF1/J, Jackson Laboratories, Bar Harbor, ME) were given free access to food and water and were maintained on a 12 h light/dark cycle. Mice were superovulated with 10 IU pregnant mare serum gonadotropin (Sigma, St. Louis, MO, USA) per animal followed 48 h later by 10 IU human chorionic gonadotropin (hCG, Sigma) per animal. The hormones were delivered by intraperitoneal injection. Immediately following the hCG injection female mice were mated with males overnight. Mating was confirmed by identification of a vaginal plug. The morning of the vaginal plug is referred to as embryonic day 0.5 (E0.5).

Mice were sacrificed at E0.5, E1.5, E2.5, and E3.5 to isolate embryos at the 1-cell, 2-cell, 4-cell, morula, and blastocyst stage of development. All procedures described above were reviewed and approved by the animal studies committee at Washington University and were performed in accordance with IACUC approval.

Immunofluorescent Staining

Embryos were stained in microdroplets on Superfrost Plus microscope slides (ThermoFisher Scientific Inc., Waltham, MA, USA). Embryos were fixed in 3% paraformaldehyde (Sigma) for 20 min, washed with phosphate-buffered saline (PBS, Sigma), and then permeabilized in 0.1% Tween 20 (Sigma) for 20 min. All incubations were performed at room temperature. The embryos were blocked in PBS supplemented with 2% BSA and 20% normal goat serum (ThermoFisher Scientific, Inc.) for 1 h. The cells were then stained with one of the following antibodies: anti-mouse TLR2 (R & D Systems, Minneapolis, MN, USA) anti-mouse TLR4 (Abcam, Cambridge, MA, USA), or an irrelevant species matched control antibody (R & D Systems) at a concentration of 20 µg/ml. The embryos were then washed in PBS/2% BSA and incubated in 8 µg/ml Alexa Fluor 488 goat anti-rabbit secondary antibody for 30 min (Invitrogen, Carlsbad, CA, USA). The embryos were washed in PBS/2% BSA and incubated in 2 µM TO-PRO-3 iodide (Invitrogen), a nuclear stain, for 20 min. Finally the embryos were washed in PBS and mounted in drops of Vectashield (Vector Laboratories, Burlingame, CA). Fluorescence was detected with a Nikon C1 laser-scanning confocal microscope (Nikon Instruments Inc., Melville, NY, USA). Confocal images were taken at 60X magnification and visualized using Nikon EZ-C1 3.6 software. Staining of individual embryos was performed at least three times with a minimum of five embryos per group.

Cells

TS cells were generously provided by Dr. Janet Rossant (The Hospital for Sick Children, Toronto, Ontario, CA) and will be referred to as TS cell line 1²⁷. A second TS cell line was derived as previously described²⁸ and will be referred to as TS cell line 2. TS cells were maintained in the absence of a mouse embryonic fibroblast (MEF) feeder layer cultured instead in the presence of MEF conditioned media as previously described²⁹. RAW264.7 cells, a murine monocyte-macrophage cell line was obtained from American Type Culture Collection (Manassas, VA, USA) and cultured as described elsewhere³⁰.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from TS cell lines and RAW264.7 cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). One microgram of RNA was reverse transcribed using the Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Each PCR reaction contained 250 ng cDNA, 200 µM dNTPs (Invitrogen), 25 pMoles of each primer (Integrated DNA Technologies, San Diego, CA, USA) 1x ThermoPol reaction buffer (New England, BioLabs Inc., Ipswich, MA, USA), and 1.25 units Taq DNA

polymerase (New England BioLabs). Negative control samples (–) did not contain reverse transcriptase but did contain template and TLR specific primers. The PCR program was as follows: 94°C for 2 min, 94°C for 30 s, 52°C for 30 s and 72°C for 30 s. The PCR program contained 45 cycles followed by a 5 min extension at 72°C. The program was performed on a DNA Engine Peltier Thermal Cycler (BioRad, Hercules, CA, USA). The murine primer sequences were as follows: *TLR1* forward primer: 5'GTTGTCAGTGTCTTCAGC, *TLR1* reverse primer: 5'GCTGTACCTTAGAGAATTCTG (product size 320 bp), *TLR2* forward primer: 5'ACAACTTACCGAAACCTCAGAC, *TLR2* reverse primer: 5'ACCCCAGAAGCATCACATG (product size 145 bp), *TLR4* forward primer: 5'CAGTGGTCAGTGTGATTGTGG, *TLR4* reverse primer: 5'TTCCTGGATGATGTTGGCAGC (product size 265 bp), *TLR6* forward primer: 5'CAACTTAACGATAACTGAGAG, *TLR6* reverse primer: 5'CCAGAGAGGACATATTCTTAG (product size 365 bp), *actin* forward primer: ACCTTCTACAATGAGCTGCG, *actin* reverse primer: CTGGATGGCTACGTACATGG (product size 146 bp). PCR products were cloned and their identity confirmed by sequencing.

Flow cytometry reagents

An anti-mouse TLR1 PE antibody (eBio TR23), an anti-mouse TLR2 PE antibody (6C2), an anti-mouse TLR4/MD-2 complex PE antibody (MTS510), a rat IgG2b PE isotype control antibody (P3), and a rat IgG2a PE isotype control antibody were purchased from eBioscience (San Diego, CA, USA). An anti-mouse TLR6 PE antibody (418601) was purchased from R&D Systems (Minneapolis, MN, USA). 7-Aminoactinomycin D (7-AAD) was purchased from BD Biosciences (San Jose, CA, USA).

Flow cytometry

TLR1, TLR2, TLR4/MD2, and TLR6 staining—TS cells and RAW264.7 cells were harvested and blocked in 5% normal rat sera and 5% normal mouse sera in PBS/10% FBS for 15 min at 4°C. Cells were then stained with an anti-mouse TLR1, TLR2, TLR4/MD2, or TLR6 antibody or the appropriate isotype control antibody. Cells were incubated for 30 min on ice with gentle agitation after 15 min. The cells were washed and resuspended in PBS/10% FBS. Data was collected using a BD FACSCalibur flow cytometer (Becton Dickinson) and analyzed with FlowJo software (TreeStar, Inc., Ashland, OR, USA).

7-AAD cell staining—TS cells were plated at 250,000 cells/well in 6 well plates (ThermoFisher Scientific Inc.) and allowed to adhere overnight at 37°C and 5% CO₂. Cells were either untreated or stimulated with 10 µg/ml, 25 µg/ml, 50 µg/ml, or 100µg/ml PGN from *Staphylococcus aureus* (PGN, Sigma) for 24 h *in vitro*. The cells were then harvested, washed twice, and resuspended in 150 µl PBS/10%FBS with 2.5 µl 7-AAD. The cells were incubated in the dark for 10 min at 4°C and data was collected using a FACSCalibur.

Western Blot Analysis

Cleaved caspase-3—TS cells were plated and stimulated as described above for 7-AAD staining. In addition to PGN, cells were also treated with 0.2 µM staurosporine for 12 h as a positive control. Cells were harvested and lysates were generated. The lysates were quantified using the BCA protein assay (ThermoFisher Scientific, Inc.). Fifteen micrograms of protein was added to gel loading buffer and boiled for 5 min. Samples were loaded on a 12% sodium dodecyl sulphate-polyacrylamide gel. The gels were transferred onto nitrocellulose membranes (BioRad) and blocked in 5% BSA in tris-buffered saline/0.05% Tween-20 (TBS-T) for 1 h. The membranes were then incubated overnight at 4°C in blocking buffer containing an anti-cleaved caspase-3 antibody (Cell Signaling Technology,

Danvers, MA, USA). The membranes were subsequently washed and incubated for 30 min in TBS-T containing a horseradish peroxidase conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The blots were developed using Supersignal west dura (ThermoFisher Scientific, Inc.). The membranes were then stripped and reprobed with an anti-actin antibody (Millipore, Billerica, MA, USA) as a loading control.

HMGB1—TS cells were plated and stimulated as described above for 7-AAD staining. Supernatants were collected after 24h PGN treatment and centrifuged at 1,000 rpm for 5 min. The supernatants were then transferred to a new tube and an albumin/IgG removal kit was used on the supernatants according to the manufacturer's protocol (Thermo Scientific Pierce, Waltham, MA, USA). Twenty-five microliters of each supernatant was added to 5X gel loading buffer and boiled for 5 min. The samples were run on a 4–15% gradient SDS-PAGE gel and transferred to nitrocellulose. The samples were blocked as described above and incubated overnight at 4°C with an anti-HMGB1 antibody (Abcam). The membranes were then processed as previously described. As a loading control membranes were incubated with an amido black staining solution (Sigma) according to the manufacturer's protocol.

Transmission Electron Microscopy

TS cell line 1 was plated as described above for 7-AAD staining and was untreated, treated with 0.2 μ M staurosporine for 12 h *in vitro*, or treated with 50 μ g/ml PGN for 24h. Cells were fixed in 2% paraformaldehyde/2.5% glutaraldehyde and 1% osmium tetroxide as previously described³¹. Briefly, cells were embedded in Embed 812 resin, sectioned, and stained with 1% uranyl acetate and lead citrate. Samples were viewed with a Hitachi H7500 transmission electron microscope.

Cytokine Profiles

TS cell line 1 was plated at 10,000 cells per well in 96 well flat bottom plates and allowed to adhere overnight at 37°C and 5% CO₂. Cells were either untreated or stimulated with 50 μ g/ml PGN for 24 h *in vitro*. The supernatants were harvested and then centrifuged to remove nonadherent cells. The supernatants were then stored at -80°C. Cytokine and chemokine levels in the supernatants were analyzed using a Bio-Plex Mouse Cytokine 23-Plex (Bio-Rad, Hercules, CA, USA). The panel was analyzed using the Luminex 100 IS system (Millipore, Billerica, MA, USA). The 23 cytokines and chemokines analyzed include IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN- γ , KC, MCP-1, MIP-1 α , MIP-1 β , RANTES, and TNF- α . This experiment was performed three times.

Statistical Analysis

Differences between control values and experimental values were determined using a Student's *t* test or a one-way ANOVA with a Tukey's *post hoc* test when comparisons were made between more than one experimental group (PASW, IBM, Chicago, IL, USA).

Results

TLR2 and TLR4 Protein are Expressed in Preimplantation Embryos while only TLR2 is Present on the Surface of Trophoblast Stem (TS) Cells

The preimplantation period of embryo development extends from the time of fertilization through the 1-cell, 2-cell, 4-cell, morula, and finally the blastocyst stage. During the transition from morula to blastocyst the totipotent cells of the embryo differentiate into two

cell lineages. The trophoblast develops into the fetal portion of the placenta and the inner cell mass gives rise to the embryo proper³². To determine whether preimplantation embryos have the potential to sense bacterial infections we examined TLR2 and TLR4 protein expression from the 1-cell through the blastocyst stage of murine embryo development by immunofluorescent confocal microscopy. Both TLR2 and TLR4 protein were detected at the 1-cell, 2-cell, 4-cell, morula, and blastocyst stages (Fig. 1). TLR2 and TLR4 protein were also identified on the cumulus cells remaining around the fertilized one cell embryo as previously described³³.

TS cells are derived from the trophoblast of blastocysts and possess the ability to develop into each of the four-trophoblast cell lineages²⁷. TLR expression among trophoblasts differs by lineage and gestational age¹⁰. To determine whether TLR1, TLR2, TLR4, and TLR6 are expressed on trophoblast progenitor cells we examined the expression pattern of these proteins in two independently generated trophoblast stem (TS) cell lines. We also examined TLR1 and TLR6 expression as these proteins form heterodimers with TLR2 expanding the number of ligands recognized by this receptor³⁴. RT-PCR was performed to establish the expression pattern of TLR1, TLR2, TLR4, and TLR6 in the TS cell lines. TLR2 and TLR6 but not TLR1 or TLR4 RNA was expressed in both TS cell lines (Fig. 2). TLR4 and TLR1 transcripts were however detected in the RAW264.7 positive control cell line. To confirm this expression pattern at the protein level the TS cell lines were stained with an anti-TLR1, TLR2, TLR4/MD-2, or TLR6 antibody or the appropriate isotype control antibody and analyzed by flow cytometry. MD-2 associates with the extracellular domain of TLR4 and its expression is required for LPS recognition³⁵. TLR2 but not TLR1, TLR4/MD-2, or TLR6 protein was detected at the plasma membrane of the TS cell lines (Fig. 3). TLR1, TLR4/MD2, and TLR6 protein was detected on the RAW264.7 positive control cell line. Thus TLR2 and TLR6 RNA are expressed in TS cells but only TLR2 protein was detected at the plasma membrane.

Peptidoglycan Induces TS Cell Death

Previous studies demonstrated that PGN, a TLR2 ligand, induces apoptosis in first trimester human trophoblast cells *in vitro* and in placental trophoblasts of pregnant mice *in vivo*^{21, 22}. We next wanted to determine whether PGN induces TS cell death. TS cells were incubated with increasing concentrations of PGN for 24 h and then stained with 7-AAD, which is excluded by live cells but penetrates the plasma membrane of dead or dying cells. The TS cells were then analyzed by flow cytometry. PGN induced a dose dependent increase in the number of 7-AAD positive TS cells (Fig. 4A). The geometric mean fluorescence intensity (GMFI) was determined for each treatment group (Fig. 4B). TS line 1: 0 µg/ml PGN: 100 ± 0, 10 µg/ml PGN: 112.6 ± 1.5, 25 µg/ml PGN: 152.8 ± 9.7 (p<0.001), 50 µg/ml PGN: 179.4 ± 2.4 (p<0.001), 100 µg/ml PGN: 239.6 ± 6.8 (p<0.001). TS line 2: 0 µg/ml PGN: 100 ± 0, 10 µg/ml PGN: 125.0 ± 4.8, 25 µg/ml PGN: 157.7 ± 4.5, 50 µg/ml PGN: 227.5 ± 14.5 (p<0.001), 100 µg/ml PGN: 350.4 ± 23.4 (p<0.001). Thus TS cell line 1 treated with 25, 50, and 100 µg/ml PGN demonstrated significantly increased numbers of 7-AAD positive cells as compared to untreated controls. TS cell line 2 demonstrated significant increases in the number of 7-AAD positive cells at the 50 µg/ml and 100 µg/ml doses of PGN.

Peptidoglycan-Induced TS Cell Death is Consistent with Necrosis

To begin to establish the mechanism involved in PGN-induced TS cell death we examined whether PGN treatment leads to the induction of apoptosis in TS cells. Caspase-3 is a key mediator of apoptosis. Caspase-3 exists as an inactive zymogen and must be proteolytically processed to its activated form³⁶. To determine if PGN treatment leads to the generation of cleaved caspase-3 TS cells were treated with increasing concentrations of PGN for 24 h or 0.2 µM staurosporine a known inducer of apoptosis for 12 h. Whole cell lysates were

generated and the presence of cleaved caspase-3 was examined by western blot. PGN treatment did not induce caspase-3 cleavage products in either TS cell line (Fig. 5A and 5B). Cleaved caspase-3 was also not detected in PGN treated lysates generated at earlier time points (data not shown). In contrast, lysates derived from staurosporine treated TS cells demonstrated the presence of cleaved caspase-3. Low levels of cleaved caspase-3 could be detected in all TS cell samples upon prolonged exposure of the western blot reflecting low constitutive levels of apoptosis in the culture. The low level of cleaved caspase-3 detected was unaltered by the addition of PGN (data not shown). As a loading control western blots were stripped and re probed with an anti-mouse actin antibody.

Given that caspase-3 was not activated by PGN we next sought to determine whether PGN-induced TS cell death was morphologically consistent with necrosis. TS cell line 1 was untreated, exposed to 50 $\mu\text{g/ml}$ PGN for 24 h *in vitro*, or 0.2 μM staurosporine for 12h. The cells were then examined morphologically by transmission electron microscopy. Cells treated with PGN exhibited morphological features consistent with necrosis. PGN treated TS cells demonstrated degradation of the plasma membrane with loss of cytoplasmic components (Fig. 6C and 6D respectively). In addition, PGN treated cells demonstrated a lack of chromatin condensation as compared to staurosporine treated cells (Fig. 6B). These results suggest that PGN-induced TS cells death is consistent with necrosis³⁷.

PGN Induces HMGB1 Release from TS Cells

Necrotic cell death can lead to the induction of an inflammatory response through the release of cellular debris. Cellular components released by necrotic cells are able to activate the innate immune system through molecules containing damage-associated molecular patterns or DAMPs³⁸. High mobility group box 1 (HMGB1) belongs to the DAMP family of endogenous molecules³⁸. To determine whether PGN treatment leads to the release of HMGB1 TS cells were either untreated or treated with increasing concentrations of PGN for 24 h *in vitro*. Supernatants were then collected and centrifuged to remove cellular components. The presence of HMGB1 in the TS cell supernatants was analyzed by western blot. HMGB1 was not detected in supernatants derived from untreated cells nor was it readily detected in supernatants collected from cells treated with lower doses of PGN (Fig. 7A and B). HMGB1 was clearly present in supernatants derived from TS cells treated with either 50 $\mu\text{g/ml}$ or 100 $\mu\text{g/ml}$ PGN. The release of HMGB1 into the supernatant was dose dependent. Amido black staining of the nitrocellulose membranes was used as a loading control.

PGN Differentially Regulates Chemokine Production by TS Cells

Previous reports demonstrated that activation of TLR2 in human first trimester trophoblast cells induced apoptosis and suppressed cytokine production^{21, 22}. Thus we wanted to determine whether PGN modulates TS cell cytokine production in a similar manner. TS line 1 was either untreated or treated with 50 $\mu\text{g/ml}$ PGN for 24 h *in vitro*. Supernatants were then collected and the levels of cytokines and chemokines produced by untreated or PGN treated TS cells were analyzed using multiplex technology. TS cells were found to constitutively secrete a number of cytokines. The most highly secreted cytokines included IL-6 (266 ± 32 pg/ml), Eotaxin (679 ± 30 pg/ml), KC ($8,749 \pm 1,930$ pg/ml), MCP-1 ($12,175 \pm 2,986$ pg/ml) and RANTES (845 ± 118 pg/ml) (data not shown). Of the 23 cytokines examined 2 chemokines were significantly affected by PGN treatment. MIP-1 β (CCL4) was strongly induced by PGN treatment ($3,255 \pm 730$ pg/ml) as compared to the control (30 ± 3 pg/ml) (Fig. 8, Panel A). Conversely, PGN inhibited RANTES (CCL5) secretion (845 ± 118 pg/ml) by TS cells as compared to control cells (631 ± 99 pg/ml) (Fig. 8, Panel B). Thus PGN differentially regulated MIP-1 β and RANTES secretion by TS cells. We also examined

cytokine levels in supernatants derived from TS line 2 however the majority of the cytokines were undetectable (data not shown).

Discussion

Intrauterine infections are clinically associated with preterm birth, intrauterine growth restriction, and preeclampsia¹. It is thought that an excessive innate immune response to invading microbes contributes to adverse pregnancy outcomes associated with intrauterine infection¹. The ability to sense the presence of pathogens is the initial event that triggers an inflammatory response. Previous studies demonstrated that trophoblasts detect and respond to microbes through the TLR family of pattern recognition receptors^{21–24, 39–44}. TLR mediated trophoblast responses may affect pregnancy outcome through the production of cytokines, chemokines, and the regulation of immune cell migration⁹. While the role of trophoblast TLRs in detecting danger signals during the first trimester of pregnancy is increasingly well defined relatively little is known regarding the ability of trophoblast stem cells to detect and respond to pathogens at the maternal-fetal interface.

The human placenta expresses transcripts for TLRs 1 through 10⁹. Interestingly, TLR expression patterns are dynamic and appear to be regulated during gestation¹⁰. In humans, TLR4 protein expression is higher in term placenta as compared to placenta from the first trimester of pregnancy⁴⁵. Furthermore, TLR6 is not expressed by first trimester trophoblasts²² but it is expressed in term trophoblasts⁴³. In addition to temporal regulation TLR proteins are differentially expressed depending on trophoblast lineage. First trimester trophoblasts express TLR2 and TLR4 in extravillous and villous cytotrophoblasts but not in syncytiotrophoblasts²². Thus this data suggests that not all placental cells express TLRs and placental expression of TLRs varies with gestational age.

As compared to human placenta TLR expression in murine trophoblasts is less well defined. Trophoblast cells of the placenta are derived from the trophoblast layer of blastocysts³². In this current study we demonstrated that TLR2 and TLR4 protein are expressed from the 1-cell through the blastocyst stage of murine embryo development. Thus preimplantation embryos possess the potential to respond to foreign microbes that may be encountered in the reproductive tract prior to implantation. One limitation of this study is that at the blastocyst stage we were unable to determine by confocal microscopy whether TLR expression is largely in the inner-cell mass, the trophoblast, or both. As TS cells are derived from the trophoblast layer of blastocysts TLR expression patterns on TS cells may provide insight into which blastocyst lineage(s) express these proteins.

Herein, we demonstrated that TS cells express TLR2 but not TLR1, TLR4/MD-2, or TLR6 protein at the plasma membrane. As TLR6 transcripts but not cell surface protein was detected in the TS cell lines perhaps TLR6 mRNA stability or translation is regulated in these cells. Given the limited expression pattern of TLRs on TS cells this primitive trophoblast may be less able to detect pathogenic stimuli than differentiated trophoblasts. Although it is difficult to be certain as the expression pattern of TLRs on murine trophoblast cells throughout pregnancy has not been well characterized.

Our data demonstrated that PGN treatment induces a loss of membrane integrity in TS cells as determined by 7-AAD staining. In addition, we demonstrated that PGN exposed TS cells undergo a form of cell death that is morphologically consistent with necrosis. Cells can die by several mechanisms each having distinct effects on immune cells. Apoptosis, or programmed cell death, is an energy dependent process that results in the removal of dysfunctional or unwanted cells without activation of the immune response⁴⁶. Apoptotic cell death is a normal part of placental development^{2, 47}. However, excessive placental

apoptosis is associated with pregnancy complications including preeclampsia^{48–51} and intrauterine growth restriction^{49, 52–54}. Necrosis is a form of cell death characterized by cytoplasmic swelling, organelle dysfunction, and plasma membrane permeabilization⁵⁵. Necrosis has been reported to occur at low levels in villous cytotrophoblast cells during normal human pregnancy⁵⁶. Similarly to apoptosis, necrosis is often associated with cell death in human pathologies but unlike apoptosis it can lead to local inflammation due to the release of cellular factors such as HMGB1 that activate the innate immune system^{55, 57–63}.

Previous studies demonstrated that TLR ligands induce necrosis in gestational tissues of pregnant mice. Pregnant mice injected with the TLR3 ligand poly (I:C) demonstrated necrosis in the chorion and amniotic membrane as well as other pregnancy-associated tissues¹⁴. In addition, these mice underwent preterm birth¹⁴. In a separate study pregnant mice infected with MHV-68 demonstrated necrosis in the labyrinth and giant cells of the placenta¹¹. Similarly pregnant mice infected with either *Listeria monocytogenes* an intracellular, Gram-positive bacterium^{64, 65} or with *Fusobacterium nucleatum* a Gram-negative bacteria demonstrated placental necrosis^{66, 67}. Thus viruses, Gram-positive, and Gram-negative bacteria, which activate distinct TLRs can induce necrosis in the mouse placenta.

PGN was previously shown to induce apoptosis in first trimester human trophoblast cells²². Moreover, PGN induced apoptosis in the placenta of pregnant mice²¹. Herein we demonstrated that murine TS cells treated with PGN undergo cell death consistent with necrosis. The difference in cell death mechanism employed by first trimester human trophoblast cells and murine trophoblast stem cells may be due to an intrinsic difference in the manner in which the stem cell responds to PGN. Alternately TLR1 and TLR6 have been shown to regulate TLR2 responses^{21, 68} perhaps the lack of TLR1 and TLR6 expression on TS cells alters the TLR2 response to PGN. Finally it is possible that the effects of PGN on trophoblast cell death may be species specific. In short, it is clear that PGN induces cell death in trophoblast cells and that both apoptosis and necrosis are associated with human pregnancy pathologies.

HMGB1 is a DNA-binding protein that is expressed in virtually all cells of human term placenta^{69, 70}. HMGB1 is often passively released by necrotic cells or actively secreted by activated immune cells⁷¹. HMGB1 binds multiple receptors that induce inflammation including TLR2, TLR4, TLR9, and RAGE^{72–76}. Indeed, HMGB1 induces NF- κ B activation and the secretion of proinflammatory cytokines⁷⁷. Thus the release of HMGB1 by TS cells at the maternal-fetal interface may have important immunomodulatory effects that could potentially impact pregnancy outcome.

The data presented herein demonstrates that PGN differentially regulates the secretion of two chemokines, MIP-1 β and RANTES by TS cells. Interestingly, PGN induced increased MIP-1 β secretion while inhibiting the constitutive release of RANTES. Similarly, a previous report demonstrated that infection with *Chlamydia trachomatis* differentially regulated trophoblast chemokine production⁷⁸. In this study *Chlamydia* induced the secretion of the proinflammatory cytokines IL-1 β and IL-8 while inhibiting the release of RANTES, MCP-1, and GRO α . Increased MIP-1 β secretion by TS cells at the maternal-fetal interface could lead to an influx of T-cells, monocytes, dendritic cells, NK cells, and to a lesser extent neutrophils and eosinophils⁷⁹. RANTES has been shown to recruit monocytes, NK cells, T cells, basophils, eosinophils, and dendritic cells⁸⁰. In addition, RANTES was shown to affect trophoblast migration and was reported to play a role in peripheral tolerance in immune-privileged tissues through its ability to recruit regulatory T cells⁸¹. Importantly, RANTES has been implicated in the induction of maternal tolerance to fetal alloantigens⁸¹. Thus the ability of PGN to regulate TS cell chemokine responses could significantly alter

the composition of immune cells at the maternal-fetal interface, which may ultimately have adverse effects on pregnancy outcome.

In summary, we have demonstrated the expression pattern of specific TLRs on murine preimplantation embryos and trophoblast stem cells. In addition, we demonstrated the functional consequences of exposing TS cells to the TLR2 ligand peptidoglycan. PGN induced necrosis in TS cells, triggered the release of HMGB1, and increased the secretion of MIP-1 β while RANTES production was suppressed. We suggest that intrauterine infection during early pregnancy may adversely affect placentation by inducing necrosis in trophoblast progenitor cells and by modulating the release of proinflammatory molecules by TS cells. The release of proinflammatory molecules might alter the composition of immune cells at the maternal-fetal interface or their activation status, which may adversely affect pregnancy outcome.

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Fig. 1.

TLR2 and TLR4 protein are expressed from the 1-cell through the blastocyst stage of murine embryo development. Embryos were recovered from B6SJLF1/J mice at E0.5, E1.5, E2.5, and E3.5 to isolate embryos at the 1-cell, 2-cell, 4-cell, morula, and blastocyst stage of development. The embryos were stained with 20 $\mu\text{g}/\text{ml}$ of an anti-mouse TLR2 antibody, an anti-mouse TLR4 antibody or the proper irrelevant control antibody and then incubated with 8 $\mu\text{g}/\text{ml}$ of the appropriate Alexa Fluor 488 secondary antibody. The embryos were stained with 2 μM TO-PRO-3 iodide, a nuclear dye and analyzed by confocal microscopy (magnification 60 $\times$).

Fig. 2.

TLR2 and TLR6 but not TLR1 and TLR4 RNA is expressed in TS cells. RT-PCR was performed using RNA isolated from TS cell lines and RAW264.7 cells. Primers specific for murine *TLR1*, *TLR2*, *TLR4*, or *TLR6* were employed. Negative control samples (-) did not contain reverse transcriptase but did contain template and TLR specific primers.

Fig. 3.

TS cells express TLR2 but not TLR1, TLR4/MD-2, or TLR6 protein at the plasma membrane. (B) TS cell line 1, (C) TS cell line 2, or (A) the RAW264.7 positive control were blocked in 5% normal rat sera and 5% normal mouse sera in PBS/10% FBS for 15 min at 4°C. Cells were then stained with an anti-mouse TLR1 antibody, an anti-mouse TLR2 antibody, an anti-mouse TLR4/MD2 antibody, an anti-mouse TLR6 antibody (black histogram) or the appropriate isotype control antibody (grey histogram). Cells were incubated for 30 min on ice. Data was collected using a BD FACSCalibur.

A. 7-AAD

B. 7-AAD

Fig. 4.

PGN treatment induces TS cell death. (A) TS cell lines were untreated (grey histogram) or stimulated with 10 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$ or 100 $\mu\text{g/ml}$ peptidoglycan (black histogram) for 24 h *in vitro*. The cells were then incubated with 7-AAD and the level of cell death was analyzed by flow cytometry. (B) 7-AAD geometric mean fluorescence intensity (GMFI) for treatments described in (A). Values are a mean $\pm$ SEM of three independent experiments. A p value < 0.001 was considered statistically significant.

Fig. 5.

PGN does not lead to caspase-3 activation. (A) TS cell line 1 or (B) TS cell line 2 was untreated or stimulated with 10 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, or 100 $\mu\text{g/ml}$ PGN for 24 h *in vitro*. In addition, cells were treated with 0.2 μM staurosporine for 12 h as a positive control. The western blot membranes were probed with an anti-cleaved caspase-3 antibody or with an anti-actin antibody as a loading control.

Fig. 6.

PGN induces TS cell death consistent with necrosis. (A) TS cell line 1 was untreated, (B) treated with 0.2 μ M staurosporine for 12 h as a positive control, or (C and D) treated with 50 μ g/ml PGN for 24h. Cells were processed and the samples analyzed using a Hitachi H7500 transmission electron microscope. Panel B depicts staurosporine induced chromatin condensation a hallmark of apoptosis. Panel C and D depict a loss of plasma membrane integrity and lack of chromatin condensation which are morphological features consistent with necrosis.

Fig. 7. TS cells treated with PGN released HMGB1, a proinflammatory molecule. (A) TS cell line 1 or (B) TS cell line 2 were untreated or treated with 10 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, or 100 $\mu\text{g/ml}$ PGN for 24 h *in vitro*. The western blot membranes were probed with an anti-HMGB1 antibody. As a loading control the membranes were then stained with amido black.

Fig. 8.

PGN differentially regulates MIP-1 α and RANTES production by TS cells. TS line 1 was untreated or treated with 50 μ g/ml PGN for 24 h *in vitro*. Cytokine levels in the supernatants were determined by multiplex analysis. Depicted are levels of A) MIP-1 β or B) RANTES secretion by untreated and PGN treated TS cells. Values are a mean $\pm$ SEM of three independent experiments. A *p* value < 0.05 was considered statistically significant.