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Regulation of Apoptosis and Innate Immune Stimuli in Inflammation-Induced Preterm Labor

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An innate immune response is required for successful implantation and placentation. This is regulated, in part, by the a2 isoform of V-ATPase (a2V) and the concurrent infiltration of M1 (inflammatory) and M2 (anti-inflammatory) macrophages to the uterus and placenta. The objective of the present study was to identify the role of a2V during inflammation-induced preterm labor in mice and its relationship to the regulation of apoptosis and innate immune responses. Using a mouse model of infection-induced preterm delivery, gestational tissues were collected 8 h after intrauterine inoculation on day 14.5 of pregnancy with either saline or peptidoglycan (PGN; a TLR 2 agonist) and polyinosinic-polycytidylic acid [poly(I:C); a TLR3 agonist], modeling Gram-positive bacterial and viral infections, respectively. Expression of a2V decreased significantly in the placenta, uterus, and fetal membranes during PGN+poly(I:C)-induced preterm labor. Expression of inducible NO synthase was significantly upregulated in PGN+poly(I:C)-treated placenta and uterus. PGN+poly(I:C) treatment disturbed adherens junction proteins and increased apoptotic cell death via an extrinsic pathway of apoptosis among uterine decidual cells and spongiosotrophoblasts. F4/80+ macrophages were increased and polarization was skewed in PGN+poly(I:C)-treated uterus toward double-positive CD11c+ (M1) and CD206+ (M2) cells, which are critical for the clearance of dying cells and rapid resolution of inflammation. Expression of Nlrp3 and activation of caspase-1 were increased in PGN+poly(I:C)-treated uterus, which could induce pyroptosis. These results suggest that the double hit of PGN+poly(I:C) induces preterm labor via reduction of a2V expression and simultaneous activation of apoptosis and inflammatory processes. The Journal of Immunology, 2013, 191: 5702–5713.

Preterm birth is considered the most important cause of neonatal morbidity and mortality not due to congenital anomalies in the developed world (1). Up to 40% of cases of preterm births occur in association with microbial invasion of the gestational compartment (2). Although it may be difficult to determine whether infection is a cause or a consequence of labor in individual cases, it has become clear that infection and inflammation represent important and frequent mechanisms of disease.

TLRs are a family of membrane-bound proteins that recognize pathogen-associated molecular patterns and mediate innate immune responses (3–6). Binding of TLRs is the initial event in activation of the innate immune system that leads, among other events, to the nuclear translocation of the transcription factor NF-κB and the elaboration of a network of inflammatory mediators. We showed that preterm labor can be induced in mice by pathogen-derived TLR ligands for TLR2 (peptidoglycan [(PGN), 22%], TLR3 (polyinosinic-polycytidylic acid [poly(I:C)], 14%), and, in a synergistic fashion, TLR2 plus TLR3 (100%) (7). Using this well-validated mouse model of infection-induced preterm delivery, we (7, 8) and other investigators (9, 10) demonstrated that combined activation of TLR2 and TLR3 using PGN and poly(I:C) yields a dramatic synergy in the labor response and expression of inflammatory mediators in gestational tissues. Such combined stimulation might occur in nature in at least four scenarios: engagement of TLR4; activation of TLR3 and another TLR simultaneously by a single organism [e.g., murine CMV, HSV, and Schistosoma mansoni (11, 12)]; superinfection, in which a host is infected simultaneously by more than one microorganism, such as a virus and a bacterium (13); and activation of TLRs by one of several known, endogenously produced TLR ligands together with an exogenous pathogen (14, 15).

a2V-ATPase is a protein that is expressed in many mammalian cells and is involved in immune regulation and apoptosis. a2V-ATPase is the a2 isoform of the “a” subunit of vacuolar ATPase, the enzyme that is present in intracellular vesicles and in the plasma membrane of specialized cells (16). One main function of V-ATPases is the acidification of intracellular compartments, a process that is crucial for many cellular functions (16). The a2 isoform of V-ATPase (a2V) is required for normal implantation, placental development, and spermatogenesis (17–19). Expression of V-ATPase subunits in the bovine endometrium is crucial for trophoblast invasion and cellular communication (20). Our previous studies (17, 18) showed that for successful implantation and placentation, a definite innate immune response is generated, which is regulated, in part, by a2V with the concurrent infiltration of M1 (inflammatory) and M2 (anti-inflammatory) macrophages.
in the uterus and placenta. We also showed that during LPS-induced fetal resorption, the decrease in placental a2V expression was associated with upregulation of proinflammatory cytokines (17).

The internal acidification of intracellular compartments, such as lysosomes, endosomes, the Golgi complex, and secretory granules, was suggested to play an important role in the mechanism of cell survival. V-ATPase plays an important role in the regulation of activity in organelles of the central vacuolar system. The V-ATPase inhibitors, such as concanamycin A (21) or bafilomycin A1 (22), induce apoptosis in various cells, such as a pancreatic cancer cell line (23) and RAW 264.7 (mouse macrophage) cells (24). Specifically, our laboratory also showed that anti-a2V Ab induces activation of caspase-3 and is associated with apoptosis in T lymphocytes (25). Two major apoptotic pathways (intrinsically and extrinsically) are active in cellular apoptosis (26). The intrinsic pathway involves the release of cytochrome c from the mitochondria into the cytosol where it binds to apoptotic protease activating factor-1. This results in the activation of caspase-9; induction of proteolytic activity by the executioner caspses (caspase-3, -6, and -7); and, finally, cleavage of poly (ADP-ribose) polymerase (26-28). The extrinsic pathway for apoptosis involves death receptors, such as Fas, which binds to its ligand, FasL. The binding of FasL to Fas induces recruitment of the Fas/FADD complex. This Fas/FADD complex recruits the initiator caspses (caspase-8, -10), which also can activate the effector caspses (caspase-3, -6, and -7) and cleavage of poly (ADP-ribose) polymerase (29, 30).

a2V regulates apoptosis and delicate cytokine and chemokine networks for successful implantation (18, 31), placental development, and growth at the fetio–maternal interface (17). We hypothesized that a2V is an important factor in the regulation of the immune response in pregnancy and may be involved, in part, in infection-induced preterm labor. In this study, we examined the role of a2V in PGN-poly(I:C)-induced preterm labor in the mouse model and characterized its association with apoptosis and innate immune responses. We find that PGN-poly(I:C) induces preterm labor via simultaneous activation of apoptosis and inflammatory processes and that a2V might be a bridge between these two processes.

Materials and Methods

Mice

For pregnancy-outcome experiments, CD-1 female mice in estrus were selected by the gross appearance of the vaginal epithelium and were impregnated naturally. Mating was confirmed by the presence of a vaginal plug. Intrauterine injections were performed on day 14.5 of a 19-20-d gestation, as previously described (32). Briefly, animals were anesthetized with 0.015 mg/kg body weight Avertin (2.5% tribromoethanol alcohol and 2.5% tert-amyl alcohol in PBS). A 1.5-cm midline incision was made in the lower abdomen. In the mouse, the uterus is a bicornuate structure in which the fetuses are arranged in a "heads-on-a-string" pattern. Mice underwent injection of either PGN (0.3 mg/mouse) plus poly(I:C) (1.0 mg/mouse) or saline alone. PGN and poly(I:C) were combined because we showed previously that this results in synergistic effects (both preterm delivery and inflammatory responses), a phenomenon that is mediated by both the MyD88-dependent- and MyD88-independent-signaling pathways downstream of the TLR receptors (7). Each of the above intrauterine injections was performed in the midsection of the right uterine horn at a site between two adjacent fetuses, taking care not to inject individual fetal sacs. This dose of PGN+poly(I:C) causes death delivery within 12–18 h after injection (7). Delivery of one or more pups in the cage or lower vagina within 48 h was considered preterm. This model was shown to reproduce faithfully many aspects of infection-induced preterm labor in women, including the expression of cytokines, PGs, and other mediators; the lack of dependence on a decrease in circulating progesterone; neonatal brain injury; and other characteristics (32–34). Surgical procedures lasted ~10 min. The abdomen was closed in two layers, with 4-0 polyglactin sutures at the peritoneum and wound clips at the skin.

Tissue harvest

Animals were euthanized 8 h after surgery to examine tissue-level phenomena in an intrapartum state. Mice injected with PGN+poly(I:C) deliver in 12–18 h. The inoculated horn was incised longitudinally along the antimesenteric border. Gestational tissues (uteri [full-thickness biopsies from the middle region], fetal membranes [pooled from all conceptuses in the injected uterine horn], fetuses, and placentas) were harvested, washed in ice-cold PBS, and either flash-frozen in liquid nitrogen and stored at −85°C for later RNA or protein extraction or fixed in 10% neutral-buffered formalin for immunohistochemistry.

Cell culture

RAW 264.7 cells were cultured in DMEM High Glucose (Life Technologies, cat no. 11965-092) supplemented with 10% FBS, 1% streptomycin, and 1% penicillin in tissue culture flasks at 37°C in 5% CO2/95% air and were passed every 2 or 3 d to maintain logarithmic growth. Prior to each experiment, cells (4 × 10⁶ cells/well) were plated in triplicate in 24-well plates and cultured overnight.

Cells were initially incubated for 2 h with either PBS or PGN plus poly(I:C) (1 μg/ml plus 10 μg/ml) and then sequentially incubated for 3 h with ATP (1 mM), IgG (3 μg/ml), anti-a2V (3 μg/ml), or ATP plus anti-a2V. The experiment was conducted in triplicate and repeated twice.

Real-time PCR

Total RNA from gestational tissues was extracted after homogenization in TRizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol. For the cells, at the end of cell culture experiments, medium was aspirated, and cells were washed with PBS and lysed in the wells with TRizol reagent. Quantity and integrity of RNA were confirmed by the ratio at 260:280 nm, and electrophoresis was performed on 1.5% native agarose gel to visualize 18S and 28S rRNA subunits. Samples were stored at −80°C until further use. Two micrograms of RNA was used as a template for cDNA synthesis. cDNA was prepared using random primers and the Moloney Murine Leukemia Virus Reverse Transcriptase system (Invitrogen).

Duplex RT-PCR was performed, with one primer pair amplifying the gene of interest and the other as an internal reference (GAPDH) in the same tube, using the Applied Biosystems Step One Real-Time PCR system. The prevalidated TaqMan gene-expression assays for a2V (Hs00414184_m1); trophoblast development markers, Hox10 (Mm00432549_m1) and Hbegf (Mm00439306_m1); cytokines Mcp1 (Mm00441242_m1), No2 (Mm0040502_m1) and TNF (Mm00443258); macrophage markers F4/80 (Envi; Mm00802529_m1), CD11c (Mm00409696_m1), CD206 (Mm00485148_m1), CD209a (Mm00460067_m1), and Arg1 (Mm00473988_m1); inflammotin markersNrps3 (Mm00849004_m1) and caspase-1 (Mm00438023_m1); and internal control Gdpd (Mm00433933_m1) were purchased from Applied Biosystems (Foster City, CA). Real-time PCR was performed using Universal PCR Master Mix reagent (Applied Biosystems). The use of TaqMan PCR Reagent Kits was in accordance with the manufacturer’s manual. Reactions were performed in a 10-μl mixture containing 0.5-μl cDNA. PCR assays were performed in duplicate for each of the tissue samples.

Abs

The following primary Abs were used: mouse anti-a2V, rabbit anti-a2NTD (Covance, Denver, PA); rabbit anti-GAPDH (Cell Signaling, Danvers, MA); mouse anti-inducible NO synthase (NOS), rabbit anti-FAS, rabbit anti-FasL, rabbit anti-FADD, rabbit anti-Bax, mouse anti-Bcl2, rabbit anti-E-cadherin, rabbit anti-N-cadherin, rabbit anti-B-catenin, rabbit anti-active caspase-3 (recognizes the p17 fragment of the active caspase 3), rabbit anti-caspase-1, rat anti-F4/80, mouse anti-ITGAX (CD11c), mouse anti-CD206 (Abcam, Cambridge, MA); and mouse anti-cleaved caspase-8 (recognizes the cleaved fragment of caspase-8 resulting from cleavage at Asp384) and rabbit anti-cleaved caspase-9 (recognizes the cleaved fragment of caspase-9 resulting from cleavage at Asp382) (Cell Signaling Technology). Rat IgG isotype, mouse IgG isotype, and rabbit IgG isotype (Abcam) were used as isotype control Abs. Secondary Abs used were goat anti-rabbit IgG-FTC, goat anti-rabbit IgG AF-594 (Invitrogen), rabbit anti-rat IgG-FTC (Abcam), goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA), donkey anti-rabbit IRDye-800CW (LI-COR Bioscience, Lincoln, NE), and EnVision + Dual Link System-HRP (Dako, Carpenteria, CA).

Immunohistochemistry and immunofluorescence

The placenta and uterine horns were collected from control and PGN+poly(I:C)-treated groups. Tissues were fixed in 10% neutral-buffered formalin at 4°C overnight, rinsed in PBS, and infused with 30% sucrose solution at

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4°C overnight or until the tissue sank. The tissues were snap-frozen in OCT (Tissue-Tek, Sakura Finetek USA, Torrance, CA) in liquid nitrogen. Frozen tissue was stored at −80°C until further use. Five-micrometer sections from frozen tissues were mounted onto saline-coated glass slides (Dako) and stored at −80°C until used. The Dako EnVision + Dual Link System-HRP (DAB+) was used to stain the frozen sections, according to the manufacturer’s instructions with slight modifications. The sections were heated in the microwave in sodium citrate buffer (pH = 6) for Ag retrieval.

For the detection of a2V protein, sections were incubated with 20 μg/ml Ab in 1% BSA-PBS for 1 h at room temperature. The following Ab concentrations were used: 2 μg/ml for proapoptotic marker Bax, antiapoptotic marker Bcl2, and cytokine iNOS; 10 μg/ml for gap-junction proteins E-cadherin and N-cadherin; a 1:1000 dilution for FAS, FasL, and FADD; and a 1:100 dilution for active caspase-8 and -9 overnight at 4°C. After washing, sections were incubated with secondary Ab EnVision + Dual Link System-HRP–labeled polymer anti-mouse and anti-rabbit IgG. The chromogen 3,3′-diaminobenzidine was used as substrate for the EnVision + Dual Link System-HRP, according to the manufacturer’s instructions. The sections were counterstained with Mayer’s hematoxylin and mounted in Faramount aqueous mounting medium (Dako). The immunostaining was evaluated by light photomicroscopy (Carl Zeiss, Weesp, The Netherlands) using a high-resolution camera (Canon G10, Canon, Tokyo, Japan).

For detection of β-catenin and active caspase-3, tissue sections were incubated with 10 μg/ml Ab overnight at 4°C and the appropriate secondary Ab labeled with FITC for 45 min at room temperature. To visualize the nuclei, cells were fixed in ProLong Gold Antifade reagent with DAPI (Invitrogen). After mounting the specimens on slides with VECTASHIELD, Ag distribution was examined under a Nikon Eclipse TE2000-S florescence microscope (Nikon Instruments, Melville, NY). Mouse/rabbit isotype-control Abs (Abcam) were used at the same concentration as were the primary Abs, and sections were incubated simultaneously with isotype-control Abs for all primary Abs used.

For macrophage staining, we used Abs targeted to F4/80, ITGAX (CD11c), and MRC1 (CD206), which identify macrophages and M1 and M2 macrophage subtypes, respectively (18, 35, 36). The Dako EnVision G2 Doublestain System was used to stain the frozen sections, according to the manufacturer’s instructions with slight modifications. Dilutions of 1:200 and 1:25 were used for ITGAX and MRC1 (Abcam), respectively. The visualization of ITGAX and MRC1 was done by horseradish peroxidase using diaminobenzidine chromogen and alkaline phosphatase using permanent red chromogen, respectively. Macrophage staining was also evaluated by light photomicroscopy (Carl Zeiss, Weesp, The Netherlands) using a high-resolution camera (Canon G10, Canon, Tokyo, Japan).

Table I. Decrease in gene expression and ISIS of a2V in uterus and placenta correlated with preterm labor

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tissues</th>
<th>Relative Gene Expression of a2V</th>
<th>Protein Level of a2V</th>
<th>Preterm Delivery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Uterus</td>
<td>28.40 ± 3.7</td>
<td>10.92 ± 0.4</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td></td>
<td>Placenta</td>
<td>26.08 ± 1.6</td>
<td>10.70 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fetal Membrane</td>
<td>12.15 ± 2.1</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fetus</td>
<td>28.33 ± 2.44</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>PGN+poly (I:C)</td>
<td>Uterus</td>
<td>12.20 ± 1.4*</td>
<td>6.62 ± 0.5**</td>
<td>11/15 (73)</td>
</tr>
<tr>
<td></td>
<td>Placenta</td>
<td>16.8 ± 2.2*</td>
<td>7.7 ± 0.4**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fetal Membrane</td>
<td>5.89 ± 1.45*</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fetus</td>
<td>28 ± 2.63</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

*p ≤ 0.05, **p ≤ 0.01 versus respective control.
performed in reverse order to confirm the specificity. The number of macrophages was counted in $10$ randomly chosen areas/uterine section at $40\times$ view. Four to six mice were included in each group, and six sections/animal were analyzed. Macrophage staining was also confirmed by immunofluorescence (same concentration of Abs as for immunohistochemistry) using the methods described for active caspase-3.

The tissue immunostaining results were scored negative if no immunopositive tissue was present. The total score was based on the percentage of stained tissue and immunostaining intensity. The percentage of stained tissue and immunostaining intensity were calculated according to the method described by Teixeira Gomes et al. (37). Then, the immunostaining index score (ISIS) was calculated by multiplying the stained area score by the immunostaining intensity score.

**Protein extraction**

For cytokine/chemokine assays, uterus and placenta were homogenized in ice-cold $1\times$ radioimmune precipitation assay buffer (Santa Cruz Biotechnology) containing protease and phosphatase inhibitor (Roche Applied Science, Indianapolis, IN). Lysates were incubated on ice for $30$ min and centrifuged at $10,000 \times g$ for $10$ min at $4^\circ C$. Supernatant fluid was collected and used as total-cell lysates for protein assays. Protein concentration was measured spectrophotometrically (Nanodrop 2000, Thermo Scientific, Hanover Park, IL) at $A_{280}$.

**Caspase activity measurement**

Activity of caspase-9, -8, -3, and -1 was measured using a SensoLyte AFC Caspase Profiling Kit (AnaSpec, Fremont, CA) in total-cell lysates from uterus (38) and assayed on a fluorescence microplate reader (BioTek Instruments, Winooski, VT), as per the instructions provided by the manufacturer. Equal amounts of protein ($50 \mu g$) from total-cell lysates were used for the assay. Caspase activity was measured as relative fluorescence unit/$\mu g$ protein. The assay was run with duplicates, with $n = 4$ in each group.

**Western blot analysis**

Equal amounts of protein ($50 \mu g$) from total-cell lysates were separated by $4$–$20\%$ SDS-PAGE and blotted onto polyvinylidene difluoride transfer membranes. The membranes were blocked at room temperature for $1$ h in $5\%$ nonfat dry milk in TBS-T. Blots were incubated with rabbit anti-a2NTD, rabbit anti-FAS, rabbit anti-FADD, and rabbit anti-caspase-1 primary Ab overnight at $4^\circ C$ and with the appropriate secondary Ab for $1$ h at room temperature. Fluorescent blots were imaged on the Odyssey Infrared Imaging System (LI-COR Biosciences). After detection of signal

**FIGURE 2.** Decreased a2V is associated with induction of iNOS. (A and B) mRNA expression of iNOS. Error bars represent SEM. (C–F) Immunolocalization of iNOS (DAB staining, brown) in the uterus and placenta recovered from the control and PGN+poly(I:C)-treated groups. Staining (DAB staining, brown) with isotype-control Abs for mouse IgG (G) and rabbit IgG (H). Original magnification $\times 400$. $n = 6–11$ mice/group; six sections were analyzed per animal. *$p \leq 0.01$, versus control.

**FIGURE 3.** Loosening of adherens junctions in PGN+poly(I:C)-treated uterus and placenta. Immunolocalization of E-cadherins (A–D), N-cadherins (E–H), and $\beta$-catenin (I–L) in the uterus and placenta recovered from the control and PGN+poly(I:C)-treated groups. (A–H) DAB staining, brown. (M–P) Merge images. DAPI nuclear stain (blue) merged with FITC (stain for $\beta$-catenin) for (I–L), respectively. Original magnification $\times 400$ (A–H), $\times 200$ (I–P). $n = 6$ mice/group; six sections were analyzed per animal.
for the target protein, membranes were reprobed for GAPDH. Each experiment was done twice in duplicate.

Cytokine/chemokine bioassay

The secretion of a panel of mouse cytokine/chemokines and FasL was analyzed using a MILLIPLEX MAP kit (Millipore, St. Charles, MO) in the supernatant of RAW 264.7 cells and total-cell lysates from uterus or placenta and assayed on a MAGPIX instrument (Millipore), as per the instructions provided by the manufacturer. Equal amounts of protein (50 μg) from total-cell lysates were used for the assay, which was repeated three times with duplicates.

Statistical analysis

Continuous variables (e.g., relative mRNA levels) were assessed with the Student t test or ANOVA. Mann–Whitney U test when data were not normally distributed and two groups were compared.

Results

a2V expression decreases in PGN+poly(I:C)-induced preterm labor

To test whether a2V plays a role in infection-induced preterm labor, we used a well-validated mouse model that combines stimulation by bacterial and viral products (7). The right uterine horn was injected with either PGN+poly(I:C) or saline on day 14.5 of pregnancy. Real-time PCR was performed in tissues harvested 8 h after surgery. The mRNA expression of a2V was significantly decreased with PGN+poly(I:C) treatment in uterus, placenta, and fetal membranes but not in the fetus (Fig. 1A–C, Supplemental Fig. 1A). Immunohistochemistry correlated with the mRNA levels and showed that expression of a2V protein was decreased in both myometrium and decidua due to PGN+poly(I:C) treatment. Overall ISIS of a2V in the uterus and placenta was decreased significantly after PGN+poly(I:C) treatment compared with the respective control: ISIS [uterus] = 6.62 ± 0.5 versus 10.92 ± 0.4 (p ≤ 0.01) (Fig. 1D, 1E) and ISIS [placenta] = 7.7 ± 0.4 versus 10.70 ± 0.3 (p ≤ 0.01) (Fig. 1D, 1F), respectively.

Using an Ab that detects the cleaved N terminus domain of a2V protein (i.e., a2NTD), Western blot was performed to evaluate cleavage of a2V protein in uterus and placenta of control and PGN+poly(I:C)-treated animals. Compared with uterus and placenta from control animals, decreased levels of a2NTD were noted in the uterus and placenta from PGN+poly(I:C)-treated animals (Fig. 1G). We also determined the mRNA expression of trophoblast development markers Hbegf and Hox10, which remained the same in control and PGN+poly(I:C)-treated placenta (Supplemental Fig. 1B, 1C).

As seen with mRNA and protein localization, a2V was highly expressed in control uterus and placenta. This expression is required for normal spongiotrophoblast development and invasion. However, in PGN+poly(I:C)-treated uterus and placenta, a2V was significantly reduced, which could disturb the integrity and normal physiological function of decidual cells and spongiotrophoblasts and lead to premature pregnancy loss. The resulting preterm labor in the PGN+poly(I:C)-treated group was associated with lower
density (ISIS) of α2V⁺ cells in decidua cells and spongiontrophoblasts (Fig. 1E, 1F, Table I). Staining profile of the whole uteroplacental unit is shown with the demarcation of the different zones (Supplemental Fig. 2).

Decrease in α2V is associated with induction of iNOS and altered integrity of adherens junctions in PGN+poly(I:C)-treated uterus and placenta

We observed that blocking of α2V by anti-α2V induces iNOS and TNF-α mRNA expression and TNF-α secretion in vitro. Moreover, anti-α2V enhances PGN+poly(I:C)-induced iNOS and TNF-α in comparison with respective controls (Supplemental Fig. 3). It was reported that blocking of V-ATPase induces iNOS expression (39). In this study, using the mouse preterm labor model we show that the decrease in α2V in PGN+poly(I:C)-treated animals is associated with the induction of iNOS mRNA in both uterus and placenta (Fig. 2A, 2B). The expression of iNOS protein was significantly increased in uterine decidual and mononuclear cells (Fig. 2C, 2D) and placental spongiontrophoblasts (Fig. 2E, 2F) after PGN+poly(I:C) treatment compared with control.

NOS is structurally associated with cadherin/β-catenin/actin complex (40) and its activation is a key regulator of adherens junctions (41). Structural assessment of the uterus and placenta by immunolocalization of E-cadherin and N-cadherin revealed that they were localized on the plasma membrane of uterine decidual cells and placental spongiontrophoblasts of the control group (Fig. 3A, 3C, 3E, 3G). However, in the PGN+poly(I:C)-treated group, uterine decidual cells and placental spongiontrophoblasts underwent a morphologic shift in the expression of membrane E-cadherin and N-cadherin, consistent with loss or internalization (Fig. 3B, 3D, 3F, 3H).

The extent to which treatment with PGN+poly(I:C) affects other plasma membrane proteins is not known. Because other cell-adhesion molecules, such as β-catenin, are associated with cadherins, we examined the membrane expression of β-catenin using immunofluorescence. Similar to cadherins, both the level of surface expression and the proportion of cells expressing surface β-catenin was low in PGN+poly(I:C)-treated uterus and placenta (Fig. 3I–P). Isotype controls are also shown (Fig. 2G, 2H, Supplemental Fig. 4A, 4B). These results suggest that disruption of α2V in the PGN+poly(I:C)-treated uterus and placenta is associated with iNOS activation, which ultimately causes the weakening of adherens junctions among uterine decidual cells and placental spongiontrophoblasts.

FIGURE 5. PGN+poly(I:C) induces apoptosis in the placenta via the extrinsic pathway. Immunolocalization of Bax (A, B), Bcl2 (C, D), active caspase-9 (E, F), active caspase-8 (G, H), and active caspase-3 (I, J) in placenta recovered from the control and PGN+poly(I:C)-treated groups. (A–H) DAB staining, brown. (K and L) Merged images. DAPI nuclear stain (blue) merged with FITC (stain for activated caspase-3). Original magnification ×400. (M–P) ISIS of Bax, Bcl2, active caspase-9, and active caspase-8 in placentas recovered from control and PGN+poly(I:C)-treated groups. n = 6 mice/group; six sections were analyzed per animal. Error bars represent SEM. *p ≤ 0.05, versus control.
PGN+poly(I:C)-induced preterm labor. The immunolocalization of the proapoptotic marker Bax was significantly higher in control uterus compared with PGN+poly(I:C)-treated uterus (Fig. 4A, 4B, 4M). However, no difference was observed in placental Bax expression (Fig. 5A, 5B, 5M).

The expression of the antiapoptotic marker Bcl2 was similar in uterus and placenta of both groups (Figs. 4C, 4D, 4N, 5C, 5D, 5N). Cleaved caspase-9 also remained unchanged in uterus and placenta of both groups (Figs. 4E, 4F, 4O, 5F, 5O). In control uterus, some decidual cells had higher levels of cleaved caspase-9 (Fig. 4E, 4F). Cleaved caspase-8 (Figs. 4G, 4H, 4P, 5G, 5H, 5P) and caspase-3 (Figs. 4I–L, 5I–L) were significantly increased in PGN+poly(I:C)-treated uterus and placenta compared with control. Isotype controls are also shown (Fig. 2G, 2H, Supplemental Fig. 4A, 4B). Activity of caspase-9,-8, and -3 was measured in uterus using fluorometry. Activity of caspase-9 remained the same in both groups, whereas the activity of caspase-8 and -3 was higher in the PGN+poly(I:C)-treated group compared with control (Fig. 4Q), as shown by immunohistochemistry.

These data indicate that apoptosis induced by PGN+poly(I:C) treatment is regulated by the extrinsic pathway of apoptosis, as reflected by the increased activation of caspase-8 and -3. To further verify that PGN+poly(I:C) induces apoptosis through the extrinsic pathway, the levels of death receptor Fas, its ligand FasL, and FADD were investigated in the uterus and placenta. Immunohistochemistry results showed that the expression levels of Fas (Fig. 6A–C, 6J–L), FasL (Fig. 6D–F, 6M–O), and FADD (Fig. 6G–I, 6P–R) were increased significantly in PGN+poly(I:C)-treated uterus and placenta compared with control. Western blot analysis of Fas and FADD (Fig. 6S, 6U, 6V) and Luminex assay of FasL (Fig. 6T) also confirmed the higher expression levels of FAS, FasL, and FADD in PGN+poly(I:C)-treated uterus and placenta.

**FIGURE 6.** PGN+poly(I:C) induces apoptosis in the uterus and placenta via the extrinsic pathway. Immunolocalization of Fas (A, B, J, K), FasL (D, E, M, N), and FADD (G, H, P, Q) (DAB staining, brown) in uterus and placenta recovered from the control and PGN+poly(I:C)-treated groups. (C, F, I, L, O, R) ISIS in uterus and placenta recovered from control and PGN+poly(I:C)-treated groups. (W) Isotype controls (DAB staining, brown). (S and V) Western blot analysis of Fas in uterus and placenta. (T) Luminex assay of FasL in uterus and placenta. (U and V) Western blot analysis of FADD with GAPDH loading control in uterus and placenta recovered from the control and PGN+poly(I:C)-treated groups. n = 4–6 mice/group; six sections were analyzed per animal. Error bars represent SEM. **p ≤ 0.01, versus control. Original magnification ×400.
M2-like macrophage markers are increased in the PGN+poly(I:C)-treated uterus. mRNA expression of M2-like macrophage markers in uterus but not in placenta

Proinflammatory chemokines were reported to be associated with preterm birth (42). Therefore, we determined the expression of the proinflammatory chemokine MCP-1 in uterus and placenta of control and PGN+poly(I:C)-treated groups. Its expression was significantly increased in uterus and placenta of the PGN+poly(I:C)-treated group, with more robust expression in the PGN+poly(I:C)-treated uterus (Fig. 7A). MCP-1 is capable of recruiting monocytes/macrophages into sites of inflammation, as well as stimulating the respiratory burst required for macrophage activation. MCP-1 transcripts and immunoreactivity were expressed by uterine tissues (i.e., decidual cells and myometrium) and, thus, may participate in the process of labor (42). Macrophages are the cells responsible for phagocytosis and elimination of apoptotic cells, and they exhibit an inflammatory response or express anti-inflammatory cytokines for the resolution of inflammation (43). Therefore, we examined the markers associated with macrophage polarization during PGN+poly(I:C)-induced preterm labor. F4/80 (general macrophage marker) was increased significantly in PGN+poly(I:C)-treated uterus but remained unchanged in placenta compared with controls (Fig. 7B). The mRNA expression of the M1 macrophage marker CD11c remained unchanged in uterus and placenta of controls and in the PGN+poly(I:C)-treated group (Fig. 7C). However, the mRNA expression of the M2 macrophage markers Mrc1 (CD206), Arg1, and CD209a was significantly upregulated in PGN+poly(I:C)-treated uterus compared with controls (Fig. 7D), and Arg1 and CD209a were undetectable (Fig. 7E, 7F). These results indicate that M2 macrophage markers are increased in PGN+poly(I:C)-treated uterus but not in placenta.

M1 and M2 polarization during preterm labor

We determined the distribution of M1- and M2-like macrophages in control and PGN+poly(I:C)-treated uteri. The presence of macrophages in the uterus was confirmed by F4/80 localization using immunohistochemistry and immunofluorescence (Fig. 8A, 8B, 8C–F). Immunostaining showed that the number of F4/80⁺ macrophages increased in PGN+poly(I:C)-treated uterus compared with control uterus (Fig. 8M). Double immunostaining on serial sections for CD11c (brown, M1 macrophage marker) and CD206 (Mrc1; red, M2 macrophage marker) showed that macrophages in control uterus preferentially expressed CD11c (Fig. 8G), whereas those in PGN+poly(I:C)-treated uterus expressed both CD11c and CD206 (Fig. 8H). To validate double immunostaining, serial sections were immunostained with reverse staining of CD11c (red) and CD206 (brown), yielding the same result (Fig. 8I, 8J). Isotype controls are also shown (Fig. 8K, L).

This result was also confirmed by the double-immunofluorescence staining of F4/80/CD11c and F4/80/CD206 in serial sections of uterus. In control uterus, few F4/80⁺ macrophages preferentially expressed CD11c (Fig. 9A–9F). However, in PGN+poly(I:C)-treated uterus, ∼85% of F4/80⁺ macrophages expressed both CD11c and CD206 (Fig. 9G–L). Isotype controls are also shown (Supplemental Fig. 4C–H). These results suggest that, in the PGN+poly(I:C)-treated uterus, the number of F4/80⁺ macrophages was increased, and these macrophages became double positive for CD11c and CD206 (Fig. 8M).

M1 and M2 macrophage–associated cytokines during preterm labor

To further determine M1 and M2 polarization, the levels of M1 macrophage–associated cytokines (IFN-γ, IL-1β, IL-6, IL-12p70, and TNF-α), M2 macrophage–associated cytokine (IL-10), chemokines (G-CSF, GM-CSF, MCP1, and MIP-1α), and other cytokines IL-2 and LIF were checked by the Luminex assay. M1 macrophage–associated proinflammatory cytokines were significantly elevated in PGN+poly(I:C)-treated uterus (Fig. 10A) and placenta (Fig. 10B) compared with control. The M2 macrophage–associated anti-inflammatory cytokine, IL-10, was significantly increased in PGN+poly(I:C)-treated uterus (Fig. 10A) but not in placenta (Fig. 10B). The chemokines were significantly elevated in PGN+poly(I:C)-treated uterus (Fig. 10A) and placenta (Fig. 10B) compared with the control group. There was no change in IL-2 level. LIF was significantly increased in PGN+poly(I:C)-treated uterus and placenta. These observations indicate that during PGN+poly(I:C)-induced preterm labor, both M1 and M2
Macrophage–associated cytokines are increased in the uterus, whereas few M1 macrophage–associated cytokines are increased in the placenta.

**Inflammasome activation during preterm labor**

a2V functions as an ATPase on the cell surface and prevents the danger associated molecular pattern ATP from activating the inflammasomes (44). ATP can trigger the activation of NLRP3 inflammasome in response to pathogen-associated molecular patterns, such as PGN and LPS (45). It stimulates the caspase-1–dependent cleavage and secretion of IL-1β from cells stimulated with pathogen-associated molecular patterns (46). We observed low expression of a2V in PGN+poly(I:C)-treated uterus; therefore, we examined the total ATP concentration in control and PGN+poly(I:C)-treated uterus. It was increased significantly in PGN+poly(I:C)-treated uterus compared with control (Fig. 11A). We further examined the mRNA expression of Nlrp3 and caspase-1, which was significantly increased in the PGN+poly(I:C)-treated uterus compared with control (Fig. 11B, 11C). Cleavage of pro-caspase-1 into active caspase-1 and activity of caspase-1 also were increased significantly in the PGN+poly(I:C)-treated uterus compared with control (Fig. 11D, 11E). Nlrp3 and caspase-1 gene expression and cleavage of pro-caspase-1 were undetectable in placenta. These results demonstrate the activation of inflammasomes in response to PGN+poly(I:C) treatment.

**Discussion**

The internal acidification of intracellular compartments, such as lysosomes, endosomes, Golgi complexes, and secretary granules, was suggested to be critical to cell survival and function (47). V-ATPase plays a critical role in the maintenance of intracellular compartments and extracellular pH by pumping protons to the extracellular environment. This low acidic pH triggers proteases, which results in the dissolution of extracellular matrix. This process is well known for the contribution of tumor invasion and dissemination (48). We showed that placental a2V-ATPase (a2V)...
plays a critical role in the development of the trophoblast and its invasion in normal pregnancy (17). In contrast, decreased placental a2V is associated with upregulation of proinflammatory cytokines (e.g., IL-1β, TNF-α) during LPS-induced abortion (17). Other investigators (49–51) described the fundamental role of V-ATPase during cytokine trafficking and secretion. Our previous studies showed that a2NTD signaling of a2V participates in the induction of an immune-tolerogenic state during tumorigenesis (52, 53).

In the current article, we report that during PGN+poly(I:C)-induced preterm labor, a2V expression and cleaved a2NTD levels were significantly reduced in the uterus and placenta. This decreased a2V expression might halt the further development of the placental trophoblast, and it hinders the production of one of a2V’s main target products, cleaved a2NTD, which is responsible, in part, for the maintenance of the immune tolerogenic state (52). Loss of this tolerance may manifest preterm labor.

NO synthase is structurally associated with the cadherin/β-catenin/actin complex (40), and its activation is a key regulator of the adherens junction (41). Several reports suggested that V-ATPase inhibitors prevent cell growth and survival through iNOS

FIGURE 10. Cytokine and chemokine pattern during PGN+poly(I:C) induced preterm labor. M1 and M2 macrophage associated cytokines and other chemokines level were measured by luminex assay in uterus (A) and placenta (B) recovered from control and PGN+poly(I:C) treated animals. n = 6 each group. Error bars = ±SEM. *p ≤ 0.05, **p ≤ 0.01 Significant difference versus respective control.

FIGURE 11. PGN+poly(I:C) induces inflammasome activation in uterus. Total ATP concentration (A) and expression of Nlrp3 (B) and caspase-1 (C) in control and PGN+poly(I:C)-treated uterus (n = 6–11 mice/group). (D) Level of activated caspase-1 in control (lane 1) and PGN+poly(I:C)-treated (lane 2) uterus. (E) Caspase-1 activity. n = 4–6 each group. Error bars represent SEM. *p ≤ 0.05, **p ≤ 0.01, versus control.
via NO production, which is a potent inducer of apoptosis in various cell types (39, 54–56). We observed that the decreased expression of a2V is allied with upregulation of iNOS in both myeloid and nonmyeloid cells of PGN+poly(I:C)-treated uterus and placenta. Our in vitro data also showed that blocking of a2V by anti-a2V is directly associated with the induction of iNOS gene expression. It was reported that upregulation of iNOS is associated with disruption or opening of cell-to-cell contacts and induction of apoptosis (40, 41, 57). Our data showed that adherens junctions in uterine decidua and placental trophoblasts are disrupted, as evidenced by the disassembly of E-cadherin, N-cadherin, and β-catenin during PGN+poly(I:C)-induced preterm labor. We suggest that downregulation of a2V in the PGN+poly(I:C)-treated uterus and placenta is associated with iNOS activation, which ultimately causes the weakening of adherens junctions among uterine decidual cells and placental spongiotrophoblasts.

A V-ATPase inhibitor (bafilomycin A1) induces apoptosis in various mammalian cell lines (22–24). We showed that apoptosis induced by PGN+poly(I:C) treatment is regulated by the extrinsic pathway of apoptosis, as indicated by the increased activation of caspase-8 followed by caspase-3. Further investigation revealed that PGN+poly(I:C) can also activate the FAS/Fasl/FADD death pathway in the uterus and placenta. It was reported that blocking of V-ATPase enhances TNF-α–induced DNA fragmentation and activation of caspase-8 (58). Our in vitro data showed that PGN+poly(I:C)-induced TNF-α and iNOS were significantly enhanced in the presence of anti-a2V. We also showed that TNF-α and iNOS were significantly upregulated in PGN+poly(I:C)-treated uterus and placenta. Therefore, it is possible that, in the presence of PGN+poly(I:C), which itself induces the upregulation of TNF-α and iNOS, this upregulation might be enhanced because of the decreased levels of a2V in uterus and in placenta undergoing preterm labor. Interaction of TNF-α with its receptor (TNFR1) induces recruitment of the death domain–containing cytosolic adapter proteins TRADD and FADD, as well as procaspase-8, which triggers the caspase-activation cascade and apoptosis (59).

Thus, our results show that activation of the extrinsic apoptotic pathway might be associated with the decreased expression of a2V and enhanced Fas signaling and levels of iNOS and TNF-α in the PGN+poly(I:C)-treated uterus and placenta.

Cell death by apoptosis may trigger inflammation, and it also suppresses inflammatory responses (43). Intracellular mechanisms underlying inflammatory responses induced by PGN+poly(I:C) involve direct activation of NF-κB or degradation of IkBα in the macrophage cell line (8). The inflammatory response induced by PGN+poly(I:C) also involves induction of a second signaling event downstream of TLR activation is phosphorylation of ERK-1 and ERK-2, that play important roles in cell proliferation, apoptosis, and inflammation (3, 8).

In this study, we identify apoptosis and the innate immune response as dual pathogenic factors in the uterus and placenta undergoing preterm labor during a double hit of infection initiated by PGN+poly(I:C). The simultaneous activation of apoptosis and a specific innate immune response represents a critical pathogenic step in the induction of preterm labor. We showed that the number of macrophages (F4/80+) was increased in the PGN+poly(I:C)-treated uterus. Gonzalez et al. (60) demonstrated the increased infiltration of macrophages in mice that deliver preterm in response to LPS treatment. Depletion of F4/80+ macrophages prevented cervical remodeling and preterm delivery in LPS-treated mice. Our macrophage phenotypes study showed that these increased macrophages (F4/80+) were double-positive for CD11c and CD206. We could not identify separate populations of M1 (CD11c+) or M2 (CD206) macrophages in the PGN+poly(I:C)-treated uterus, although uterine macrophages in control animals were mainly M1-like (CD11c+). We propose that the deployment of double-positive (CD11c+ and CD206+) macrophages and their respective cytokine responses (both M1 and M2 cytokines) may contribute to recruitment and activation of innate immune defenses when uterine cell death occurs at infected or inflamed sites. We further propose that this combinational pro- and anti-inflammatory effect of double-positive macrophages on the uterine apoptotic cells is critical for the clearance of dying cells and then rapidly abetting the resolution of inflammation.

Previously, we showed that, under chronic pathologic conditions like LPS-induced abortion, placental a2V is decreased and linked with upregulation of proinflammatory cytokines (e.g., IL-1β, TNF-α, Mcp1) (17). Derks and Beaman (44) showed that the secretion of IL-1β from human monocytes was enhanced with anti-a2V in combination with exogenous ATP. It was shown that a2V expression occurs on the cell surface, where it functions as an ATPase and prevents extracellular ATP from activating the inflammasome through the P2X7 channel. ATP binding to P2X7 causes the efflux of potassium ions and activation of inflammasomes (61). Our study showed that PGN+poly(I:C) treatment activates caspase-1 via Nlrp3 inflammasome, although it is possible that there is a contribution from decreased a2V activity leading to elevated ATP levels, which activate the inflammasome through the P2X7 channel. The overexpression and activation of caspase-1 during a lethal response to LPS are crucial mediators of apoptosis and maturation of cytokines IL-1 and IL-18 (62, 63). In this study, we showed that PGN+poly(I:C) exposure also resulted in overexpression and activation of caspase-1, which could induce pyroptosis and lead to preterm labor.

Therefore, the double hit of PGN and poly(I:C) induces preterm labor in the murine model via simultaneous activation of apoptosis and inflammmatory processes. a2V might be a bridge linking these apoptotic and inflammatory processes, and it may be a useful therapeutic target for preterm labor.

Disclosures
The authors have no financial conflicts of interest.

References


