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# Human Endometrial Epithelial Cells Cyclically Express Toll-Like Receptor 3 (TLR3) and Exhibit TLR3-Dependent Responses to dsRNA

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## Abstract

Toll-like receptor 3 (TLR3) responds to dsRNA, a product of most viral life cycles, and initiates production of proinflammatory and antiviral cytokines. The role of TLR3 in human mucosal immunity of the endometrium has not been examined. The effects of TLR3 ligation in endometrial epithelium could be significant as the endometrium is a significant site for viral entry and infection. Additionally, the cytokine milieu plays an essential role in normal functions of the endometrium such as uterine cycle progression, epithelial proliferation and shedding, and embryo implantation. In this study, we demonstrated cycle dependent expression of functional TLR3 in primary endometrial epithelial tissue and expression of intracellular TLR3 in human endometrial epithelial cell lines. We established that stimulation of TLR3-positive cell lines and primary human endometrial epithelial cells with dsRNA leads to TLR3-dependent expression of interleukin (IL)-6, IL-8, interferon (IFN)-inducible protein 10, RANTES, and IFN-β. These results indicate that the cytokine profile of human endometrial epithelial cells can be modified through TLR3 stimulation. Our findings suggest that TLR3 is involved in the immune responses of endometrial epithelial cells after exposure to dsRNA and has the potential to alter the cytokine milieu and influence the outcome and consequences of infection.

### Keywords

human; innate immunity; mucosa; reproductive immunology; Toll-like receptor

## INTRODUCTION

Toll-like receptors (TLRs) are a family of innate pattern recognition receptors characterized by amino-terminal leucine-rich repeat domains and carboxy-terminal Toll/interleukin (IL)-1 receptor (TIR) signaling domains [1] (for reviews, see [2,3]). Toll-like receptor family

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members recognize unique pathogen-associated molecular patterns to initiate innate and shape adaptive immune responses [4-7]. Alexopoulou *et al*. [8] established that TLR3 is critical for cellular responses to dsRNA, a product of most viral life cycles. Induction of TLR3 signaling via dsRNA activates transcription factors such as NF-κB and interferon (IFN) regulatory factor 3, resulting in the production of proinflammatory and antiviral cytokines [8-14].

In humans, TLR3 expression was thought to be restricted to human dendritic cells [2,15]. However, Matsumoto *et al*. demonstrated that the lung fibroblast cell line, MRC-5, expresses functional TLR3 [16]. This discovery was followed by numerous groups establishing epithelial cell line expression of TLR3 [17,18]. Carrio *et al*. demonstrated expression of TLR3 by primary intestinal epithelial cells [19], whereas Sha *et al*. found expression in airway epithelial cell lines [20]. Fichorova *et al*. [21] demonstrated cervicovaginal epithelial cell line expression of TLR3. Our group established that endometrial epithelial tissues and cell lines express TLR3 as well as other TLRs [22]. Schaefer *et al*. [23] independently confirmed this observation. These findings establish that TLR3 is expressed in mucosal epithelial cells, but its function in innate mucosal immunity is not known [24].

In the gut, TLRs can distinguish between commensal and pathogenic organisms, possibly because of polarized expression patterns, reduced sensitivity to pathogen-associated molecular patterns, or low expression levels [25-28]. Inappropriate TLR activation in the intestine results in proinflammatory cytokine production that can be linked to inflammatory bowel disorders [29,30]. Differences in localization of TLR3 are seen in different cell types, and it is not known how this affects the function of TLR3 in mucosal epithelium [16,31,32]. The importance of other TLRs, like TLR4 and TLR5, in responses of gut mucosal epithelium to bacteria may suggest that TLR3 expression in mucosal epithelium could play a similar role in determining mucosal responses to viral infections in the endometrium [25-29,33].

We have previously demonstrated expression of TLR3 mRNA in the endometrium [22]. However, expression of TLR3 protein and the importance of TLR3 in endometrial function and immune responses have not been examined. It is known that cytokines play an essential role in normal functions of the endometrium such as embryo implantation and epithelial proliferation and shedding [34-37]. Cytokine imbalances and immune dysfunction have been implicated in problems such as spontaneous abortion and endometriosis, but the trigger of these cytokine imbalances is not known [35,38-40]. Because TLR3 activation impacts cytokine production, recognition of dsRNA in the endometrial epithelium could be a critical event in endometrial dysfunction [41,42]. Little is known regarding responses to dsRNA by endometrial epithelial tissue and cell lines or the impact of immune responses to viral infection on the endometrium.

The role of TLR3 in antiviral immunity *in vivo* is unresolved, possibly because of redundancy of function between TLR3, TLR4, and dsRNA binding proteins such as the double-stranded RNA-activated protein kinase (PKR). TLR4 initiates antiviral responses to viral proteins, whereas PKR can bind directly to dsRNA and is required for NF-κB activation during infection with viruses such as herpes simplex virus 1 [13,43-48]. Double-stranded RNA-activated protein kinase has been demonstrated to be required for innate immunity to viral infections such as vesicular stomatitis virus and influenza virus [49]. In the endometrium, PKR is expressed throughout the uterine cycle [50]. The contribution of PKR in direct binding and response to dsRNA is often ignored in the investigation of cellular response to dsRNA even though TLR3-independent responses to dsRNA have been observed [51].

In this study, we investigated the immune response of primary endometrial epithelial cells and cell lines to dsRNA. We found that endometrial epithelial tissue cyclically expresses TLR3 mRNA, and protein expression was demonstrated to be cycle dependent in primary tissue and

intracellular in the endometrial epithelial cell line, RL95-2. On stimulation with polyinosinic-polycytidylic acid (Poly I:C), endometrial tissues and cell lines produce proinflammatory and antiviral cytokines. We were able to demonstrate that this response is dependent on TLR3 through the use of TLR3-specific small inhibitory RNAs (siRNAs). Additionally, transfection of a TLR3 expression vector in an endometrial epithelial cell line (Ishikawa) that does not express TLR3 is sufficient to confer Poly I:C responsiveness to the Ishikawa cell line. These novel findings indicate the cytokine profiles of endometrial epithelial cells can be modified through TLR stimulation and suggests a role for TLRs in modulating the cytokine milieu during the uterine cycle and mediating antiviral responses in the endometrial epithelium.

## MATERIALS AND METHODS

### Cell Lines and Cell Culture

The endometrial epithelial cell line, RL95-2, and the lung fibroblast cell line, MRC-5, were obtained from ATCC (Manassas, VA). The endometrial epithelial cell line, Ishikawa, was a gift from Bruce Lessey (Greenville Hospital, Greenville, SC). RL95-2 cells were maintained in phenol-red free DMEM-F12 and Ishikawa and MRC-5 cells in MEM. RL95-2 and Ishikawa cells were supplemented with 2 mM L-glutamate and 50 μg/ml gentamicin (American Pharmaceutical Partners, Schaumburg, IL). MRC-5 cells were additionally supplemented with 1 mM sodium pyruvate and 1 mM nonessential amino acids. All media contained 10% fetal bovine serum (FBS) (US Bio-Technologies, Parkerfield, PA). Adherent cell lines were harvested with 0.05% trypsin/0.53 mM EDTA in HBSS. Unless otherwise indicated, all reagents were obtained from Invitrogen (Carlsbad, CA).

### Tissue Collection and Culture

Endometrial biopsy specimens were obtained under institutional review board–approved protocols from 18–38-year-old volunteers with normal regular 26–34-day menstrual cycles. Subjects receiving any medication that would affect hormone levels or those with any uterine disease were excluded. A portion of the biopsy specimen was fixed in formalin and embedded in paraffin; the remainder was flash-frozen or placed in RNA Later (Ambion, Austin, Texas, USA). Menstrual cycle stage was determined by morphologic evaluation of a hematoxylinand-eosin-stained fixed section following the criteria of Noyes *et al.* [52]. In some cases, a portion of the biopsy specimen was also separated as previously described into epithelial and stromal cells in the laboratory of Dr. Kathy Timms (University Hospital, Columbia, MO) [52]. Cell purity was assessed by cytokeratin and vimentin immunostaining, typically demonstrating greater than 98% purity [53]. Endometrial epithelial cells were plated at $0.2 \times 10^6$ cells/well/ml in 12-well plates. Some plates were precoated with Matrigel Matrix (BD Biosciences). Cells were maintained under the same conditions as RL95-2 cells except that media were supplemented with 5% FBS instead of 10% FBS.

### RNA Isolation

Endometrial epithelial cell lines were grown to confluence in 12-well plates, and total RNA was isolated with the RNAaqueous-4PCR kit (Ambion, Austin, TX) per manufacturer's instructions, except RNA was treated with DNase I twice. The second digestion was performed with twice the amount of DNase I enzyme and DNase inactivation reagent. RNA was quantified with the RiboGreen RNA Quantitation kit (Molecular Probes, Eugene, OR), and fluorescence was measured with a 485-nm excitation filter and a 535-nm emission filter on a Fusion Universal Microplate Reader (Perkin-Elmer, Wellesley, MA). RNA concentration was determined by interpolating from a standard curve. Samples were measured in triplicate. RNA from primary tissues was provided by Bruce Lessey (Greenville Hospital, Greenville, SC).

### End point Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) and Electrophoresis

A total of 100 ng of RNA was used to synthesize cDNA by using the random hexamers provided in the 1st Strand Synthesis kit (Roche, Indianapolis, IN). cDNA was synthesized according to manufacturer's instructions. Briefly, 100 ng of RNA was denatured at 65°C for 15 minutes and immediately placed on ice for 5 minutes. RNA was combined with master mix containing or not containing AMV RT in a total reaction volume of 20 μl. The reaction was run on an Eppendorf Master Gradient Thermocycler (Brinkman, Westbury, NJ). Polymerase chain reaction that used gene-specific primers was performed with 1 μl of cDNA in 25 μl total reaction volume. Polymerase chain reaction amplification was performed by Taq Gold polymerase (Perkin-Elmer, Foster City, CA). TLR3, β-glucuronidase (GUS), IFN-β, and IFN-inducible protein 10 (IP-10) were amplified by using 30 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. IL-6 and IL-8 were amplified by using 33 cycles. TLR5 was amplified by using 30 cycles, but the annealing step was performed at 59°C for 30 seconds. Primers for TLR3, TLR5, PKR, GUS, IL-6, and IL-8 were obtained from the literature [22, 54-56]. Primers for IFN-β and IP-10 were designed by Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) (Whitehead Institute for Biomedical Research, Cambridge, MA). Primer sequences for IFN-β and IP-10 were as follows: 5′-AATTGAATGGGAGGCTTGAA-3′, 5′-AGCCAGGAGGTTCTCAACAA-3′, and IP-10, 5′-TCTCACCCTTCTTTTTCATTGTAG-3′, 5′-AATTGCCTTATCTTTCTG-3′. Loading dye (5 μl) was added to the reaction and 10 μl of sample was run on a 2% agarose 3:1 (Amresco, Solon, OH) gel in order to separate PCR products. Gels were stained with SYBR Green (Bio-Whittaker, Rockland, ME) for 45 minutes and visualized by ultraviolet transillumination at 302 nm. Digital images were obtained with a GelLogic 100 (Kodak, Rochester, NY).

### Real-Time RT-PCR

Real-time RT-PCR was performed with cDNA synthesized as for end point RT-PCR. cDNA was combined with primer/probe sets and Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Primer/probe sets for TLR3, TLR5, GUS, and hypoxanthine guanine phosphoribosyl transferase (HPRT) were purchased and designed by Assays-on-Demand (Applied Biosystems, Foster City, CA). Real-time assays were run on an ABI 7000 (Applied Biosystems, Foster City, CA). For primary cells, samples were normalized internally by using the cycle threshold (CT) of the housekeeping gene GUS as follows: ΔCT = (CT TLR3) − (CT GUS). The mean CT of RNA from proliferative phase tissue (Norm 1) was set to a relative quantity (RQ) value of 1 by using the ΔΔCT calculated as follows: ΔΔCT = mean ΔCT (Norm 1) − mean ΔCT (Norm 1) and RQ = $2^{(-1\times\Delta\Delta CT)}$. All other samples were compared with the mean proliferative phase RQ value by using the following equation; ΔΔCT = ΔCT (sample) − mean ΔCT (Norm 1). Relative quantity values were calculated as follows; RQ = $2^{(-1\times\Delta\Delta CT)}$. In the siRNA experiments, samples were normalized internally by using CT of the housekeeping gene HPRT as follows; ΔCT = mean (CT gene of interest) − mean (CT HPRT). The normalized value of the RNA from cells not treated with siRNAs (Norm 1) was set to an RQ value of 1, and all other samples were compared with that value as described for the primary tissue RNA samples.

### Immunohistochemistry

Frozen and paraffin-embedded tissue sections from primary endometrial epithelial tissue were provided by Kathy Timms (University Hospital, Columbia, MO). Frozen tissue sections were air dried, fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes, and washed in PBS, then water. Endogenous peroxides were quenched with 0.3% $H_2O_2$ in methanol and washed in PBS. Slides were then permeablized in 0.4% Triton X-100 in PBS for 10 minutes, washed with PBS, and blocked with PBS containing 1% normal horse serum (NHS) in PBS for 20 minutes. Slides were incubated with primary antibody (αTLR3.7 monoclonal

antibody [mAb]) at a 1:25 dilution overnight, followed by wash and incubation with biotin-conjugated antimouse immunoglobulin (Ig) G1 secondary antibody at 1:200 for 1 hour. Slides were incubated with ABC reagent for 30 minutes and stained with DAB for 10 minutes. Slides were then counterstained with 1:4 hematoxylin/water. Pictures of the slides were taken with a Nikon Cool Pix 990 and analyzed by Nikon View 3.1 software (Nikon, Melville, NY). Formalin-fixed, paraffin-embedded tissue sections were deparaffinized by incubation in xylene twice for 5 minutes, followed by washes in 95% and 70% ethanol. Slides were then washed in water and stained as frozen tissue sections after fixation in 4% paraformaldehyde.

### Cell Stimulation

Cells were plated at $0.2 \times 10^6$ cells/well/ml in 12-well plates. The next day (~12 hours), cells were stimulated with ligand. At indicated times, supernatants or cells were harvested. Cells were stimulated with Poly I:C (Amersham Pharmaceutical Biotech, Piscataway, NJ), polydeoxyinosinic-deoxycytidylic acid (Poly dI:dC) (Amersham Pharmaceutical Biotech, Piscataway, NJ), lipopolysaccharide (LPS) (Calbiochem, San Diego, CA), Flagellin (Apotech, Epalinges, Switzerland), or phorbol-12-myristate-13-acetate (Calbiochem) plus Ionomycin (Calbiochem) (PMA/I). Supernatants were centrifuged at 1400 rpm for 8 minutes at 4°C, transferred to a fresh tube, and stored at −20°C until use. Cells were washed once with 1× PBS before use.

### Flow Cytometry

Cells were plated in 12-well plates, grown to confluence, and harvested for labeling with either phycoerythrin (PE)-labeled monoclonal [m]IgG1αhTLR3 antibody (clone TLR3.7) (eBioscience, San Diego, CA) or PE-labeled mIgG1 isotype control (BD Biosciences). For intracellular staining, cells were fixed with Cytofix/Cytoperm buffer (BD Biosciences), and all washes and incubation were performed in the saponin containing Perm/Wash buffer (BD Biosciences). Cells used for surface staining were not fixed, and all washes were with PBS containing 1% BSA. Cells were analyzed with a fluorescence activated cell sorter [FACS]can instrument, and analysis was performed with CellQuest software (BD Biosciences). Ten thousand cells were counted, and viability was determined by generating forward scatter versus side scatter density plots and setting the gate to exclude dead cells. Histogram plots included only gated cells. Markers indicating TLR3-positive cells were set so that less than 10% of isotype control cells were included in the positive marker.

### Enzyme-Linked Immunosorbent Assay (ELISA)

IL-6, IL-8, and IP-10 Duoset kits were purchased from R&D Systems (Minneapolis, MN), and ELISA was performed according to manufacturer's instructions with 100 μl of cell free supernatant. IL-6 detection limit was 5 pg/ml, IL-8 was 8 pg/ml, and IP-10 was 10 pg/ml (the lowest standard used in each standard curve). Absorbance at 450 nm was read with the SPECTRAMax 190 microplate spectrophotometer, and results were analyzed by SOFTMax Pro software (Molecular Devices, Sunnyvale, CA). Sample concentrations were determined by interpolation from the standard curve. Samples were read in triplicate.

### Cytometric Bead Array (CBA)

The human inflammation CBA kit and the human chemokine CBA kit were purchased from BD Biosciences and used according to manufacturer's instructions. Briefly, 50 μl of supernatant was incubated with capture antibody-linked beads and PE-labeled detection beads. Mean fluorescent intensities of samples were read on a FACSVantage instrument and analyzed by software provided by the manufacturer (BD Biosciences). Concentrations were determined by interpolation from a standard curve.

### siRNA Treatment

Cells were plated with regular growth media at $0.2 \times 10^6$ cells/well/ml in 12-well plates. The next day (~12 hours), media were replaced with antibiotic-free media, and 10 pmol of TLR3 or GUS-specific siRNAs and 6 μl of oligofectamine (Invitrogen, Carlsbad, CA) were added as described by manufacturer. HPLC-purified siRNAs designed by the manufacturer were purchased from Ambion (Austin, TX). Sequence information can be found on the manufacturer's Web site (http://www.ambion.com). The siRNA ID number for TLR3 and GUS are 4626 and 8128, respectively. Forty-eight hours later, media were replaced with fresh growth media and stimulated with TLR ligands. At 18 hours after stimulation, cells and supernatants were collected. RNA was collected and examined by real-time RT-PCR for expression of TLR3, TLR5, GUS, and HPRT mRNA. Supernatant was analyzed for IL-6 and IL-8 production by ELISA. All experiments were performed in triplicate.

### Transfection with pUNO-hTLR3

The pUNO-hTLR3 (pTLR3) and pZERO-hLTR3-TIR-(pTIR) expression vectors were purchased from Invivogen (San Diego, CA). Vector DNA was purified from *Escherichia coli* grown in FastMedia + Blasticidin or Fast Media + Puromycin (Invitrogen), respectively, with the Qiaprep Spin Minprep kit (Qiagen, Valencia, CA) and quantified by spectrophotometry with the Eppendorf biophotometer (Brinkman, Westbury, NJ). Ishikawa cells were plated at $0.1 \times 10^6$ cells/well/0.5 ml in 24-well plates. The next day (~12 hours) media were replaced with OptiMEM (Invitrogen, Carlsbad, CA) and 0.4 μg of vector and 4 μl lipofectamine (Invitrogen) were added to each well per manufacturer's instructions. At 12 hours, 400 μl of growth media containing 2× serum was added. Twenty-four hours after transfection, media were replaced with normal growth media. At 48 hours after transfection, cells were stimulated with TLR ligands for 18 hours. RNA, cells, and supernatant were collected. RNA was used to analyze for the expression of TLR3, TLR5, and GUS mRNA. Cells were used for FACS analysis of expression of TLR3 protein. Supernatants were used to measure production of IL-6 and IL-8 by ELISA.

## RESULTS

### RL95-2 Cells Express TLR3 Intracellularly

We have previously demonstrated expression of TLR3 but not TLR4 mRNA by the RL95-2 cells [22]. We confirmed that RL95-2 cells, but not Ishikawa cells, expressed TLR3 mRNA; MRC-5 cells, previously demonstrated to express TLR3, were included as a positive control for TLR3 (data not shown) [16]. However, RL95-2 cell expression of TLR3 mRNA does not ensure that protein is expressed by these cells. We used FACS analysis of RL95-2 cells to examine TLR3 protein expression. TLR3 expression was characterized by using the mIgG1 antihuman TLR3 mAb TLR 3.7 (αTLR3.7 mAb). We found that RL95-2 cells express intracellular but not surface TLR3 protein (Figure 1). RL95-2 cells incubated with isotype antibody and Ishikawa cells incubated with αTLR3.7 mAb were included as negative controls. RL95-2 cell surface expression of TLR3 as compared with the isotype control was only 1% (Figure 1A), whereas expression of intracellular TLR3 was 66% (Figure 1B). These results were confirmed by immunohistochemistry (data not shown). These results demonstrate that TLR3 protein is expressed by endometrial epithelial cells, and, unlike the lung fibroblast cell line MRC-5, this expression is primarily intracellular [16].

### RL95-2 Cells Produce Inflammatory and Antiviral Cytokines and Chemokines in Response to Poly I:C Stimulation

We have previously demonstrated IL-8 production in response to stimulation of RL95-2 cells with dsRNA [22]. Cytometric bead array was performed in order to further investigate the

production of proinflammatory and antiviral cytokines and chemokines in response to dsRNA stimulation. Cytometric bead arrays allow the measurement of up to six cytokines from 50 μl of supernatant. RL95-2, Ishikawa, and MRC-5 cells were stimulated Poly I:C, LPS, Poly dI:dC, and PMA/I for 18 hours. Supernatants were collected and examined for cytokine production by CBA. The human inflammation CBA detected production of IL-6 and IL-8 but not IL-1β, IL-12p70, IL-10, or TNFα by RL95-2 cells in response to stimulation with Poly I:C and PMA/I only (Figure 2A). The response to dsRNA was specific because stimulation with dsDNA, Poly dI:dC, and LPS (TLR4 is not expressed by RL95-2 cells) had no effect. MRC-5 cells included as a positive control produced IL-6 and IL-8 in response to Poly I:C and PMA/I (data not shown). As expected, Ishikawa cells produced IL-8 only in response to PMA/I stimulation (data not shown).

In addition to IL-8 production, the human chemokine CBA detected RL95-2 production of the macrophage chemoattractant RANTES and the antiviral chemokine IP-10 but not MCP-1 or MIG (Figure 2B). MRC-5 cells stimulated with Poly I:C and PMA/I produced IP-10, MCP-1 and IL-8, but did not produce RANTES or MIG (data not shown). Ishikawa cells produced only IL-8 in response to PMA/I stimulation (data not shown). In order to confirm these results, ELISA was performed with the same supernatants to detect IL-6, IL-8, TNF-α, RANTES, and IP-10. In all cases, ELISA results confirmed the CBA results (data not shown). The detection of RANTES (in addition to IL-6 and IL-8) and IP-10 suggests activation of both proinflammatory responses and antiviral responses on Poly I:C stimulation of RL95-2 cells.

## Endometrial Epithelium Cyclically Express Functional TLR3

We next examined expression and function of TLR3 in primary endometrial tissue. We have previously demonstrated that primary endometrial epithelium express TLR3 mRNA [22]. We wanted to determine whether this expression is altered as the endometrium undergoes changes associated with the uterine cycle. RNA collected from endometrial tissue during the proliferative, early, mid-, and late secretory phases was used to examine expression of TLR3 mRNA via real-time RT-PCR. Surprisingly, we found the TLR3 mRNA levels were cycle dependent. TLR3 mRNA was expressed throughout the uterine cycle, but was expressed at the highest levels during the mid- and late secretory phases. TLR3 was expressed at the lowest level during the proliferative phase (Figure 3A).

We next ascertained whether TLR3 protein regulation was cycle dependent. We examined endometrial epithelial tissue via immunohistochemistry. Frozen or paraffin embedded tissue sections were stained with αTLR3.7 mAb. We found that TLR3 protein expression, like TLR3 mRNA expression, is cycle dependent. TLR3 protein was expressed at very low levels in proliferative tissues (Figure 3B). However, mid- and late secretory tissue strongly expressed TLR3 protein. Expression of TLR3 in paraffin-embedded tissue indicated expression was highest in the luminal epithelium, but could also be detected at slightly lower level in glandular tissue (Figures 3C,E). Adjacent tissue sections stained with isotype antibody were included as a negative control (Figures 3D,F).

We then investigated whether TLR3 expressed by primary endometrial epithelium was functional. We examined responsiveness of TLR3-expressing primary endometrial epithelial cells to TLR ligands *in vitro.* Separated endometrial epithelial cells were plated in wells precoated with matrigel (a substance used to mimic basement membrane) and stimulated for 18 hours. On stimulation with Poly I:C, primary endometrial epithelial cells produced IL-6, IL-8, and IP-10 (Figure 3G). RANTES and tumor necrosis factor α were not produced (data not shown). When stimulated with LPS, the cells produced significant amounts of only IL-6. Small amounts of cytokine were detected in response to PMA/I stimulation (Figure 3G). The same results were seen when cells were plated on wells not coated with matrigel (data not shown). Our results demonstrate that functional TLR3 protein is cyclically expressed by

primary endometrial epithelial cells and that dsRNA can induce cytokine production by endometrial epithelial cells.

### RL95-2 Cells Have a Rapid and Sustained Response to Low Doses of Poly I:C

A dose response to Poly I:C by RL95-2 cells was performed in order to examine sensitivity of endometrial epithelial cells to Poly I:C stimulation. RL95-2 cells were treated with doses of Poly I:C ranging between 5 ng/ml and 250 ng/ml (Figures 4A, B). Surprisingly, a dose-dependent response to Poly I:C was observed at doses as low as 10 ng/ml. Both IL-6 (Figure 4A) and IL-8 (Figure 4B) reach maximal production by 50 ng/ml. MRC-5 cells included as a positive control do not exhibit responses to Poly I:C until a dose of 1 μg/ml, as previously reported [16]. Ishikawa cells do not respond to Poly I:C even at doses as high as 250 ng/ml (data not shown). Our results suggest that RL95-2 cells are more sensitive to Poly I:C stimulation than other cell lines previously described to express TLR3.

A time course was performed to determine the rapidity of response and verify that supernatants were analyzed at the appropriate time point. RL95-2 cells stimulated with Poly I:C start secreting IL-6 and IL-8 4 hours after stimulation and sustain production through 72 hours (Figure 4C). Ishikawa cells do not produce any cytokine. These results verify that peak responses are seen at 18 hours and supernatants were analyzed at the appropriate time point. Also, a rapid and sustained response of RL95-2 cells to Poly I:C stimulation was observed on exposure to dsRNA.

### Poly I:C Stimulation Leads to Rapid Induction of Proinflammatory and Antiviral Cytokines by RL95-2 Cells

The kinetics of the production of proinflammatory and antiviral cytokines is important in determining the signaling events leading to cytokine production. For example, the detection of the viral response protein IP-10 by RL95-2 cells in response to Poly I:C stimulation could be caused by TLR3 signaling or from autocrine activation of the interferon α/β receptor by IFN-β [12,57]. To investigate the kinetics of proinflammatory and antiviral cytokine production in RL95-2 cells, we compared RL95-2 expression of mRNA for IL-6, IL-8, IFN-β, and IP-10 by using end point RT-PCR. RNA was collected from RL95-2 cells stimulated with Poly I:C for 30 minutes, 1 hour, or 2 hours. Production of IFN-β and IL-8 mRNA was detected at 1 hour after stimulation; IP-10 and IL-6 mRNA was induced by 2 hours after stimulation (Figure 5). The housekeeping gene GUS was measured to control between samples. To demonstrate that expression was not from genomic contamination, a no RT control was included for each sample. These data suggest that the proinflammatory and antiviral responses arise concurrently and do not require autocrine action of one response to stimulate the other. Additionally, the induction of mRNA for IFN-β and IP-10 at similar time points (1 and 2 hours, respectively) suggests that treatment of endometrial epithelial cells with Poly I:C initiates production of IP-10 as a part of the primary antiviral response rather than a part of the secondary antiviral response as seen in other systems [10,13,14].

### TLR3 siRNAs Reduce Expression of TLR3 and Decrease RL95-2 Responses to Poly I:C

The production of cytokines in response to Poly I:C exclusively by TLR3-expressing cell lines strongly implicates TLR3 as the receptor for Poly I:C and suggests that the antiviral and proinflammatory responses in endometrial tissue are TLR3-dependent. However, PKR is also known to bind dsRNA and initiate an antiviral response [49,58]. To further investigate the role of TLR3 in responsiveness of endometrial epithelial cells to dsRNA, siRNAs were used to knock down the expression of TLR3. siRNAs are known to reduce expression of protein in a gene-specific manner [59] (for reviews, see [60,61]).

RL95-2 cells were transfected with TLR3- or GUS-specific siRNAs for 48 hours. RNA was collected from the cells and used to measure TLR3, TLR5, and GUS mRNA expression by real time RT-PCR. Our data demonstrated that gene-specific siRNAs knocked down expression only of the corresponding gene. TLR3-specific siRNAs reduced expression of TLR3 mRNA to 12% of the level seen in untreated cells while having little effect on the expression of GUS or TLR5 mRNA (Figure 6A). GUS-specific siRNAs did not significantly affect expression of TLR3 or TLR5 mRNA but reduced expression of GUS mRNA to 36% of the level seen with untreated cells (Figure 6A). As expected, Ishikawa cells, included as a negative control, did not express TLR3 mRNA but did express GUS and TLR5 mRNA as compared with untreated RL95-2 cells (Figure 6A). These data indicate the action of the TLR3 and GUS siRNAs were gene specific.

In order to verify that protein expression was suppressed, RL95-2 cells treated with TLR3-specific siRNAs were examined for expression of TLR3 by flow cytometry. RL95-2 cells were stained with PE αTLR3.7 mAb or with isotype antibody and analyzed for intracellular TLR3 expression. TLR3 expression levels were reduced to 29.04% in TLR3 siRNA-treated cells, as demonstrated by FACS analysis (Figure 6B).

When supernatants from cells treated with siRNAs and then stimulated with Poly I:C were analyzed by ELISA, our data demonstrated that addition of TLR3-but not GUS-specific siRNAs to RL95-2 cells reduced the production of IL-6 (Figure 6C) and IL-8 (Figure 6D) to near background levels. Because RL95-2 cells express TLR5 (Figure 6A) and respond to stimulation with the TLR5 ligand (Figure 6D), flagellin and PMA/I were used to control for nonspecific effects of the siRNAs. RL95-2 cells were still able to produce cytokines in response to stimulation with both ligands (Figures 6C,D). Cells treated with GUS-specific siRNAs were able to respond to Poly I:C stimulation (Figures 6C,D). siRNAs did have some nonspecific effects as both TLR3- and GUS-specific siRNAs decreased RL95-2 responses to flagellin and increased the response to PMA/I (Figures 6C,D). Although addition of TLR3-specific siRNAs decreased the response of RL95-2 cells to flagellin, this effect was nonspecific as GUS siRNAs had the same effect on the RL95-2 cells (Figure 6D). It is unclear why these effects occur, although Kariko *et al.* [62] reported that siRNAs can stimulate cytokine responses and induce sequence-independent mRNA degradation and suppression of protein synthesis in TLR3-expressing cells. These data indicate that TLR3 is required for RL95-2 cells to respond to Poly I:C stimulation and eliminate the possibility that PKR is responsible for the dsRNA response in endome-trial epithelial cells.

### Transfection of Ishikawa Cells with the TLR3 Gene Confers Responsiveness to Poly I:C

We next determined whether transfection of the TLR3 gene was sufficient for Ishikawa cells to become responsive to Poly I:C stimulation. Ishikawa cells were transfected with the expression vector pUNO-hTLR3 (pTLR3) for 48 hours. pTLR3 contains the cDNA for TLR3 under the control of the HTLV promoter. Transfected cells strongly express TLR3 mRNA despite unaltered expression of TLR5 mRNA by end point RT-PCR (Figure 7A). Approximately 29.5% of the cells transfected expressed TLR3 intracellular protein (Figure 7B). Surface-expressed protein was not detected (Figure 7B). On transfection with pTLR3, Ishikawa cells became Poly I:C responsive. Ishikawa cells produced IL-8 and IP-10 after Poly I:C stimulation, even though only IL-8 production was seen with PMA/I stimulation (Figure 7C). As expected, transfection with pTLR3 did not alter the response to LPS stimulation by Ishikawa cells. As a negative control, Ishikawa cells were transfected with the pZERO-hTLR3-TIR- (pTIR) expression vector. pTIR encodes a truncated cDNA for TLR3 that is missing the portion of the cDNA encoding the TIR signaling domain. Ishikawa cells transfected with this expression vector did not respond to Poly I:C stimulation (Figure 7C). These data make clear the unexpected result that TLR3 is sufficient to confer Poly I:C-responsiveness to Ishikawa

cells. This suggests that Ishikawa cells express TLR3-associated adaptor proteins and signaling molecules even in the absence of TLR3 expression.

## DISCUSSION

In this study, we provide the first demonstration that primary endometrial epithelial cells cyclically express TLR3 protein and exhibit TLR3-dependent proinflammatory and antiviral responses. Endometrial epithelial tissue and cell lines rapidly produce cytokines in response to dsRNA stimulation. These responses were demonstrated to be TLR3-dependent because expression of TLR3 mRNA and protein were both required and sufficient for cytokine production to occur.

Surprisingly, we found that TLR3 mRNA and protein are differentially regulated in endometrial epithelial tissue depending on the location of the cells and the stage of the menstrual cycle. Expression was highest during the mid- and late secretory phases and lowest during the proliferative phase. TLR3 was expressed more strongly in luminal epithelium than glandular epithelium. Additionally, endometrial epithelial cell lines differentially express TLR3. Our data indicated that RL95-2 cells express intracellular TLR3, whereas Ishikawa cells do not express any TLR3. This is noteworthy because MRC-5 cells express cell surface TLR3 [16]. Additionally, other endometrial epithelial cells (such as the KLE cell line) express both surface and intracellular TLR3 (unpublished observation). The consequences of these variances in TLR3 expression patterns are unclear. Variances in TLR expression patterns could prevent inappropriate immune responses in the endometrial epithelium. It is essential that immune responses are generated only in response to pathogenic organisms while foreign antigens such as sperm and the conceptus are ignored [63]. The former is particularly difficult because pathogens such as *Chlamydia trachomatis* and *Mycoplasma genitalium* can be carried by sperm [64-66]. Controlling TLR activation in mucosal tissue by restricting TLR expression has been observed in intestinal epithelial tissues [24-29]. In the gut, Gewirtz *et al*. [25] demonstrated that restriction of TLR5 expression to basolateral intestine helps prevent activation of TLR5 by commensal bacteria. Cyclic regulation and restricted TLR3 localization and expression patterns could prevent inappropriate activation of TLR3 responses in the endometrium.

We have demonstrated that RL95-2 cell and primary endometrial cell stimulation with Poly I:C results in TLR3-dependent production of the proinflammatory cytokines IL-6 and IL-8 and the antiviral cytokines IP-10. RL95-2 cells were revealed to produce RANTES and IFN-β as well. This response was demonstrated to be TLR3 dependent by means of RNA inhibition. Treatment with TLR3-specific siRNAs eliminated RL95-2 TLR3 gene and protein expression, and renders RL95-2 cells unresponsive to Poly I:C stimulation while remaining responsive to flagellin and PMA/I. This response occurs rapidly at doses of dsRNA approximately 100-fold lower than the dose required by MRC-5 cells. It is unclear why RL95-2 cells are extremely sensitive to Poly I:C stimulation. It is possible that because the uterus is exposed to and required to tolerate foreign molecules, TLRs in the endometrium may be hyporesponsive to stimuli such as LPS, thus requiring a higher sensitivity to other stimuli such as dsRNA. This is not unprecedented because many investigators have observed induction of homo- and heterotolerance to secondary stimulation with LPS [67-70]. Additionally, Abreu *et al*. [28] documented TLR4 hyporesponsiveness to LPS in intestinal epithelial cells, which must tolerate exposure to commensal-associated molecular patterns to prevent inflammatory bowel disease.

The hypothesis that endometrial tissues are able to generate rapid immune responses to dsRNA is supported by the results obtained when Ishikawa cells were transfected with the TLR3 gene. Our data indicate that the transfection of the TLR3 gene into non–TLR3-expressing cell lines is sufficient to permit responses to dsRNA. This evidence suggests that endometrial cells that

do not express TLR3 retain expression of TLR adaptor proteins and signaling molecules and could rapidly upregulate TLR3 expression and contribute to the proinflammatory and antiviral response when necessary.

Our finding that TLR3 expression is cycle dependent has important implication in estrogen and progesterone effects on TLR3 expression and responses. Future studies will examine the relationship between cycle stage and TLR3 expression as well as hormone regulation of TLR3 responses. Our data establish TLR3-dependent endometrial epithelial tissue and cell line production of proinflammatory and antiviral cytokines in response to stimulation with dsRNA. It is unknown how TLR3 functions *in vivo* during viral infection or what role it plays in determining the health of the endometrium. Our findings will allow examination of the responses of TLR3 during viral infection of endometrial epithelial cells, and the role that TLR3 activation plays in endometrial dys-functions such as endometriosis, recurrent miscarriage, or infertility can be examined. Future studies examining the role TLR3 plays during *in vitro* viral infection that use viruses known to infect the endometrium can investigate the balance between beneficial and detrimental TLR-mediated immune responses in the endometrium.

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## ABBREVIATIONS

CBA, cytometric bead array
CT, cycle threshold
ELISA, enzyme-linked immunosorbent assay
FACS, fluorescence activated cell sorter
FBS, fetal bovine serum
GUS, β-glucuronidase
HPRT, hypoxanthine guanine phosphoribosyl transferase
IFN, interferon
Ig, immunoglobulin
IL, interleukin
IP-10, interferon-inducible protein 10
LPS, lipopolysaccharide
mAb, monoclonal antibody
mIg, monoclonal immunoglobulin
PBS, phosphate buffered saline
PE, phycoerithrin
PKR, double-stranded RNA-activated protein kinase
PMA-I, phorbol-12-myristate-13-acetate plus ionomycin
Poly dI:dC polydeoxyinosinic-deoxycytidylic acid
Poly I:C polyinosinic-polycytidylic acid
RT-PCR, reverse transcriptase–polymerase chain reaction
RQ, relative quantity
siRNA, small inhibitory RNA
TLR, Toll-like receptor

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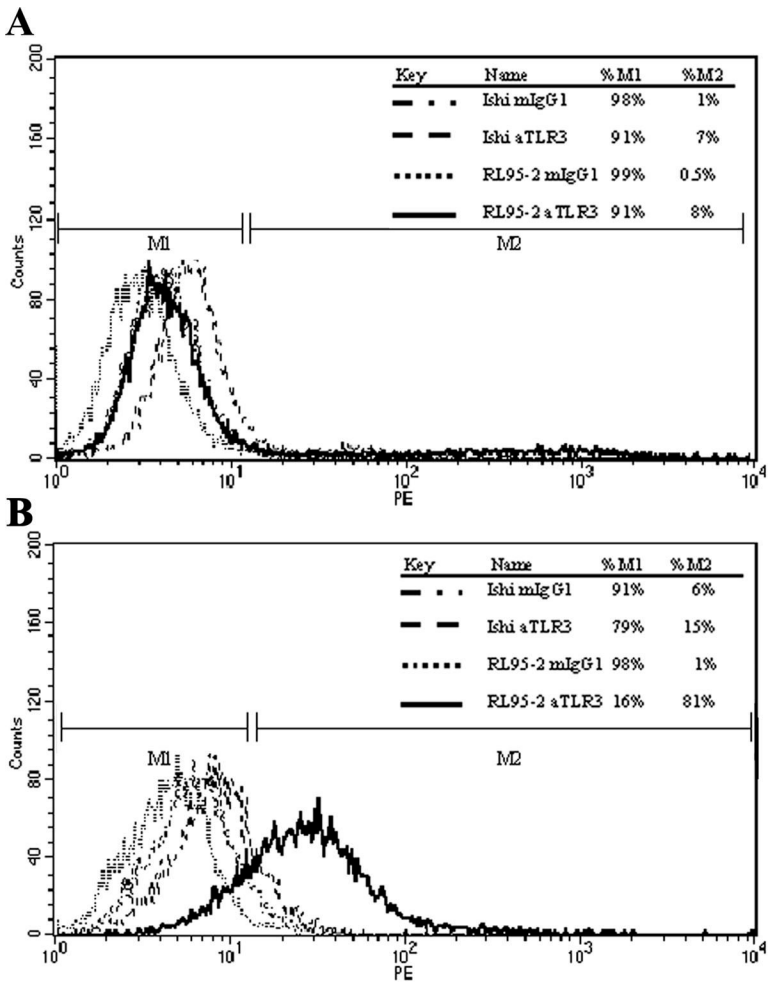


**FIGURE 1.**

RL95-2 cells express Toll-like receptor 3 (TLR3) intracellularly. RL95-2 or Ishikawa cells were labeled with PEαTLR3.7 monoclonal antibody in order to detect surface (A) or intracellular (B) expression of TLR3 protein by fluorescence activated cell sorter analysis. phycoertherin-conjugated monoclonal immunoglobulinG1 was used as an isotype control. Histogram plots were generated by gating on live cells. M1 was used to determine percentage of TLR3-negative cells, and M2 was used to determine the percentage of TLR3-positive cells. Experiments were repeated three times. Representative data are shown.

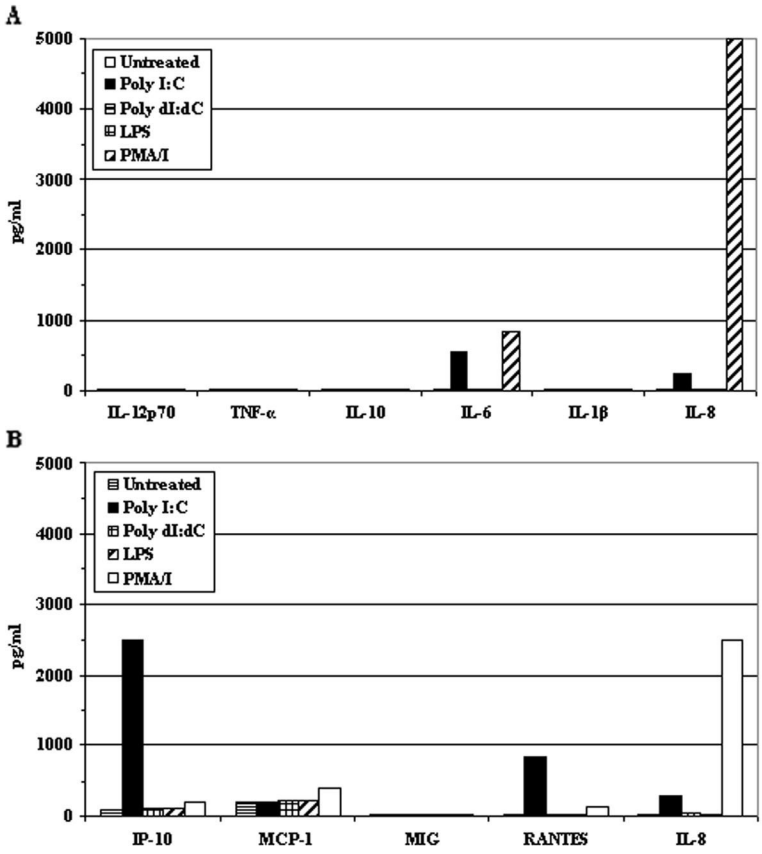


**FIGURE 2.**

RL95-2 cells produce inflammatory and antiviral cytokines and chemokines in response to polyinosinic-polycytidylic acid (Poly I:C) stimulation. RL95-2 cells were plated at $0.2 \times 10^6$ cells/well/ml and stimulated with Poly I:C (1 μg/ml), polydeoxyinosinic-deoxycytidylic acid (Poly dI:dC) (1 μg/ml), lipopolysaccharide (LPS) (100 ng/ml), or phorbol-12-myristate-13 acetate plus (PMA) (10 ng/ml)/ionomycin (I) (500 ng/ml) for 18 hours. A total of 50 μl of cell-free supernatant was used to detect cytokine by fluorescence activated cell sorter analysis by using the human inflammation cytometric bead array (CBA) (A) or the human chemokine CBA (B). Experiments were repeated twice. Data shown represent one experiment.

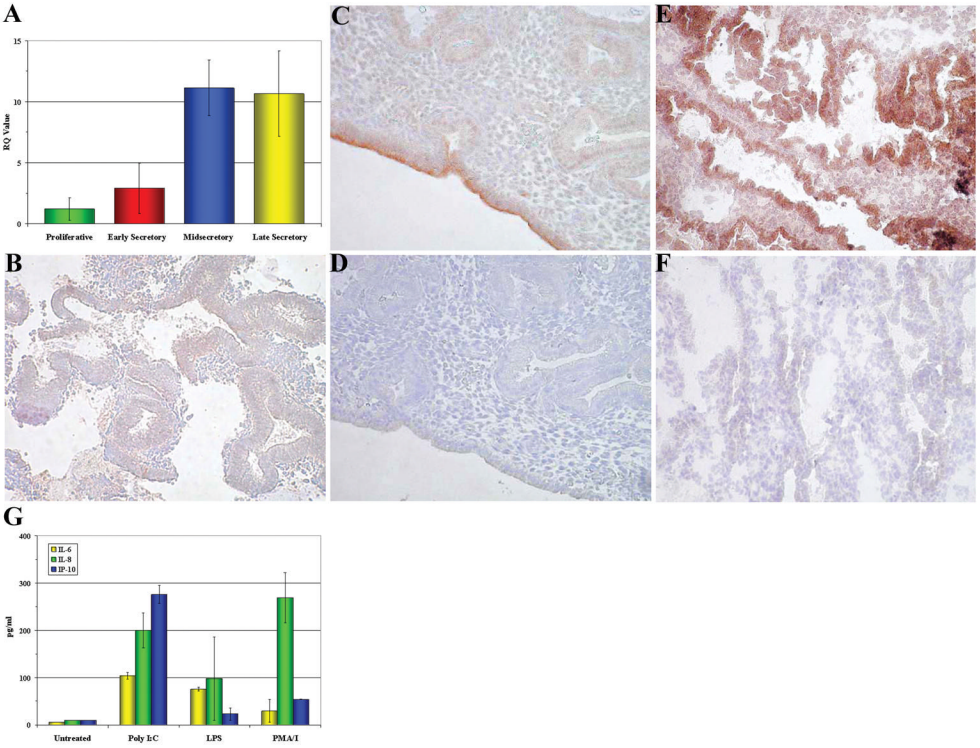


**FIGURE 3.**

Endometrial epithelium cyclically express functional Toll-like receptor 3 (TLR3). Total RNA (100 ng/ml) from primary endometrial tissue was used to detect relative expression levels of TLR3 mRNA by real-time reverse transcriptase-polymerase chain reaction (A). Data are shown as average relative TLR3 quantities (relative quantity [RQ] values) from three patients run in triplicate by using the housekeeping gene β-glucuronidase to normalize samples. Error bars indicate standard deviation between patients. Frozen (B, E, F) or paraffin-embedded (C, D) tissue sections from proliferative phase (B), midsecretory phase (C, D), and late secretory phase (E, F) endometrium were stained with αTLR3.7 monoclonal antibody (B, C, E) or monoclonal immunoglobulinG1 isotype control (D, F). Staining was visualized with DAB substrate followed by a hematoxylin/water counterstain. Separated endometrial epithelial cells were plated on matrigel-coated 12-well plates and stimulated with polyinosinic-polycytodylic acid (Poly I:C) (1 μg/ml), lipopolysaccharide (LPS) (100 ng/ml), or phorbol-12-myristate-13 acetate plus (PMA) (10 ng/ml)/ionomycin (I) (500 ng/ml) for 18 hours (G). A total of 100 μl of cell-free supernatant was used to detect IL-6, IL-8, and IP-10 production by enzyme-linked immunosorbent assay. Background values (untreated cells) were subtracted to calculate cytokine production. Experiments were performed in duplicate from three patients. Data shown indicate the average of duplicate samples from one patient.

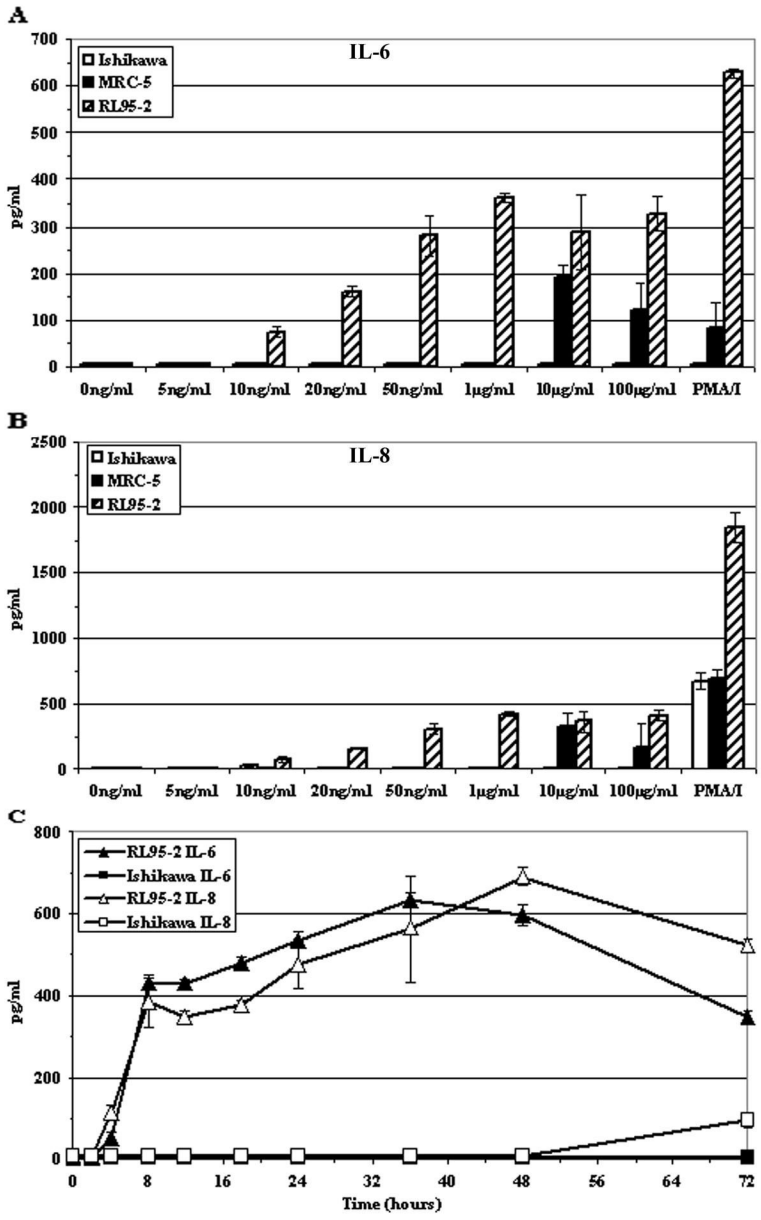


**FIGURE 4.**

RL95-2 cells have a rapid and sustained response to low doses of polyinosinic-polycytidylic acid (Poly I:C.) RL95-2, Ishikawa, and MRC-5 cells were plated as in Figure 2 and stimulated with indicated doses of Poly I:C for 18 hours (a and b). RL95-2 and Ishikawa cells were plated as in Figure 2 and stimulated with 1 μg/ml of Poly I:C for indicated times (C). A total of 100 μl of cell-free supernatant was used to detect interleukin (IL)-6 and IL-8 by enzyme-linked immunosorbent assay. Experiments were performed in triplicate and repeated three times. Data shown represent the average of one experiment.

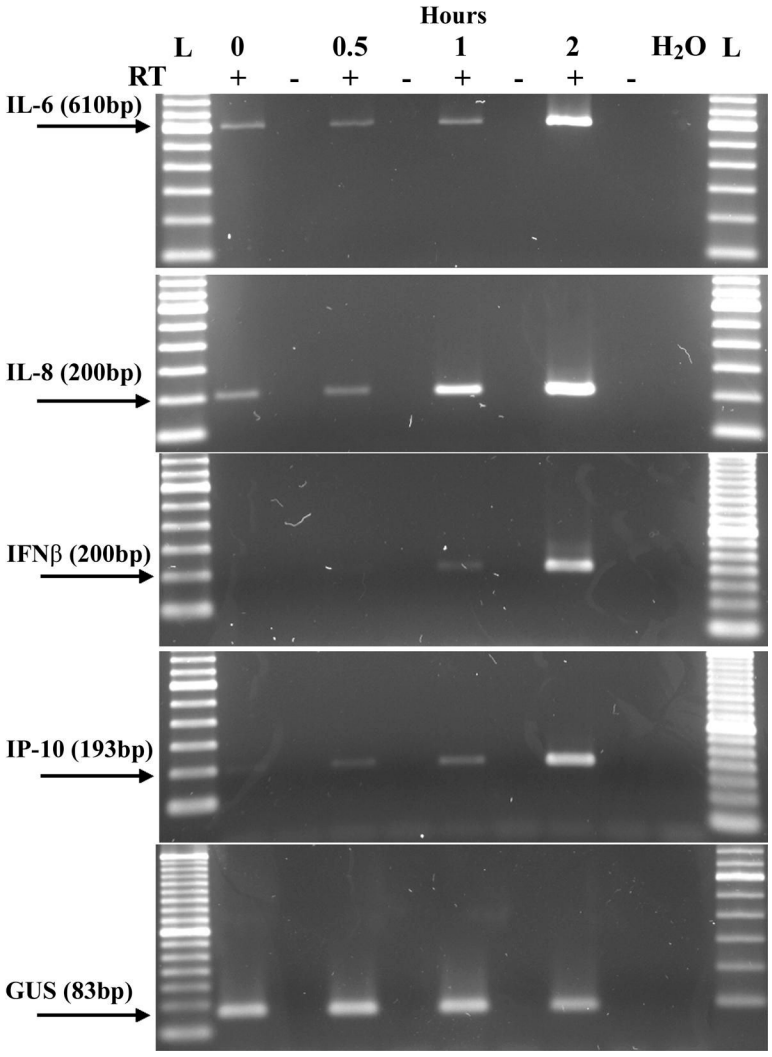


**FIGURE 5.**

Polyinosinic-polycytidylic acid (Poly I:C) stimulation leads to rapid induction of proinflammatory and antiviral cytokine mRNA by RL95-2 cells. Total RNA (100 ng) was used to generate cDNA for measurement of expression of interleukin (IL)-6 (610 bp), IL-8 (200 bp), interferon (IFN)-β (200-bp product), IFN-inducible protein (IP)-10 (193-bp product) and β-glucuronidase (GUS) (83-bp product) mRNA by RL95-2 cells at 0 hours, 0.5 hours, 1 hour, and 2 hours after stimulation with Poly I:C by using end point reverse transcriptase-polymerase chain reaction (RT-PCR). RT (reverse transcriptase) + or − indicates the presence or absence of RT during cDNA synthesis. The $H_2O$ lane indicates water was used instead of cDNA during the PCR reaction. Samples were run against a 100-bp or 50-bp DNA ladder. Experiments were performed in triplicate and repeated three times. Representative data are shown.

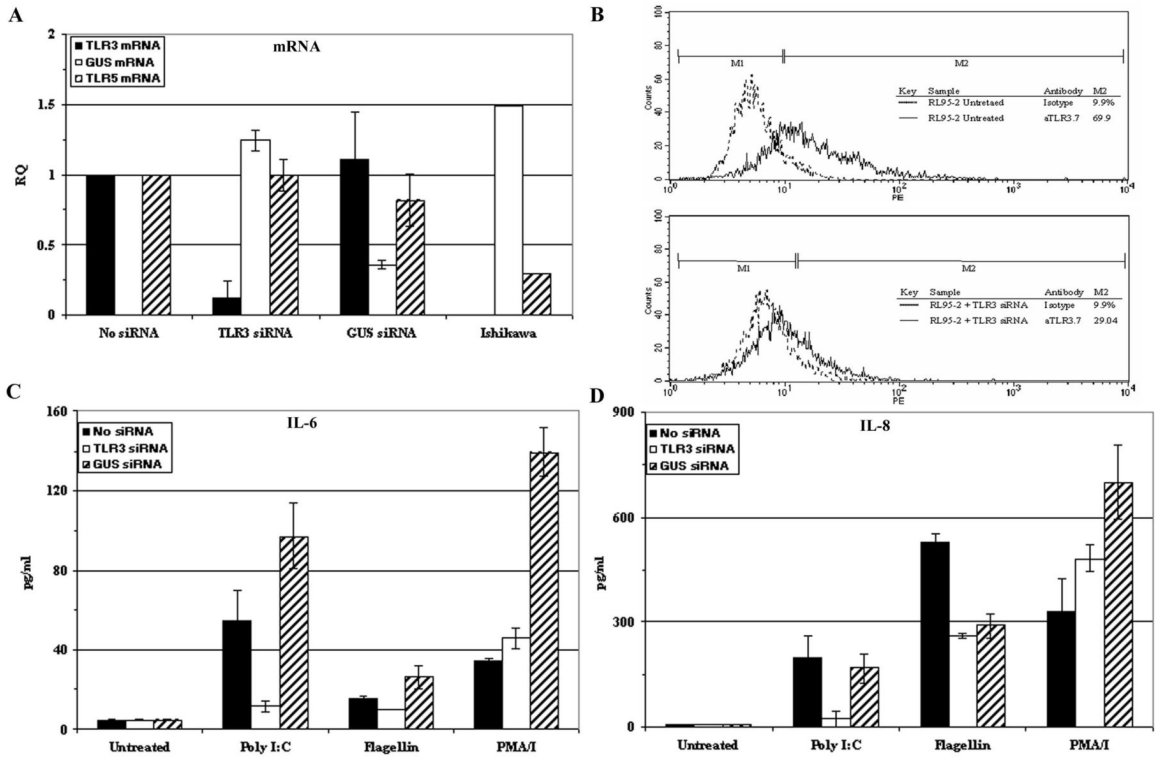


**FIGURE 6.**

Toll-like receptor 3 (TLR3) small inhibitory RNAs (siRNAs) reduce expression of TLR3 and decrease RL95-2 responses to polyinosinic-polycytidylic acid (Poly I:C). RL95-2 cells were plated at $0.2 \times 10^6$ cells/well/ml in 12-well plates and treated with TLR3 siRNA (10 pmol) or β-glucuronidase (GUS) siRNA (10 pmol) for 48 h. Cells were then treated with Poly I:C (1 μg/ml), Flagellin (50 ng/ml), or phorbol-12-myristate-13-acetate plus (PMA) (10 ng/ml)/ ionomycin (I) (500 ng/ml) for 18 h. Total RNA (100 ng/ml) was used to evaluate relative expression levels of TLR3, TLR5, or GUS mRNA by using real-time reverse transcriptase–polymerase chain reaction (A). Data are shown as average relative quantity (RQ) values of three samples, with hypoxanthine guanine phosphoribosyl transferase (HPRT) used to normalize the samples. Error bars indicate standard deviation. RNA harvested from untreated Ishikawa cells was used as a negative control. After treatment with siRNAs, cells were incubated with phycoerithrin (PE)αTLR3.7 monoclonal antibody or PE-labeled monoclonal immunoglobulinG1 antibody and analyzed for expression of TLR3 (B). Histogram plots were generated by gating on live cells. M2 was used to determine percentage of TLR3-positive cells. Experiments were repeated two times. Representative data are shown. A total of 100 μl of cell-free supernatant was used to measure interleukin (IL)-6 (C) and IL-8 (D) production by enzyme-linked immunosorbent assay. Background values (untreated cells) were subtracted to calculate cytokine production. Experiments were performed in triplicate and repeated three times. Error bars indicate standard deviation of three samples. Data shown represent the average of one experiment.

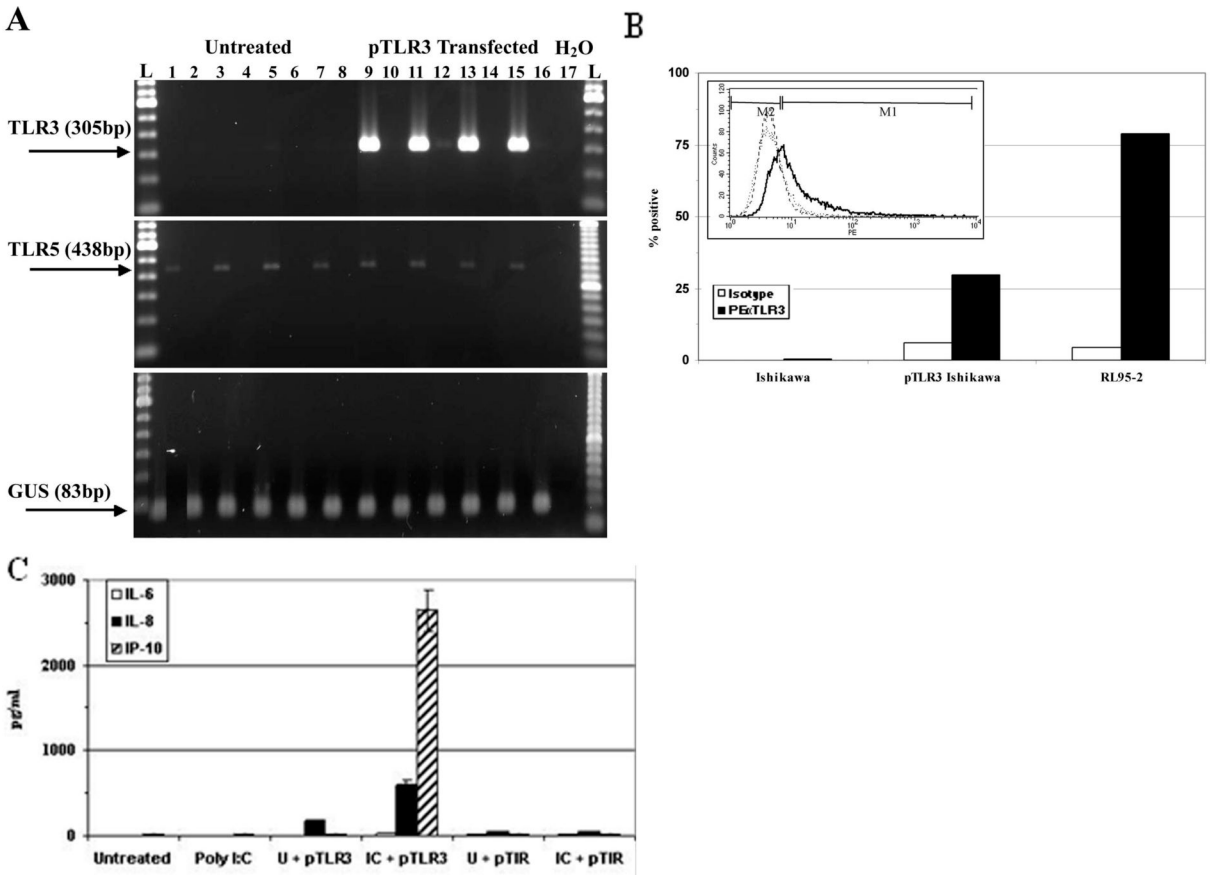


**FIGURE 7.**

Transfection of Ishikawa cells with the Toll-like receptor (TLR)3 gene confers responsiveness to polyinosinic-polycytidylic acid (Poly I:C). Ishikawa cells were plated at $0.1 \times 10^6$ cells/well in 0.5 ml of media in a 24-well plate. Cells were treated with lipofectamine (4 μl) and pUNO-hTLR3 (0.4 μg) or pZERO-hTLR3-TIR (0.4 μg) for 48 hours. Cells were stimulated with Poly I:C (1 μg/ml), lipopolysaccharide (100 ng/ml), or phorbol-12-myristate-13-acetate (PMA) (10 ng/ml)/ionomycin (I) (500 ng/ml) for 18 hours. At 18 hours, RNA (A), cells (B), or supernatants (C) were collected. Total RNA (100 ng) was used to generate cDNA for measurement of expression of TLR3 (305 bp), TLR5 (438 bp) and β-glucuronidase (GUS) (83 bp product) mRNA by nontransfected (lanes 1–9) or pUNO-hTLR3 transfected (lanes 10–17) Ishikawa cells by using end point reverse transcriptase-polymerase chain reaction (A). Results are shown in quadruplicate. Cells were collected and PEαTLR3 mAb used to detect intracellular expression of TLR3 protein by fluorescence activated cell sorter analysis (B). mIgG1 was used as an isotype control. Histogram plots were generated by gating on live cells. M1 was used to determine percentage of TLR3-negative cells, and M2 was used to determine percentage of TLR3-positive cells as demonstrated in the inset (B). Each bar represents the percentage of TLR3-positive cells. Results are representative of two experiments performed in triplicate. A total of 100 μl of cell-free supernatant was used to measure interleukin (IL)-6, IL-8, and interferon-inducible protein (IP)-10 production by enzyme-linked immunosorbent assay (C). Background values (untreated cells) were subtracted to calculate cytokine production. Experiments were performed in triplicate and repeated three times. Error bars indicate standard deviation of three samples. Data shown represent the average of one experiment.