The UDP-Glucose Receptor P2RY14 Triggers Innate Mucosal Immunity in the Female Reproductive Tract by Inducing IL-8[1]

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Innate mucosal immune responses, including recognition of pathogen-associated molecular patterns through Toll-like receptors, play an important role in preventing infection in the female reproductive tract (FRT). Damaged cells release nucleotides, including ATP and uridine 5′-diphosphoglucose (UDP-glucose), during inflammation and mechanical stress. We show in this report that P2RY14, a membrane receptor for UDP-glucose, is exclusively expressed in the epithelium, but not the stroma, of the FRT in humans and mice. P2RY14 and several proinflammatory cytokines, such as IL-8, are up-regulated in the endometria of patients with pelvic inflammatory disease. UDP-glucose stimulated IL-8 production via P2RY14 in human endometrial epithelial cells but not stromal cells. Furthermore, UDP-glucose enhanced neutrophil chemotaxis in the presence of a human endometrial epithelial cell line in an IL-8-dependent manner. Administration of UDP-glucose into the mouse uterus induced expression of macrophage inflammatory protein-2 and keratinocyte-derived cytokine, two murine chemokines that are functional homologues of IL-8, and augmented endometrial neutrophil recruitment. Reduced expression of P2RY14 by small interfering RNA gene silencing attenuated LPS- or UDP-glucose-induced leukocytosis in the mouse uterus. These results suggest that UDP-glucose and its receptor P2RY14 are key front line players able to trigger innate mucosal immune responses in the FRT bypassing the recognition of pathogen-associated molecular patterns. Our findings would significantly impact the strategic design of therapies to modulate mucosal immunity by targeting P2RY14. ***The Journal of Immunology,*** **2009, 182: 7074–7084.**

Sexually transmitted infections and their associated diseases, such as tubal infertility, ectopic pregnancy, and preterm labor, have become major worldwide health problems (1, 2). The mucosal immune system of the female reproductive tract (FRT)[3] has evolved to eliminate such sexually transmitted and environmental pathogens, thereby maintaining the ability to produce healthy offspring.

The mammalian immune system can be divided into two branches: innate and acquired immunity. The innate immune system recognizes microorganisms that invade the mammalian host through multiple families of pattern recognition molecules, such as TLRs and Nod-like receptors (NLRs) (3–5). TLRs at mucosal surfaces recognize a variety of pathogen-associated molecular patterns (PAMPs) and generate intracellular signals that induce chemokine and cytokine expression, which activate a range of host responses (3). The importance of TLRs in the FRT has recently emerged (1, 2) as well as other tissues/organs, including the respiratory, gastrointestinal, and urinary tracts (6–8). Indeed, TLRs 1–10 are spatiotemporally expressed in the nonpregnant and/or pregnant FRT, and some of them have been shown to be functional in terms of cytokine and chemokine production in different cell types throughout the FRT in in vitro experiments (1, 2). In addition to TLRs, natural antimicrobial peptides (AMPs) are released at epithelial surfaces through TLR-dependent and independent mechanisms, and AMPs, in turn, can disrupt the membranes of many microbial pathogens (9). The FRT has been shown to exhibit a spatiotemporal expression of various AMPs, including a series of defensins, secretory leukocyte protease inhibitors, and elafin (2, 10).

Besides PAMPs derived from microorganisms, endogenous danger signals, released by cells undergoing stress, damage, or abnormal death, can also induce immune responses mainly through the activation of APCs, such as dendritic cells (11, 12). These danger-associated host components include uric acid crystals, heat shock proteins, extracellular matrix degradation products, and nucleotides such as ATP, some of which are known to be recognized by TLRs, NLRs, or purinergic receptors (4, 12). For instance, TLR3 recognizes not only viral double-stranded RNA but also host-derived RNA released from damaged cells, thereby acting as an endogenous sensor of tissue necrosis during acute inflammatory events in vitro and in vivo (13, 14). Among these danger signals, nucleotides such as ATP and nucleotide sugars such as uridine 5′-diphosphoglucose (UDP-glucose) have recently emerged as potential extracellular signaling molecules (15, 16). In particular, UDP-glucose and P2RY14, a G protein-coupled P2Y purinergic receptor for UDP-glucose and related sugar nucleotides (17–19), have been shown to play a role in the regulation of immune responses, including chemokine production (20), maturation of

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[3] Abbreviations used in this paper: FRT, female reproductive tract; NLR, Nod-like receptor; PAMP, pathogen-associated molecular pattern; AMP, natural antimicrobial peptide; UDP-glucose, uridine 5′-diphosphoglucose; PID, pelvic inflammatory disease; ICR, Imprinting Control Region; PTX, pertussis toxin; siRNA, small interfering RNA; IUD, intrauterine device; KC, keratinocyte-derived cytokine; UDP-Glu*N*Ac, UDP-*N*-acetylglucosamine.

dendritic cells (21), inhibition of T lymphocyte proliferation (22), and chemotaxis of bone-marrow hematopoietic stem cells (23).

In this study, we investigated the role of UDP-glucose and P2RY14 in mucosal immunity in the FRT and provide evidence suggesting that epithelial P2RY14 behaves as a danger sensor for UDP-glucose released by damaged and dying cells. Activated P2RY14 induces IL-8 production together with neutrophil recruitment, avoiding the presumed necessity of immune recognition of PAMPs by professional APCs.

Materials and Methods

Reagents

Phenol red-free MEM, RPMI 1640, DMEM, Ham's F-12, OPTI-MEM I, FBS, antibiotic-antimycotic mixture, and TRIzol reagent were purchased from Invitrogen. Ficoll-Paque PLUS was obtained from Amersham Biosciences. HBSS, HEPES buffer solution, and DNase I were obtained from Life Technologies. Collagenase was purchased from Wako. Target Retrieval Solution was obtained from DakoCytomation. For gene transfection and sonoporation studies, Lipofectamine 2000 (Invitrogen), FlouoroGene (ImaRx Therapeutics), and Microbubble (Nepagene) were prepared. All other chemicals were purchased from Sigma-Aldrich. Abs used in this study are shown in supplemental Table I.[4]

Human subject samples

Endometria ($n = 23$), cervixes ($n = 5$), and fallopian tubes ($n = 2$) were obtained from consenting women who underwent hysterectomy because of leiomyoma or severe pelvic inflammatory diseases (PID). Placental tissues were obtained from three pregnant women after vaginal delivery. The use of these human specimens was approved by the Keio University Ethics Committee.

Animals

Eight- to nine-week-old female Crj: CD-1 Imprinting Control Region (ICR) mice were purchased from Oriental Yeast and housed in standard plastic cages under automatic 12-h light:dark cycles at 23°C with free access to food and water. This study was approved and performed in accordance with the guidelines for the care and use of laboratory animals established by the Animal Research Committee of Keio University School of Medicine.

Isolation of glandular and stromal cells from human endometrium

Human endometrial specimens were dissociated mechanically and enzymatically, filtered, isolated by gradient centrifugation using Ficoll-Paque, and separated into endometrial stromal and glandular epithelial cell fractions as described previously (24, 25).

Endometrial tissue samples were rinsed in DMEM containing 1% antibiotic-antimycotic mixture and 10% FBS ($DMEM^+$) to remove blood and debris, and weighed and cut into small pieces <1 mm^3. The tissue fragments were digested with 0.2% (w/v) collagenase and 0.05% DNase I in $DMEM^+$ for 1.5 h at 37°C on a shaking rotor. Typically, 10 ml of the cell digestion medium for each 1g of the sample was used. After the enzymatic digestion, the tissue digest was filtered through a sterile 400 μm polyethylene mesh filter to remove undigested tissues, followed by a 40 μm cell strainer (BD Biosciences). Most of the stromal cells and blood cells present as single cells or small aggregates passed through the 40-μm cell strainer and were collected in a sterile 50 ml polycarbonate tube, while the undigested fragments, mostly made up of glandular clumps, were retained in the strainer. The glandular fragments were recovered from the cell strainer by back-flushing with $DMEM^+$ into 10-cm sterile dishes.

Stromal cells prepared in a 50-ml tube were pelleted by centrifugation for 7 min at 440 $\times$ g and resuspended in 4 ml of calcium- and magnesium-free HBSS supplemented with 2% FBS, 10 mM HEPES buffer solution, and 1% antibiotic-antimycotic mixture ($HBSS^+$). RBC were removed by carefully layering the cell suspension over 3 ml of Ficoll-Paque PLUS in a 15-ml tube. The solution was centrifuged for 15 min at 780 $\times$ *g*. The medium/Ficoll interface layer containing mainly stromal and immune cells was carefully aspirated and washed.

Glandular epithelial fragments were collected again and digested with 0.05% trypsin-EDTA solution and 0.05% DNase I by pipetting for 5–10 min to dissociate the glands into single cells. Subsequently, the dispersed glandular cells were washed with $DMEM^+$ and filtered through a 40-μm cell strainer for the removal of undigested and sticky glandular tissues to yield a single-cell suspension.

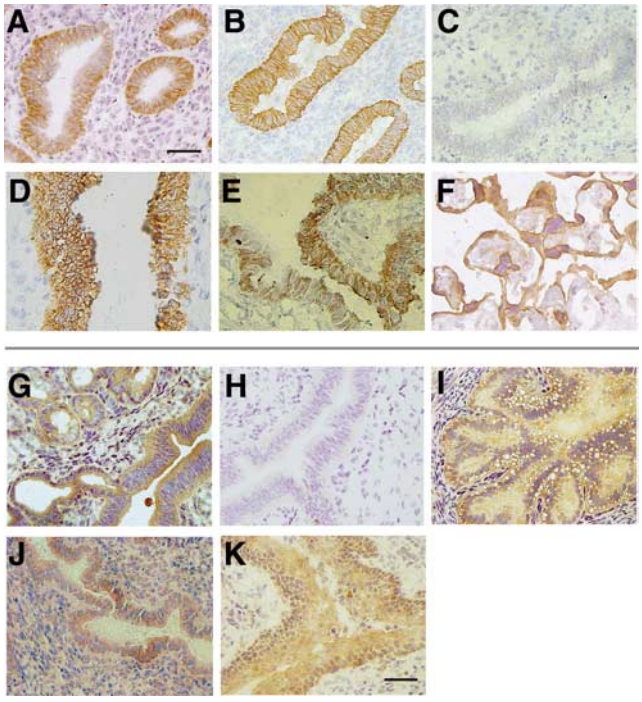

FIGURE 1. P2RY14 is exclusively and constitutively expressed in the epithelium of the human and mouse female reproductive tract (FRT). Sections from healthy human endometria (*A–C*), uterine cervix (*D*), the ampullar region of fallopian tube (*E*), and human term placenta (*F*) were stained with anti-P2RY14 Ab (*A, B, D–F*) or nonimmune serum (*C*). P2RY14 was exclusively expressed in the epithelium of human proliferative (*A*) and secretory (*B*) endometria, uterine cervix (*D*), fallopian tube (*E*), and term placenta (*F*). Sections from mouse endometria (*G* and *H*), oviduct (*I*), uterine cervix (*J*), and vagina (*K*) were stained with anti-P2RY14 Ab (*G, I–K*) or nonimmune serum (*H*). P2RY14 was similarly exclusively expressed in the epithelia of the FRT in mice. Scale bars, 30 μm.

Cell and tissue culture

The glandular and stromal cells (6×10^5 cells) isolated from human endometrium were seeded into 35-mm plates, cultured to semiconfluency, and then left untreated or treated with 10^{-8} to 10^{-6} M UDP-glucose for 24 h, and the culture medium were harvested for the measurement of IL-8 concentration.

Ishikawa cells (clone 3-H-12), a gift from Dr. M. Nishida (National Hospital Organization Kasumigaura Medical Center, Ibaraki, Japan) were used within 10 passages. HEK-293, MCF-7, and JAR cells were purchased from the American Type Culture Collection (ATCC). Ishikawa cells, HEK-293, MCF-7, and JAR cells were maintained in the recommended medium and subjected to immunofluorescent staining for P2RY14 or harvested for RNA extraction. Ishikawa cells (6×10^5 cells) were seeded into 35-mm plates, cultured to semiconfluency, and then either left untreated or treated with 10^{-8} to 10^{-3} M UDP-glucose for 30 min to 48 h, according to individual experimental designs. After treatment, RNA was extracted from the cells and subjected to RT-PCR analysis and the culture medium were harvested for the measurement of IL-8 concentration. For the pertussis toxin (PTX) inhibition assay, subconfluent Ishikawa cells were either left alone or pretreated with 1 mg/ml PTX for 4 h, then cultured with medium alone or with 10^{-5} M UDP-glucose for 1 h, harvested for RNA extraction, and subjected to RT-PCR analysis for IL-8 and GAPDH. For LPS stimulation experiments, subconfluent Ishikawa cells were either left untreated or treated with 10 ng/ml to 1 μg/ml LPS (*Escherichia coli*, O55:B5) for 12 h, followed by RNA extraction and RT-PCR analysis.

Mouse uterine tissues obtained from 8-wk-old female ICR mice were cut into 7 to 8 mm pieces under sterile conditions, cultured in DMEM/HAM's F-12 medium at 1:1 (vol/vol) in the absence or presence of 10^{-10}

[4] The online version of this article contains supplemental material.

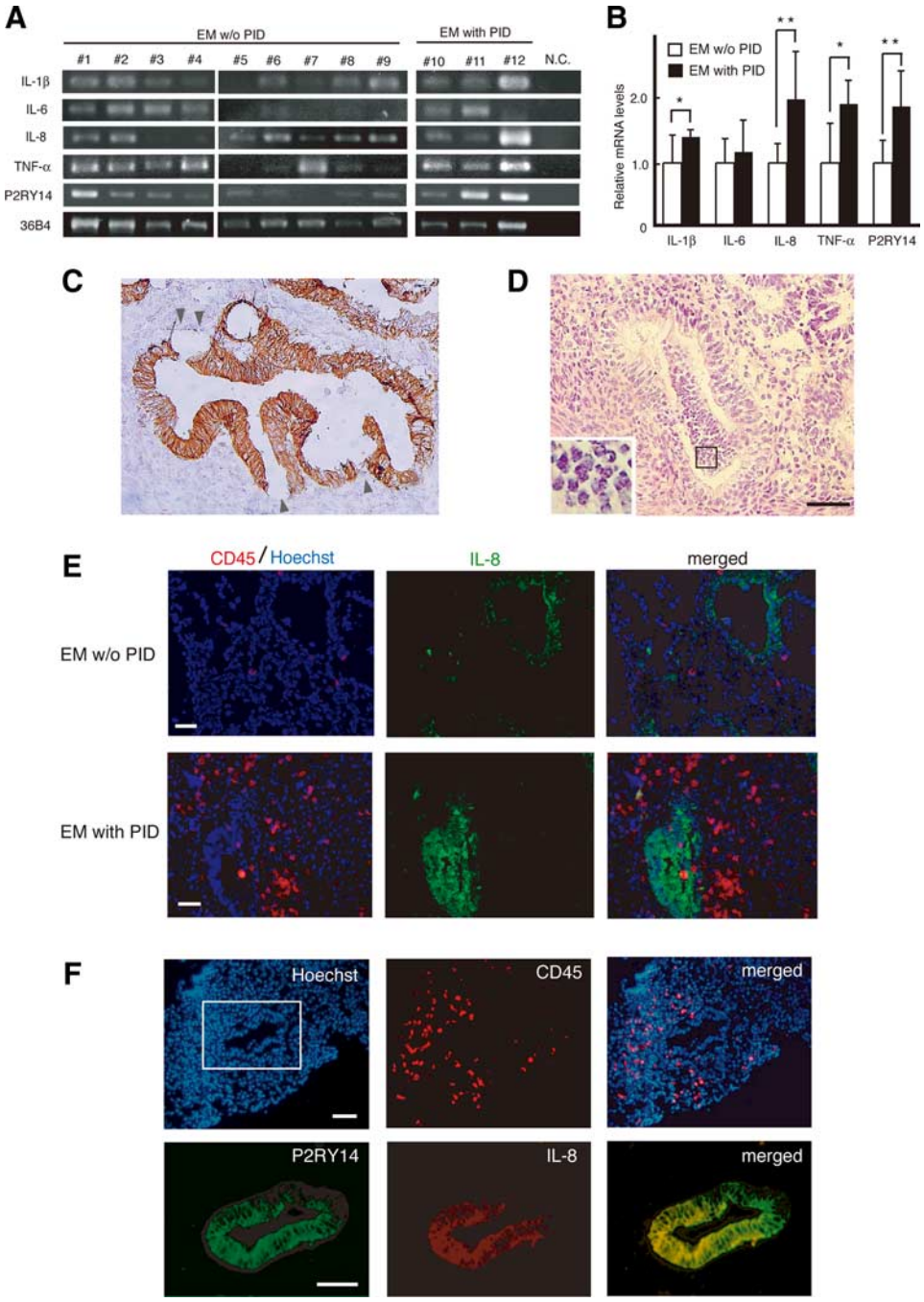

FIGURE 2. Proinflammatory cytokines and P2RY14 are up-regulated in inflammatory and menstrual endometrium. *A*, RT-PCR analyses for IL-1β, IL-6, IL-8, TNF-α, P2RY14, and 36B4 (internal control) mRNA in endometrial tissues (EM) collected from nine patients (no. 1–9) without any inflammatory diseases and three patients (no. 10–12) with PID. w/o, Without; N.C., negative control. *B*, Relative mRNA expression of the indicated proinflammatory cytokines and P2RY14 over 36B4 in endometria from the same patients as (*A*). $*$, $p < 0.05$ and $**$, $p < 0.01$. *C*, Immunohistochemical P2RY14 staining of the endometrium collected from a patient with PID. Glandular epithelial cells exclusively expressed P2RY14, some of which were partially peeled off (arrowheads). *D*, H&E staining of the same endometrium as (*C*). Numerous neutrophils infiltrated into the lumen of the uterine gland. *Inset*, A magnified image of the boxed region. Scale bar, 50 μm. *E*, Immunofluorescent CD45, IL-8, and Hoechst staining of the endometrium with or without PID. Scale bars, 30 μm. *F*, Immunofluorescent staining of the third day menstrual endometrium with Hoechst and Abs against CD45, P2RY14, and IL-8. The lower three panels derived from the serial section of the upper panels indicate the magnified image of the white boxed region. Scale bars, 50 μm.

to 10^{-6} M UDP-glucose or 10^{-10} to 10^{-6} M UDP-Glc*N*Ac for 4 h, followed by RNA extraction.

IL-8 measurement by ELISA

IL-8 levels in the culture medium were measured by ELISA (Quantikine; R&D Systems) according to the manufacturer's instructions and normalized to the number of harvested cells. The experimental ELISA data representing mean values ($\pm$SD) were obtained in duplicate from at least three independent experiments.

Immunofluorescence

We performed immunofluorescent staining as described previously (26, 27). In brief, chamber slides (Nalgene-Nunc) with cultured Ishikawa cells or glass slides mounted with cryosections derived from human endometrium or mouse uterine tissues were fixed with 4% paraformaldehyde for 20 min and washed with PBS, followed by permeablization with 0.2% Triton X-100 in PBS for 10 min. After blocking with goat or rabbit serum (Vector Laboratories), slides were successively stained with the Abs listed in supplemental Table I, followed by incubation with secondary anti-mouse, anti-rabbit, or anti-rat Abs conjugated to Cy2 or Cy3 (Jackson ImmunoResearch Laboratories) to visualize the primary Abs. Nuclei were stained by using Hoechst DNA dye (Sigma-Aldrich). Images were collected using an inverted Leica DMIRE2 fluorescent microscope (Leica Microsystems) equipped with a CCD camera (VB-700; Keyence) and a Leica TCS SP2 confocal microscopy system.

Immunohistochemistry

Cryosections derived from human endometria, cervixes, fallopian tubes, placentas and mouse uterine tissues were subjected to immunohistochemistry for P2RY14, macrophage inflammatory protein-2 (MIP-2) or keratinocyte-derived cytokine (KC) proteins using a DAB staining kit (Vector Laboratories) according to the manufacturer's instructions. We used an Ag retrieval procedure (28) for immunostaining paraformaldehyde-fixed frozen sections of mouse tissues. In brief, glass slides were placed in a heat-resistant basket containing Target Retrieval Solution (DakoCytomation)

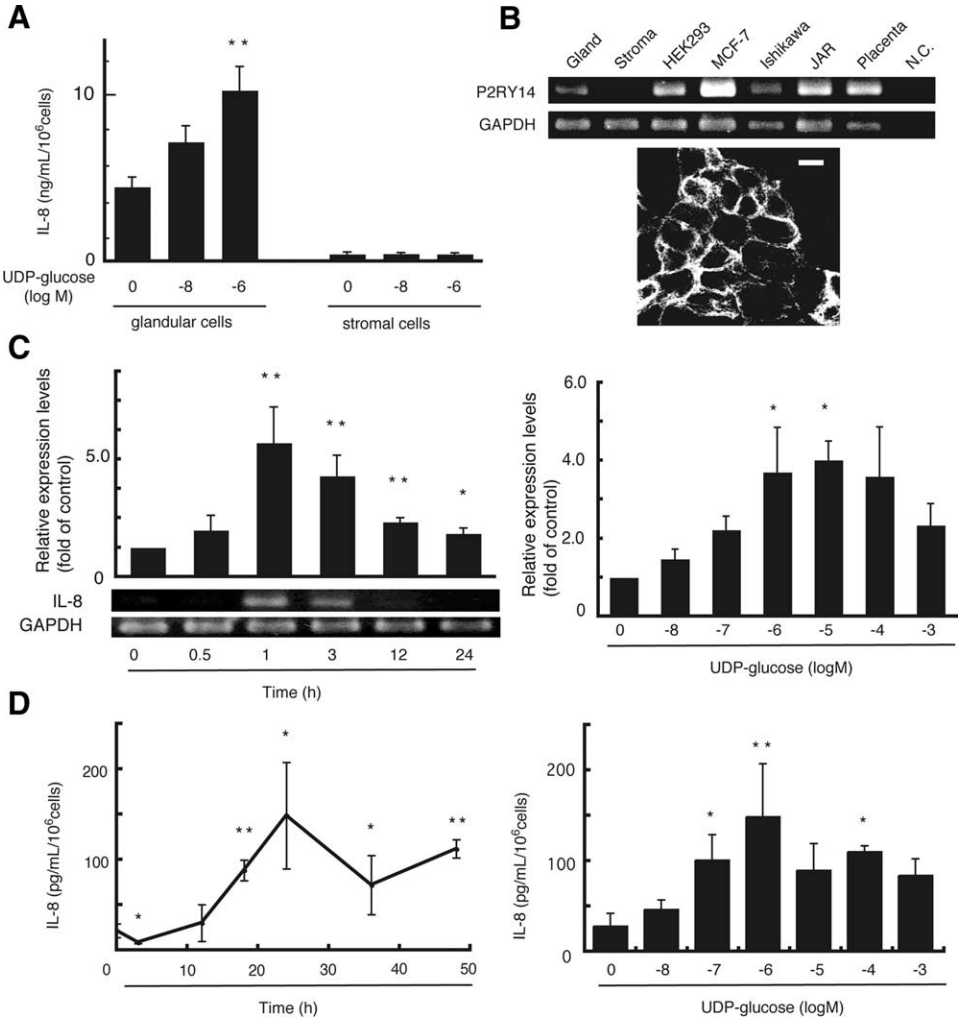

FIGURE 3. UDP-glucose stimulates IL-8 production in endometrial epithelial cells, but not stromal cells. *A*, IL-8 secretion by UDP-glucose in primary cultures of endometrial epithelial cells, but not stromal cells ($n = 3$–5). These cells were isolated from endometria of individuals different from those used in Figs. 1 and 2. **, $p < 0.01$, vs control. *B*, RT-PCR analysis for P2RY14 and GAPDH mRNA harvested from endometrial epithelial and stromal cells, and various cell lines including Ishikawa cells, as indicated. *Lower panel,* Immunofluorescence image of Ishikawa cells stained with anti-P2RY14 Ab. Scale bar, 5 μm. *C*, Time-course and dose-response induction of IL-8 mRNA by UDP-glucose in Ishikawa cells, as determined by three independent experiments using RT-PCR analysis for P2RY14 and GAPDH mRNA (*left panel*, 10^{-5} M UDP-glucose; *, $p < 0.05$ and **, $p < 0.01$, vs 0 min; *right panel*, 1 h stimulation, *, $p < 0.05$, vs control). *D*, Time-course and dose-dependent production of IL-8 protein by UDP-glucose in Ishikawa cells, as determined by three to five independent experiments by the measurement of IL-8 concentration in the culture supernatant by ELISA (*left panel*, 10^{-6} M UDP-glucose; *, $p < 0.05$ and **, $p < 0.01$, vs 0 h; *right panel*, 24 h stimulation, *, $p < 0.05$ and **, $p < 0.01$, vs control).

and heated with a microwave oven (600 W) twice each for 5 min before beginning immunohistochemistry.

RNA isolation and semiquantitative RT-PCR

Total RNA was extracted from cultured cells and human and mouse tissues using TRIzol reagent (Invitrogen) and subjected to semiquantitative RT-PCR analysis as described previously (29). Primers were designed to amplify human (h) P2RY14, hIL-1β, hIL-6, hIL-8, hTNF-α, hGAPDH, and h36B4 and mouse (m) P2RY14, mMIP-2, mKC, mGAPDH, and mβ-Actin genes as shown in supplemental Table II. Each relative ratio was calculated as the densitometric intensities divided by those of hGAPDH or h36B4 for human samples and by those of mGAPDH or mβ-actin for mouse samples.

Immunoblotting

Total proteins were extracted from cultured Ishikawa cells and mouse tissues using radioimmunoprecipitation assay buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% deoxycholate, 0.1% SDS, 1% Nonidet P-40 (NP40), 1 mM Na_3VO_4, 50 mM sodium fluoride, and 1 mM Na_2MoO_4) or NP40 buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP40, 1 mM Na_3VO_4, 50 mM sodium fluoride, and 1 mM Na_2MoO_4). Both buffers contained a protease inhibitor mixture (Roche). The extracted proteins were then subjected to SDS-PAGE and immunoblotting for the detection of P2RY14, GAPDH, MIP-2 and KC.

In brief, mouse uterine tissues and semiconfluent Ishikawa cells cultured in the indicated medium for various periods were lysed on ice with radioimmunoprecipitation assay buffer or NP40 buffer, the latter of which was only used to detect GAPDH protein. Protein concentrations were determined using the DC protein assay kit (Bio-Rad) with BSA as a standard. Samples (10 μg/lane) were separated by electrophoresis on a 12–15% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane as previously described (29). After incubation with anti-P2RY14, anti-GAPDH, anti-MAPK, anti-KC, or anti-MIP-2 Ab, followed by appropriate HRP-conjugated secondary Ab, the immunoreactive proteins were detected by the ECL method (Amersham Biosciences). All experimental data for immunoblotting represent the results obtained from three independent experiments.

Lipofection of small interfering RNA (siRNA)

All siRNAs used in this study were synthesized and purified by Ambion and the sequences are shown in supplemental Table III. GAPDH siRNA was used as a negative control according to the manufacturer's recommendation and as described previously (30–32). We first precultured Ishikawa cells for 24 h in 35 mm dishes, and changed the medium to serum-free OPTI-MEM I (Invitrogen) with the addition of cationic lipid/siRNA complexes containing 80 pmol of the indicated siRNA and 4 μl of Lipofectamine 2000 (Invitrogen). After 6 h of incubation, the medium was changed back to 10% FBS/MEM with UDP-glucose or left untreated as a control. The cells and culture medium were harvested for protein extraction and for IL-8 measurement 36 and 24 h, respectively, after lipofection.

Isolation of neutrophils

For the transmigration assay, human polymorphonuclear leukocytes (PMNs or neutrophils) were isolated from heparinized venous blood from healthy individuals as described previously (33, 34). Fresh, heparinized blood was mixed with 0.9% sodium chloride containing 3.0% dextran solution at 1:1 (w/w), which was made by diluting a 20% dextran solution (average m.w. was up to 500,000) with 0.9% sodium chloride, and incubated for 20 min at room temperature. Supernatants were centrifuged at 550 $\times$ g for 10 min and cells were suspended in 35 ml of 0.9% sodium chloride. This suspension was underlayed with 10 ml of Ficoll-Paque PLUS and centrifuged at 380 $\times$ g for 30 min to separate neutrophils from PBMC. PBMCs were removed by aspiration, the inside of the tube was wiped by a sterile cotton swab, and RBC were lysed with sterile water for

30 s, followed by immediate mixing with an equal volume of 1.7% sodium chloride. Purified neutrophils were resuspended and incubated with RPMI 1640 medium with 10 mM HEPES buffer solution. Purity of the neutrophils collected by this method was >97%.

Transwell assay

The isolated human neutrophils were incubated with RPMI 1640 medium. The transwell assay was performed using a normal Boyden chamber as described previously (35). In brief, Ishikawa cells were incubated to semiconfluence in the lower chamber and further cultured with fresh medium containing 10^{-6} M UDP-glucose for 24 h. We then placed 5×10^3 neutrophils into the upper chamber, fixed the membrane 3 h later, stained it with Hoechst DNA dye, and then counted the number of neutrophils that had migrated to the underside of the chamber in six randomly selected microscopic viewing areas (40 × magnification) in a blinded fashion using a fluorescence microscope. Goat anti-human IL-8 Ab, or goat anti-human serum Ab as a control, was also added to the lower chamber at a final concentration of 2.5 ng/ml 1 h before the introduction of neutrophils.

Intrauterine administration

We performed laparotomy on anesthetized 8- to 9-wk-old female ICR mice and placed cyanoacrylate glue (AlonAlpha gel; Toa Gosei Chemical Industry) as an "artificial vaginal plug" in the vaginal cavity to prevent cross-contamination between horns (supplemental Fig. 3*A*). We then cramped both ends of the horns and administered Hoechst dye, 60–80 μl of 10^{-8} M UDP-glucose or 50 μg of LPS into one horn and the same amount of PBS into the contralateral horn using 29-gauge needles through transuterine injection. The clamp was released 2 min after injection and the abdomen was closed. Uterine tissues were collected at 4 (for LPS experiment), 24, or 48 h postinjection and subjected to RNA isolation for RT-PCR analysis or to H&E or immunofluorescent staining. We counted the number of neutrophils per high power field in a blinded fashion and recorded the average of at least four fields for each sample, and compared the mean number across both treatments. Neutrophils were determined by H&E staining and immunofluorescence based on the morphology and expression of the mouse neutrophil marker.

In vivo siRNA sonoporation of the uterus

We prepared the siRNA solution containing 3 μg of siRNA, 40 μl of FluoroGene (ImaRx Therapeutics), 20 μl of Microbubble (Nepagene), and 1 μl of 1% lidocaine per uterine horn. We performed laparotomy on anesthetized 8- to 9-wk-old female ICR mice and placed cyanoacrylate glue in the vaginal cavity to prevent cross-contamination between horns (supplemental Fig. 3*A*). We cramped both ends of the horns and then administered the P2RY14 siRNA solution into one of the horns and GAPDH siRNA into the contralateral horn with 29-gauge needles through transuterine injection (supplemental Fig. 3*B*). The injected uterus was covered with ultrasound conducting lotion (Aloe-Sound Gel Plus, RICH-MAR) and sonoporated for 4 min at 1.0 MHz input frequency with a 20% duty cycle and 2 W/cm^2 output intensity using a Sonitron 2000 (Nepagene) with a 10 mm probe. The cramp was released and the abdomen was closed. The uterine tissues were collected 24 h after sonoporation and subjected to immunofluorescence, immunoblot analysis, and counting of infiltrated leukocytes.

Statistical analysis

All experimental data from each bioassay were compared by Student's paired *t* test or one-way ANOVA with Bonferonni's post hoc test. All the quantitative results were expressed as the mean ± SD. $p < 0.05$ was considered to reflect a statistically significant difference.

Results

P2RY14 is expressed exclusively in FRT epithelial cells

We first investigated the expression and localization of P2RY14 in human endometrium, cervix, fallopian tube, and placenta using immunohistochemistry. As shown in Fig. 1, P2RY14 was exclusively and prominently expressed in the surface epithelium, but not the stroma, of the FRT (Fig. 1, *A–E*; *C*, negative control). The staining intensity of endometrial P2RY14 was virtually constant during the menstrual cycle (data not shown). Immunohistochemical analysis of the human placenta revealed that P2RY14 was preferentially expressed in villous trophoblasts (Fig. 1*F*), which is in agreement with a previous report (23). Similarly, P2RY14 was constitutively and exclusively expressed in the surface epithelium of the endometrium, oviduct, uterine cervix, and vagina in mice (Fig. 1, *G–K*; *H*, negative control). In addition to the FRT, P2RY14 transcripts were present in various other mouse tissues, including skeletal muscle, kidney, lung, and colon (supplemental Fig. 1*A*); in particular, P2RY14 protein expression was especially strong in

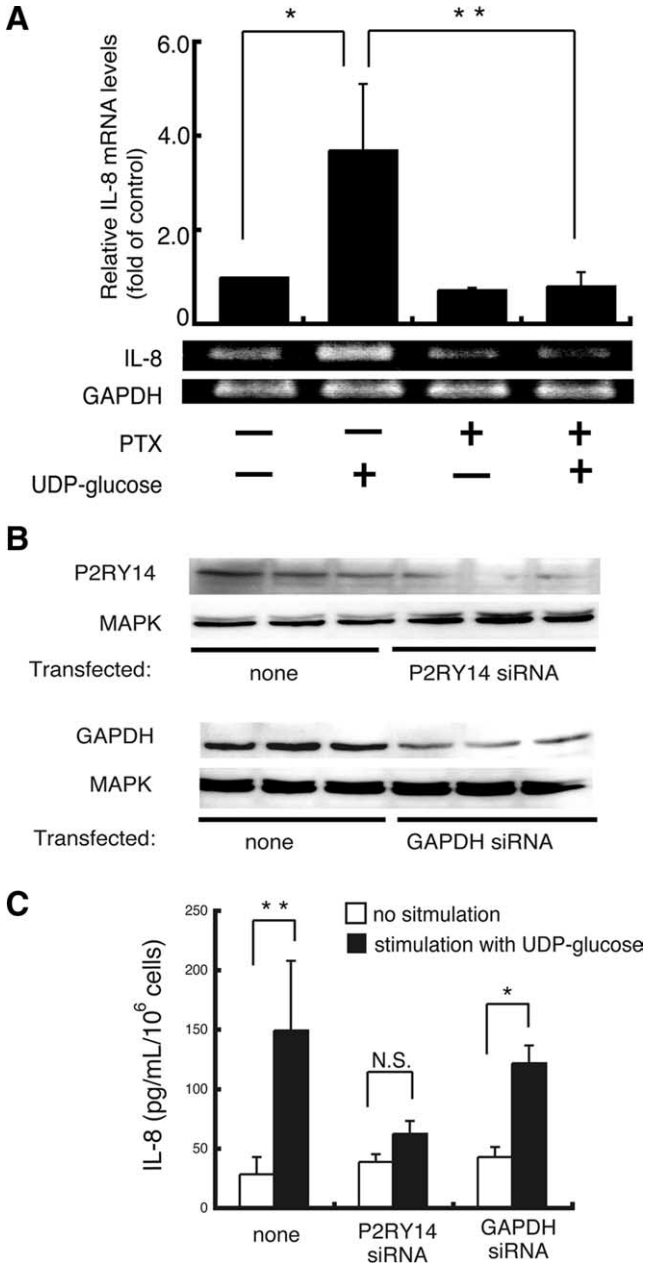

FIGURE 4. UDP-glucose-induced IL-8 production is P2RY14 dependent. *A*, Inhibition of UDP-glucose-induced IL-8 mRNA expression by PTX, an inhibitor of G proteins. Ishikawa cells preincubated with or without 1 μg/ml PTX for 4 h were treated with 10^{-5} M UDP-glucose for 1 h, harvested for RNA extraction, and subjected to RT-PCR analysis for IL-8 and GAPDH. *, $p < 0.05$; **, $p < 0.01$. *B*, Decreased expression of P2RY14 by siRNA gene silencing. Ishikawa cells transfected with or without siRNA for either P2RY14 or GAPDH were subjected to immunoblot analysis for P2RY14, GAPDH and MAPK expression. *C*, Attenuation of UDP-glucose-induced IL-8 production by treatment with P2RY14 siRNA, but not GAPDH siRNA. Culture medium of Ishikawa cells treated with or without 10^{-6} M UDP-glucose together with or without the indicated siRNA were harvested and IL-8 concentration was measured by ELISA. Results were obtained from three independent experiments (*A–C*). *, $p < 0.05$ and **, $p < 0.01$; N.S., not significant.

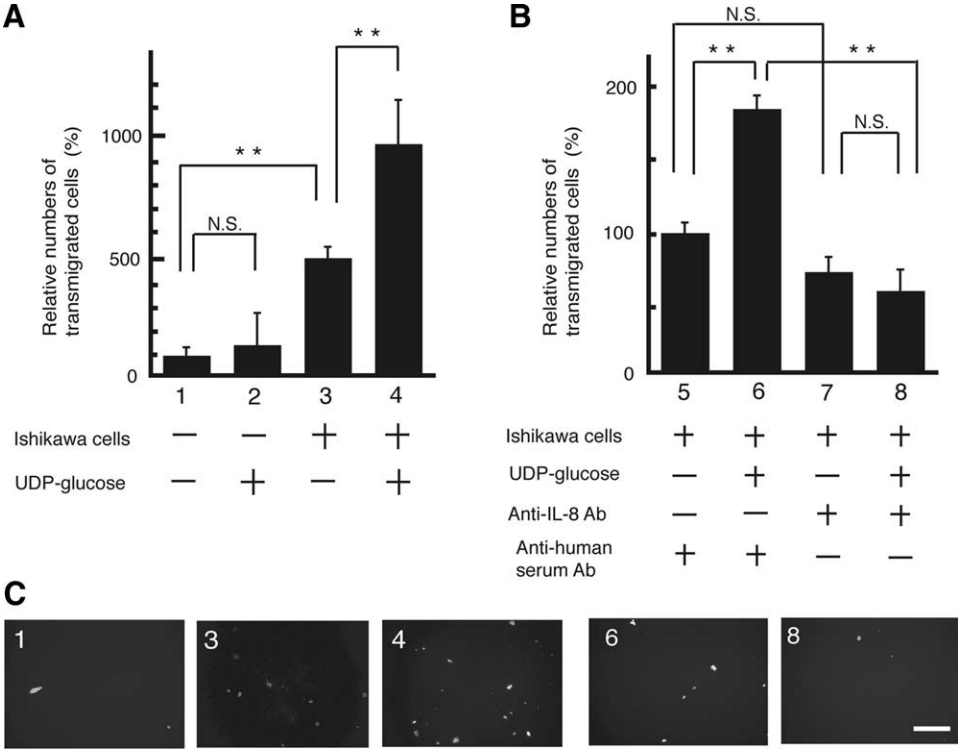

FIGURE 5. UDP-glucose enhances neutrophil chemotaxis in an IL-8-dependent manner. *A*, Enhancement of neutrophil chemotaxis by UDP-glucose. Isolated neutrophils were subjected to the transwell assay in the presence or absence of Ishikawa cells in combination with or without UDP-glucose. *B*, Attenuation of UDP-glucose-enhanced neutrophil chemotaxis by the addition of anti-IL-8 neutralizing Ab. Isolated neutrophils were subjected to the transwell assay in the presence of Ishikawa cells together with or without UDP-glucose in combination with goat anti-human serum Ab or goat anti-human IL-8 neutralizing Ab. Results were obtained from three independent experiments. *C*, Representative immunofluorescence images of Hoechst-stained neutrophils transmigrating across the membrane. Each panel number corresponds to the treatment group as indicated in *A* and *B*. Scale bar, 50 μm. **, $p < 0.01$. N.S., Not significant.

broncheolar epithelial cells, alveoli pneumocytes, mucosal epithelial cells of convoluted tubules, and enterocytes in mice (supplemental Fig. 1, *B–E*). These results collectively suggest a possible common role of epithelial P2RY14 in mucosal function.

Endometrial P2RY14 and IL-8 are up-regulated in endometritis and during menstruation

As P2RY14 has been reported to play a role in immune responses (20–23), we examined the expression level of P2RY14 and several proinflammatory cytokines in endometria with or without PID using semiquantitative RT-PCR. We found that the transcripts of P2RY14, IL-1β, IL-8, TNF-α, but not IL-6, were significantly more abundant in the endometria with PID than in those without PID (Fig. 2, *A* and *B*). Immunohistochemical and H&E staining of endometria with PID revealed that epithelial cells were partly desquamated in the endometrial glands positive for P2RY14 (Fig. 2*C*), and that numerous neutrophils had accumulated and transepithelially migrated (Fig. 2*D*). Similar to P2RY14 expression and localization, IL-8 was also exclusively expressed in the endometrial glandular cells (Fig. 2*E*, *upper panels*). IL-8 is a well known chemoattractant for neutrophils (36). Consistently, $CD45^+$ leukocytes preferentially and massively accumulated around the endometrial glands staining prominently for IL-8 in patients with PID (Fig. 2*E*, *lower panels*).

In addition to the pathological condition, $CD45^+$ leukocytes were physiologically recruited around glandular cells in the menstrual endometrium (Fig. 2*F*, *upper panels*). Importantly, those glandular cells adjacent to the infiltrated $CD45^+$ leukocytes prominently coexpressed P2RY14 and IL-8 (Fig. 2*F*, *lower panels*). Taken together, these results suggest a close association between P2RY14 and IL-8 in terms of the recruitment of neutrophils.

UDP-glucose induces IL-8 production through P2RY14 signaling in endometrial epithelial cells

The coexpression and concomitant up-regulation of P2RY14 and IL-8 prompted us to examine whether P2RY14 and its ligand UDP-glucose are involved in IL-8 induction. We tested the effect of UDP-glucose on IL-8 production in primary cultures of human endometrial glandular and stromal cells. We found that glandular cells, but not stromal cells, isolated from human cycling endometria secreted IL-8 in a dose-dependent manner in response to 24 h treatment with UDP-glucose (Fig. 3*A*), which is attributable to the unique expression of endometrial P2RY14 restricted to glandular cells, as determined by immunohistochemistry (Fig. 1) and RT-PCR analysis (Fig. 3*B*, *upper panels*). In addition to isolated endometrial glandular cells, P2RY14 mRNA was expressed in various cell lines, including Ishikawa cells (Fig. 3*B*, *upper panels*). Immunofluorescence studies showed that P2RY14 protein was predominantly present at the plasma membrane of Ishikawa cells (Fig. 3*B*, *lower panel*). Because Ishikawa cells (of endometrial epithelial origin) have been widely used for a number of studies on the physiology and pathophysiology of normal human endometrial glandular cells (37), we used Ishikawa cells to further investigate the role of UDP-glucose and P2RY14 in IL-8 production and its biological relevance. As shown in Fig. 3*C*, IL-8 mRNA was induced 1 h after stimulation with UDP-glucose (*left panels*) and the inducing effect of UDP-glucose on IL-8 expression was dose-dependent (*right panels*). Also, Ishikawa cells exhibited a time- and dose-dependent secretion of IL-8 protein in response to UDP-glucose stimulation (Fig. 3*D*, *left* and *right panels*, respectively). UDP-glucose exposure, however, did not result in the up-regulation of transcripts of other proinflammatory cytokines, including IL-1β, IL-6, and TNF-α (data not shown). LPS, which is a major component of the outer membrane of many Gram-negative bacteria and acts as an endotoxin, increased the expression of P2RY14 mRNA (supplemental Fig. 2*A*).

To address whether UDP-glucose stimulates IL-8 expression and production via P2RY14, we first examined the effect of PTX, an inhibitor of G proteins, on UDP-glucose-induced IL-8 expression in Ishikawa cells. As shown in Fig. 4*A*, PTX attenuated the stimulatory effect of UDP-glucose on IL-8 mRNA expression, which is consistent with PTX-sensitive Gi protein involvement in

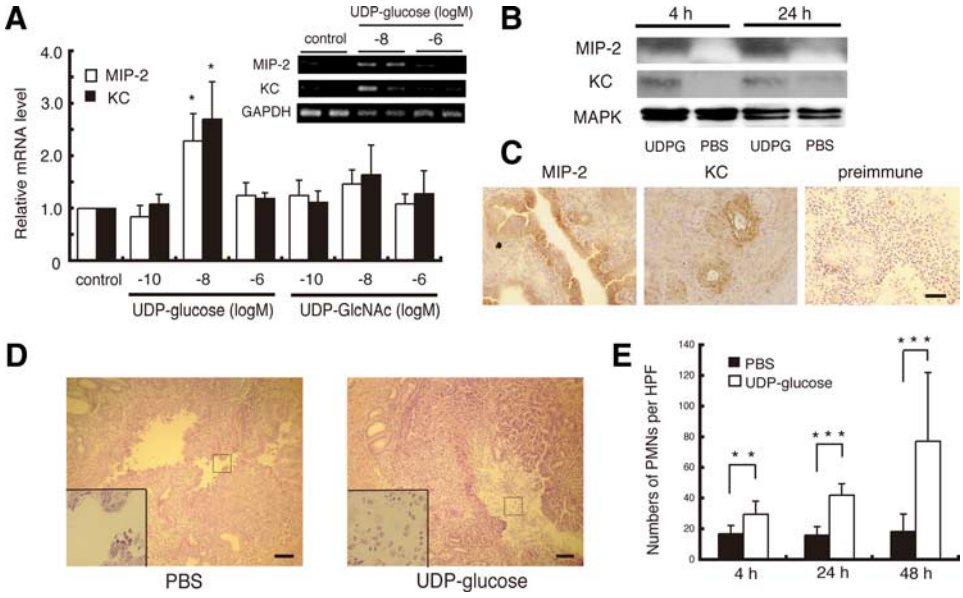

FIGURE 6. UDP-glucose induces the expression of proinflammatory chemokines and neutrophil infiltration in the mouse uterus. *A*, Up-regulation of MIP-2 and KC mRNA by treatment with UDP-glucose, but not UDP-Glc*N*Ac, in mouse endometrial tissues in vitro ($n = 6$). *Insets*, representative RT-PCR images of MIP-2, KC, and GAPDH mRNA. *, $p < 0.05$, vs control. *B*, Induction of uterine MIP-2 and KC expression by administration of UDP-glucose into the uterine cavity as determined by immunoblot analysis using anti-MIP-2, anti-KC, and anti-MAPK Abs. *C*, Induction of endometrial epithelial MIP-2 and KC by UDP-glucose. Sections of the endometrium collected from mice treated with UDP-glucose as described in *B* were stained with the indicated Abs or a nonimmune primary Ab. *D*, Enhancement of endometrial neutrophil infiltration by UDP-glucose. Sections of the endometrium obtained from a mouse whose uterine cavity was administered with either PBS or UDP-glucose and exposed for 24 h were H&E stained. *Insets*, magnified images of the boxed regions. *E*, Quantitative evaluation of the endometrial accumulation of neutrophils in mice treated as described in *A* ($n = 10$). Scale bars: 30 μm (*C*) and 100 μm (*D*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

P2RY14 activation (19). We next performed P2RY14 knockdown experiments and initially confirmed that the lipofectamin 2000-based transfection of siRNA resulted in ~80% reduction of P2RY14 and GAPDH mRNA expression (data not shown). siRNA-mediated silencing of P2RY14 resulted in the decreased expression of P2RY14 protein (Fig. 4*B*) and abrogated UDP-glucose-induced IL-8 production (Fig. 4*C*), whereas GAPDH siRNA did not affect IL-8 induction (Fig. 4*C*). The expression levels of MAPK used as an internal control were not affected by silencing of P2RY14 and GAPDH (Fig. 4*B*), as determined by three independent experiments. Collectively, these results indicate that the induction of endometrial epithelial IL-8 by UDP-glucose is mediated by P2RY14.

UDP-glucose-induced IL-8 promotes neutrophil chemotaxis

To address the pathophysiological relevance of UDP-glucose-induced IL-8 production, we next examined the effect of UDP-glucose on neutrophil chemotaxis, as IL-8 is a well-known chemoattractant for neutrophils (36). For this purpose, we used chemotactic migration assays (transwell chamber assays) using human neutrophils and Ishikawa cells to explore the chemotactic potential of UDP-glucose-stimulated Ishikawa cells. We found that UDP-glucose did not enhance the transmigration activity of neutrophils in the absence of Ishikawa cells, whereas it significantly stimulated neutrophil transmigration in the presence of Ishikawa cells (Fig. 5, *A* and *C*). The stimulatory effect of UDP-glucose on neutrophil chemotaxis was completely abrogated by the addition of anti-human IL-8 neutralizing Ab, but not a control goat Ab (Fig. 5, *B* and *C*), suggesting that UDP-glucose may act as a chemoattractant predominantly through IL-8 induction.

UDP-glucose enhances chemokine expression and leukocytosis in mice

We next determined whether UDP-glucose had a similar stimulatory effect on chemokine expression and neutrophil recruitment in the mouse uterus. First, mouse uterine tissues were incubated in the presence or absence of UDP-glucose or UDP-*N*-acetylglucosamine (UDP-Glu*N*Ac) in culture dishes, harvested for RNA extraction, and then subjected to RT-PCR analysis for the expression of MIP-2 and KC mRNA, two murine chemokines that are functional homologues of IL-8 (38). As shown in Fig. 6*A*, treatment with 10^{-8} M UDP-glucose provoked a significant induction of both MIP-2 and KC mRNA. In contrast, UDP-Glu*N*Ac had no stimulating effect on the expression of either mRNA at 10^{-6} to 10^{-10} M, which can be attributed to the low affinity of P2RY14 for UDP-Glu*N*Ac (19).

To examine whether UDP-glucose induces the expression of MIP-2 and KC in vivo, we injected UDP-glucose into one horn of the mouse uterus and PBS into the opposite horn. To avoid cross-contamination of the injected materials between the two horns, we filled both the vaginal cavity and cervix with cyanoacrylate glue (supplemental Fig. 3*A*), which, termed "an artificial vaginal plug", can prevent the leakage of Hoechst DNA dye from one horn to the other (supplemental Fig. 3*A*).

We found that expression of the MIP-2 and KC proteins was induced by administration of UDP-glucose, but not PBS, into the mouse uterine cavity, as determined by immunoblot analysis (Fig. 6*B*). Immunohistochemistry revealed that UDP-glucose-induced MIP-2 and KC proteins were both confined to the endometrial luminal and glandular cells (Fig. 6*C*), the distribution of which was similar to that of IL-8 in human endometrium (Fig. 2*E*). The expression of P2RY14 mRNA was ~40% increased 4 h after in vivo

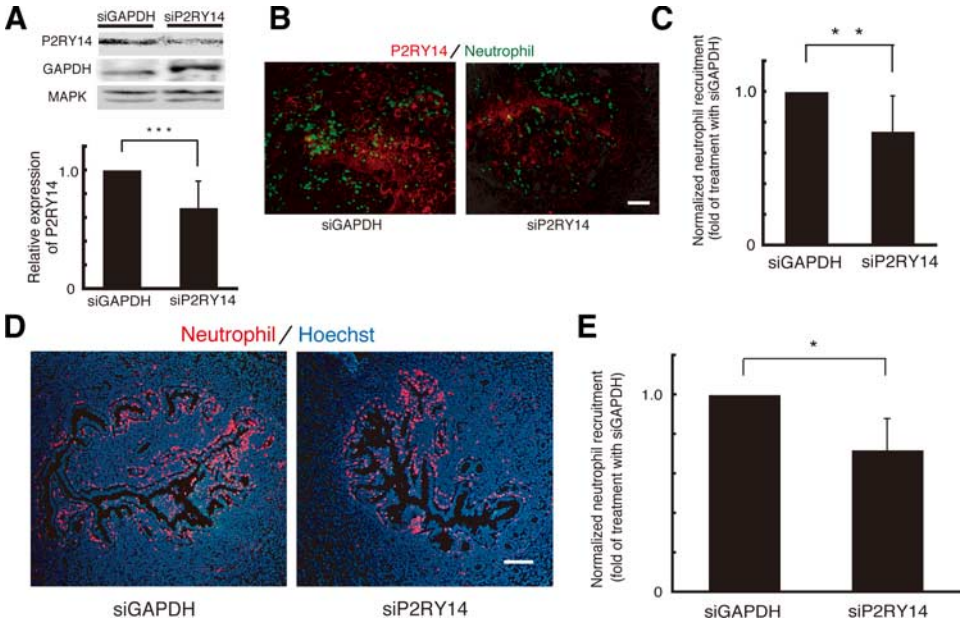

FIGURE 7. Reduced expression of P2RY14 by siRNA transfection results in attenuation of UDP-glucose- and LPS-induced leukocytosis in the mouse uterus. *A*, Decreased expression of GAPDH or P2RY14 protein by administration of the corresponding siRNA (siGAPDH or siP2RY14) in different uterine horns of the same mouse, as determined by immunoblot analysis (*upper panels*). Relative expression of P2RY14 was determined by the ratio of the band intensity of P2RY14 divided by that of MAPK (*lower panel*, $n = 13$). *B*, Inhibition of the UDP-glucose-induced recruitment of neutrophils by P2RY14 knockdown in the mouse uterus. Sections of the endometria collected from mice treated with UDP-glucose in combination with siGAPDH or siP2RY14 for 24 h were stained with Abs against P2RY14 or a neutrophil marker (clone 7/4). *C*, Quantitative evaluation of the endometrial neutrophil recruitment in mice treated as described in *B* ($n = 20$). *D*, Inhibition of the LPS-induced recruitment of neutrophils by knockdown of P2RY14 in the mouse uterus. Sections of the endometria collected from mice treated with 50 μg of LPS in combination with siGAPDH or siP2RY14 for 24 h were stained with an Ab against a neutrophil marker (clone 7/4) together with Hoechst dye. *E*, Quantitative evaluation of the endometrial neutrophil recruitment in mice treated as described in *D* ($n = 4$). Scale bars: 100 μm. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

administration of LPS (supplemental Fig. 2*B*), which may be consistent with up-regulation of P2RY14 mRNA in human inflammatory endometria (Fig. 2, *A* and *B*). We also noticed that significantly more neutrophils accumulated and migrated transepithelially in the mouse uterus treated with UDP-glucose, as compared with PBS alone (Fig. 6, *D* and *E*). The increase in the neutrophil number reached ~5-fold 48 h after UDP-glucose administration ($p < 0.001$) (Fig. 6*E*).

P2RY14 knockdown attenuates the LPS- and UDP-glucose-induced leukocytosis

Lastly, to elucidate the possible involvement of P2RY14 in the inflammatory process in the FRT, we examined the effect of P2RY14 knockdown on UDP-glucose- or LPS-induced endometrial neutrophil recruitment. For this purpose, we used ultrasound-directed gene delivery (sonoporation) to transduce P2RY14 siRNA into one horn of the mouse uterus and GAPDH siRNA into the other horn of the same mouse as a control. Using an artificial vaginal plug, we first confirmed that sonoporation-directed gene transfer of Cy3-labled P2RY14 siRNA into the mouse uterus resulted in the localization of Cy3 fluorescence to the luminal and glandular epithelial cells (supplemental Fig. 3*B*) and an ~30% decrease in the immunoblot signal intensity of P2RY14 protein (Fig. 7*A*, *upper* and *lower panels*). These results indicate the validity of the sonoporation procedure and siRNA used in this study. We next made an artificial vaginal plug, injected P2RY14 siRNA or GAPDH siRNA into each horn, exposed both horns to ultrasound, and then treated them with UDP-glucose. Immunohistochemistry revealed a decrease in the number of neutrophils recruited around the endometrial glands together with a reduction in the fluorescence intensity of P2RY14 in endometria sonoporated with P2RY14 siRNA, as compared with those sonoporated with GAPDH siRNA (Fig. 7*B*). Quantitative evaluation confirmed that the decrease in the number of recruited neutrophils was statistically significant (Fig. 7*C*). Likewise, administration of LPS into the mouse uterus induced leukocytosis (Fig. 7*D*), which was significantly abrogated by cotreatment with P2RY14 siRNA as compared with GAPDH siRNA (Fig. 7, *D* and *E*).

Discussion

Neutrophil transendothelial/transepithelial migration and accumulation at mucosal surface is a hallmark of diverse inflammatory situations. When bacterial pathogens invade intestinal mucosal sites, they elicit an innate immune response through the activation of complement pathways, eventually generating C5a and C5b (39). C5b participates in the attack of bacterial membranes, whereas the anaphylatoxin C5a serves as a potent chemoattractant for neutrophils (39). More importantly, TLRs are expressed on leukocytes and epithelial cells, where they facilitate host cellular recognition of specific PAMPs, such as LPS (TLR4) and flagellin (TLR5) (39). Bacterial stimulation of the TLR pathways induces the release of IL-8 and other chemokines from epithelial cells and leukocytes, which further recruits neutrophils to subepithelial spaces. Thus, TLRs play a pivotal role in the recruitment of neutrophils to mucosal surfaces in the gastrointestinal, respiratory, urogenital, and reproductive tracts (1, 39).

The results of this study collectively provide evidence for a non-TLR-mediated mechanism underlying neutrophil recruitment to inflamed or injured areas, specifically the FRT. As shown in Fig. 8, chemical, mechanical, or microbial insults result in epithelial cell damage in the FRT. The concentration of intracellular UDP-glucose is estimated at 100 mM (40). Given the high concentration, a certain amount of UDP-glucose can be released (or leaked)

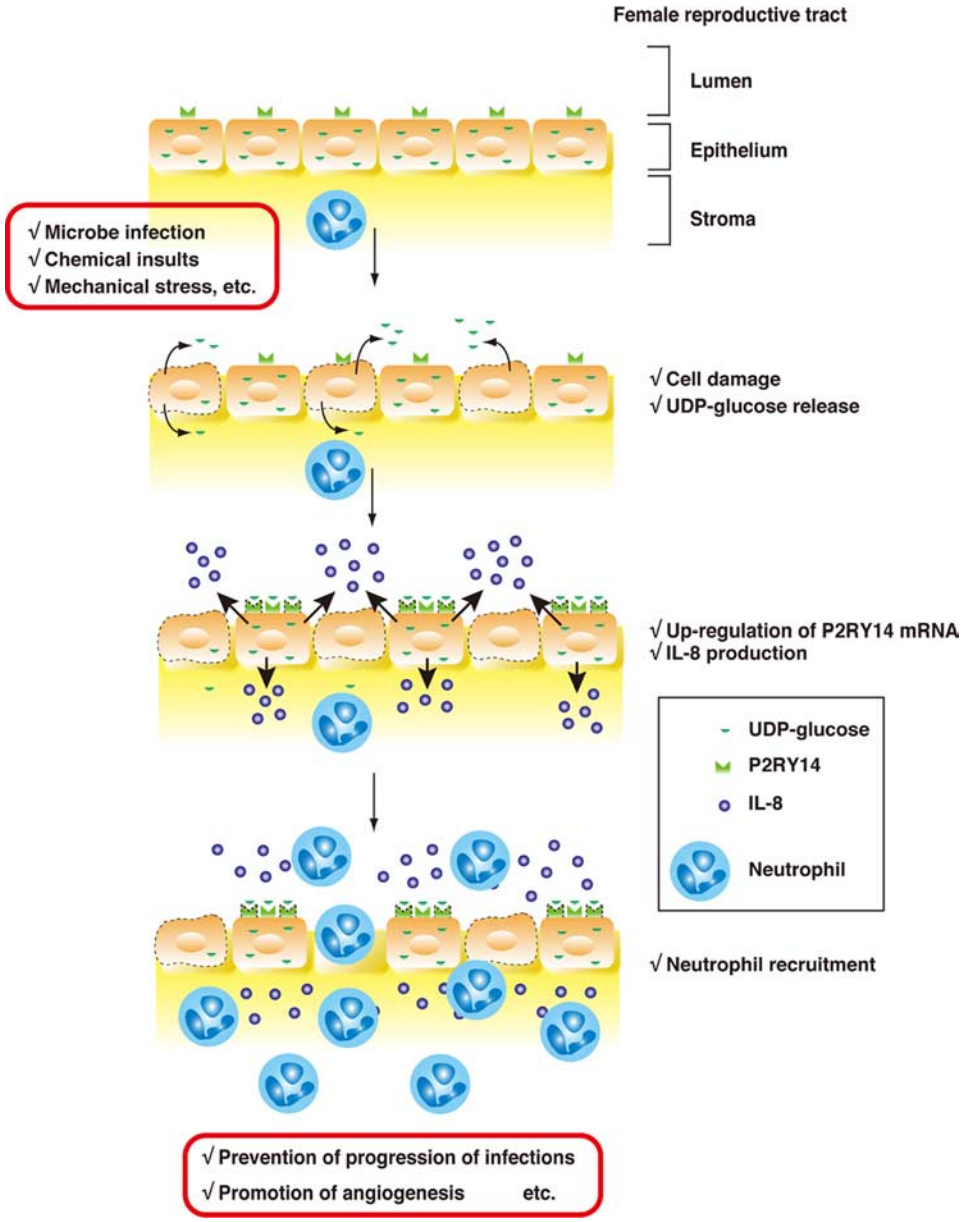

FIGURE 8. Proposed novel model for stimulating innate mucosal immunity in the FRT involving UDP-glucose and P2RY14. Chemical, mechanical, and microbial insults result in epithelial cell damage in the FRT. Damaged cells release UDP-glucose into the extracellular space, while P2RY14 expression may also be up-regulated, particularly in undamaged epithelial cells. Released UDP-glucose, in turn, immediately stimulates IL-8 via epithelial P2RY14 in a paracrine/autocrine manner, leading to the recruitment of neutrophils into the infected and/or damaged area of the FRT. Thus, UDP-glucose and P2RY14 may be novel front line players for triggering innate mucosal immunity not only in the FRT but also in the lumen of other hollow organs.

from injured and/or damaged cells and thereby the local concentration of extracellular UDP-glucose may reach effective levels ranging between 10 nM–1 mM. Indeed, even mild mechanical stimulation of primary airway epithelial cells and various cell lines including 1321N1 human astrocytoma, COS-7, CHO-K1, and C6 glyoma cells by a change of medium resulted in an increase in extracellular levels of UDP-glucose, which attained at effective concentrations ranging between 1–20 nM (16). Thus, damaged cells release UDP-glucose into the extracellular space, and P2RY14 expression is up-regulated particularly in undamaged epithelial cells. Released UDP-glucose, in turn, immediately stimulates IL-8 via epithelial P2RY14 in a paracrine/autocrine manner, leading to the recruitment of neutrophils into the infected and/or damaged area of the FRT. Thus, epithelial P2RY14 may behave as a danger sensor for UDP-glucose released by injured or dying cells, thereby activating P2RY14 to initiate an innate immune response in the absence of APCs such as dendritic cells, bypassing the process of recognition of PAMPs. Given that P2RY14 is expressed in the surface epithelium of various tissues/organs other than the FRT (supplemental Fig. 1), UDP-glucose and P2RY14 may be novel front line players able to trigger innate mucosal immunity in other luminal organs as well. Indeed, this proposed role of UDP-glucose and P2RY14 in mucosal immunity is further supported by the production of IL-8 by UDP-glucose via P2RY14 in airway epithelial cells (20) and the up-regulation of P2RY14 by immunological challenge with LPS in several types of cells (41, 42).

Stimulation of the epithelial TLR4 pathway by microbial components such as LPS activates NF-κB, which, in turn, promotes the production of inflammatory cytokines including IL-8. TLR4 recognizes LPS through a complex mechanism involving several accessory molecules including CD14 and MD-2 (43). Hirata et al. (44) reported that endometrial epithelial cells did not produce IL-8 in response to LPS, partly because they expressed TLR4, but not CD14. Such a poor response, however, could be rescued by coaddition of soluble CD14, highlighting the requirement of CD14 for IL-8 production by TLR4 (44). Indeed, endometrial stromal cells expressed both TLR4 and CD14 and, therefore, they produced IL-8 in an LPS dose-dependent manner (44). Intriguingly, Hirata et al. (44) also demonstrated that a neutralizing Ab against TLR4

completely abrogated LPS-induced NF-κB activation, but only partially inhibited IL-8 production, postulating that an alternative pathway other than the TLR4 system may participate in LPS-induced IL-8 production.

In this study we have demonstrated the potential of the P2RY14 pathway as an alternate trigger for innate immunity, responsible for endometrial epithelial IL-8 production. UDP-glucose stimulates calcium mobilization from intracellular stores and thereby up-regulates IL-8 through P2RY14 signaling, which is abrogated by cotreatment with PTX, an inhibitor of G proteins, as shown in this and other studies (20, 21). In most cases, the IL-8 gene is transcriptionally activated by the NF-κB and/or JNK pathways, and its mRNA stability is regulated by the p38 MAPK pathway (45). In some types of cells, however, the PKC pathway is involved in the up-regulation of IL-8 (45). Given the involvement of a G protein-coupled receptor and calcium influx in IL-8 induction by UDP-glucose, it is possible that P2RY14 might activate the PKC pathway through the generation of diacylglycerol and inositol triphosphate through phospholipase C activation. Thus, epithelial cells of the FRT including endometrium, and probably other luminal organs, possess at least three different cellular machineries, the TLR, NLR, and P2RY14 systems, to defend against microbial insults. Given that LPS exerts its effects through TLRs, up-regulation of P2RY14 mRNA by LPS as presented herein suggests a possible involvement of the TLR system in P2RY14 induction. Elucidation of a cross-talk between the TLR and P2RY14 pathways awaits further studies.

In addition to the elimination of pathogenic microbes, the mucosal innate immune system of the FRT is uniquely adapted to facilitate specialized physiological functions that include menstruation, fertilization, implantation, pregnancy, and parturition. In this context, it is conceivable that P2RY14 may also play role(s) in such specialized physiological functions other than as a danger sensor. On the basis of our proposed model, the physiological tissue breakdown and shedding of human endometrium during menstruation may provoke a massive release of endogenous UDP-glucose, which, in turn, may activate endometrial epithelial P2RY14, resulting in the production of IL-8 together with marked accumulation of endometrial neutrophil recruitment. Indeed, this as well as several other studies demonstrate that IL-8 is up-regulated exclusively in the menstrual endometrium together with marked accumulation of neutrophils (46), which is consistent with our hypothesis. Neutrophils are known to secrete angiogenic factors and thereby contribute to angiogenesis (47). Accordingly, endometrial IL-8-induced recruitment of neutrophils may contribute not only to elimination of tissue debris, dead cells, and any invading organisms, but also to promotion of angiogenesis and, together with IL-8, endometrial tissue remodeling and repair.

Besides massive tissue breakdown and shedding during menstruation, an implanting embryo has been shown to induce endometrial epithelial cell apoptosis to breach the epithelial barrier (48). Based on our proposed model, it is conceivable that UDP-glucose may be released from these apoptotic endometrial epithelial cells, which, in turn, may activate P2RY14 in a paracrine/autocrine manner, leading to IL-8 production. Consistent with our hypothesis, Caballero-Campo et al. (49) demonstrated using in vitro coculture experiments that both IL-8 mRNA expression and protein production were up-regulated in endometrial epithelial cells in the presence of a human blastocyst. In both menstruation and implantation, IL-8 production and infiltrated neutrophils may play a role in the angiogenesis for endometrial remodeling/regeneration, trophoblast invasion, and placentation, rather than prevention of the progression of infection (50). Further studies are needed to elucidate the role of P2RY14 in the various physiological processes of reproduction.

Intrauterine devices (IUDs) have been used worldwide as an effective contraceptive tool mainly to prevent and disrupt implantation (51). All IUDs work by inducing mild trauma in the uterine cavity through physical contact with the surface of the endometrium. IUDs also induce a foreign body reaction, which is an inflammatory response characterized by the infiltration of leukocytes and macrophages into the endometrial stroma and subsequently through the surface epithelium (52). Such features of the presence of the IUD can be explained by our proposed model whereby mechanical damage to the surface epithelium induced by direct contact with the IUD may provoke a release of UDP-glucose, which, in turn, may activate P2RY14 and stimulate IL-8 production, ultimately leading to neutrophil recruitment. In agreement with our hypothesis, it has been reported that IL-8 expression is enhanced after insertion of an IUD (53). Thus, taken together, therapeutic strategies targeting the UDP-glucose/P2RY14 system may have the potential for promoting both positive and negative regulation of implantation.

In summary, epithelial P2RY14 may behave as a novel danger sensor for UDP-glucose, released by damaged or dying cells via lytic and nonlytic pathways, thereby activating P2RY14 to stimulate IL-8 production and the recruitment of neutrophils and other immune cells through recognition of self damage, but not PAMPs. The results of this study suggest a potential role for the UDP-glucose and P2RY14 system not only in mucosal innate immunity in the FRT and other luminal organs, but also in various physiological reproductive processes, including embryo implantation, endometrial angiogenesis, and tissue remodeling.

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Disclosures

The authors have no financial conflict of interest.

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