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J Immunol 2013; 191:2226-2235; Prepublished online 2 August 2013;
doi: 10.4049/jimmunol.1300958
http://www.jimmunol.org/content/191/5/2226

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/08/06/jimmunol.1300958.DC1

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Uterine NK Cells Regulate Endometrial Bleeding in Women and Are Suppressed by the Progesterone Receptor Modulator Asoprisnil

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Received for publication April 11, 2013. Accepted for publication June 27, 2013.

This work was supported by TAP Pharmaceutical Products and in part by the Wellcome Trust and the British Medical Research Council Centre for Reproductive Health, University of Edinburgh, the Queen’s Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, U.K. E-mail address: hilary.critchley@ed.ac.uk

The online version of this article contains supplemental material.

Abbreviations used in this article: dNK, decidual NK cell; ESC, endometrial stromal cell; pNK, peripheral NK cell; PR, progesterone receptor; PRM, progesterone receptor modulator; QRT-PCR, quantitative real-time PCR; RIN, RNA integrity number; αSMA, α smooth muscle actin; uNK, uterine NK cell.

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Uterine NK cells (uNK) play a role in the regulation of placentation, but their functions in nonpregnant endometrium are not understood. We have previously reported suppression of endometrial bleeding and alteration of spiral artery morphology in women exposed to asoprisnil, a progesterone receptor modulator. We now compare global endometrial gene expression in asoprisnil-treated versus control women, and we demonstrate a statistically significant reduction of genes in the IL-15 pathway, known to play a key role in uNK development and function. Suppression of IL-15 by asoprisnil was also observed at mRNA level (p < 0.05), and immunostaining for NK cell marker CD56 revealed a striking reduction of uNK in asoprisnil-treated endometrium (p < 0.001). IL-15 levels in normal endometrium are progesterone-responsive. Progesterone receptor (PR) positive stromal cells transcribe both IL-15 and IL-15RA. Thus, the response of stromal cells to progesterone will be to increase IL-15 trans-presentation to uNK, supporting their expansion and differentiation. In asoprisnil-treated endometrium, there is a marked downregulation of stromal PR expression and virtual absence of uNK. These novel findings indicate that the IL-15 pathway provides a missing link in the complex interplay among endometrial stromal cells, uNK, and spiral arteries affecting physiologic and pathologic endometrial bleeding. The Journal of Immunology, 2013, 191: 2226–2235.

studies that function of uNK in pregnancy is to regulate placentation (2, 3). Recent data suggest that uNK contribute to the initial stages of remodeling of uterine spiral arterioles in the early stages of pregnancy before the more dramatic destruction of the media by invading trophoblast cells (4). This important observation is pertinent to pregnancy pathologies such as preeclampsia and fetal growth restriction in which the failure of spiral artery remodeling is implicated in disease pathogenesis.

In contrast, little is known about the function of uNK in the normal cycling endometrium. They are sparse in the estrogen-dominated follicular phase, but then vigorously proliferate in the luteal phase after ovulation when circulating progesterone levels increase, leading to a dense accumulation of uNK in the stroma in the late secretory phase (5, 6). Menstrual breakdown occurs a few days later unless the corpus luteum is maintained by a pregnancy, in which case, progesterone levels continue to rise and the endometrium is transformed to decidua. We have proposed that uNK have a homeostatic role in this crucial switch between menstruation and decidualization because they die a few days premenstrually, coincident with involution of the corpus luteum and falling progesterone levels (7). Indeed, many studies indicate that uNK may initiate arterial remodeling and maintain vascular stability (2, 4, 8–10). Menstrual bleeding complaints affect quality of life and comprise a substantial societal burden, including major effects on health care use and costs (11, 12). Thus, how endometrial bleeding is controlled in normal and pathologic states and how uNK contribute to this are important questions.

Progesterone receptor (PR) modulators, including PR antagonists and compounds with partial and mixed progesterone agonist and antagonist activity, have been developed over the last decade as primary therapeutics for a number of indications (13). Asoprisnil, a progesterone receptor modulator (PRM) (14), has been evaluated
as a treatment for heavy menstrual bleeding, uterine fibroids, and endometriosis (15). Marked, dose-dependent suppression of uterine bleeding has been the most profound and consistently reported clinical effect of asoprisnil, both in healthy premenopausal women (16) and in patients with heavy menstrual bleeding associated with uterine fibroids (14). The exact mechanism of action of asoprisnil on the endometrium remains to be established, but the most striking histological effect is on the spiral arteries (17), which appear unusually prominent because of abnormally thick muscular walls. This effect seems to be specific to asoprisnil, because it has not been observed with other PRMs, including mifepristone. Expression of PR is normally abundant in endometrial stroma, especially in perivascular cells (18, 19), so that asoprisnil might initiate these striking changes by affecting stromal PR expression; an effect via alteration of uNK function is also possible. Uterine NK cells do not, however, express the PR (20); therefore, any PR-associated regulation of function will be indirect. IL-15 is crucial in the regulation of uNK development, expansion, and maturation and is highly expressed in perivascular stromal cells in the nonpregnant endometrium where uNK cluster (21, 22).

To investigate the mechanisms by which asoprisnil exerts its profound suppression of uterine bleeding, we used a whole genome expression array to study gene expression profiles in asoprisnil-treated versus placebo-treated patients with bleeding owing to symptomatic uterine fibroids. Robust statistical and stringent threshold analysis identified 245 genes, predominantly associated with immune-inflammatory genes. Based on a statistical pathway analysis for functional enrichment of these genes, the IL-15 pathway was identified as the principal network altered upon asoprisnil treatment. Many of the downregulated genes fed into the IL-15 pathway, known to have a key role in uNK development and function (23). We therefore examined uNK distribution in the endometrium of asoprisnil-treated women and found that uNK were substantially reduced or absent. It is notable that these women also reported a dramatic reduction or absence of menstrual bleeding (14). These data provide convincing evidence to support a pivotal role for an IL-15–uNK axis in the regulation of endometrial bleeding.

Materials and Methods

Sample collection

Studies with asoprisnil-exposed endometrium. Samples of endometrium exposed to asoprisnil were derived from a phase II multicenter, randomized, double-blind, placebo-controlled study of asoprisnil administration in premenopausal patients with symptomatic uterine fibroids scheduled for hysterec- tomy, as described previously (14, 17). Subjects received oral doses of asoprisnil (10 mg, 25 mg, or placebo) once daily for 12 wk until hysterectomy. The study protocol was approved by the Multicentre Research Ethics Committee (LREC/2002/6/17). Endometrial tissue was collected at hysterectomy and preferably obtained from areas not overlying any fibroids.

Studies with normal endometrium. IL-15 mRNA expression was assessed (use of SYBR green-based quantitative real time PCR [QRT-PCR]) in endometrium from women not exposed to asoprisnil. Samples of normal endometrium (menstrual, n = 7; proliferative, phase n = 11; secretory phase, n = 16) were obtained from women with normal menstrual cycles and no endometrial pathology attending Addenbrooke’s Hospital, Cambridge for sterilization. Patients with endometriosis, fibroids, or a history of exogenous hormone administration were excluded.

Studies with isolated human endometrial stromal cells. Human endometrial tissue specimens were obtained from women undergoing surgery for nonmalignant gynecologic conditions; written informed consent was obtained from all subjects prior to surgery, and ethical approval was granted by the Lothian research ethics committee (LREC/05/51/04/12; LREC/1051/02/29).

Studies with first-trimester decidua. Samples of first-trimester decidua were obtained from women undergoing elective surgical termination of pregnancy in the first trimester at Addenbrooke’s Hospital. All patients provided informed consent. Peripheral blood was collected by venipuncture of healthy volunteers, with informed consent. Blood was layered directly onto

Lymphoprep (Nycomed, Oslo, Norway) and centrifuged (700 × g, 20 min) to enrich leukocytes. The interface was collected and washed in PBS. The study was approved by Cambridge Local Research Ethics Committee, study number 04/Q0101/23.

Sample preparation

RNA extraction and quality assessment for gene microarray. RNA was extracted from endometrial samples and collected from women exposed to asoprisnil, as described previously (20). Quality and integrity of each RNA sample was checked with the Agilent 2100 Bioanalyser (Agilent, Santa Clara, CA) according to manufacturer’s instructions and the RNA integrity number (RIN) determined. Only samples with an RIN > 8.5 were processed for further analysis. Prior to full array analysis, RNA was subjected to the Affymetrix Test3 Array (Affymetrix, Santa Clara, CA) to exclude degraded samples with insufficient target.

Decidualization of isolated human endometrial stromal cells. Human endometrial stromal cells (ESCs) were isolated from tissue specimens following collagenase and DNase digestion as described previously. ESCs were subsequently maintained in vitro at 37˚C under 5% CO2 in air for a maximum of four passages and cultured in RPMI 1640 media (Sigma) supplemented

FIGURE 1. Expression of PR is altered after treatment with asoprisnil. (A) Control secretory endometrium with strong expression of progesterone receptor (PR) in the endometrial stromal cells (S) and negligible expression in glandular epithelium (G). (B) PR expression is lost in endometrial stroma (S) and is upregulated in both surface and glandular epithelium (G) in asoprisnil-treated endometrium. Arrows indicate spiral arteries. (C and D) The effect of asoprisnil on CD56+ uNK and spiral arteries. Endometrial samples are immunostained for CD56 (a) and CD38 (b). Expression of CD38+ cells is not affected by asoprisnil treatment. CD56+ uNK are scattered throughout stroma in placebo-treated control secretory phase endometrium. (E) Virtually no CD56+ cells are observed following treatment with asoprisnil. (F and G) The effect of asoprisnil on uNK and spiral arteries. (H and J) Endometrium samples stained for αSMA expression and collagen. (E) αSMA expression in spiral artery after treatment with 25 mg asoprisnil shows thickening of the media. (F) Collagen in spiral arteries after treatment with 10 mg asoprisnil shows thickening of the arterial wall. (G) αSMA expression in spiral artery in normal secretory phase endometrium. (H) Collagen in spiral arteries in normal secretory phase endometrium. Scale bar, 50 μm.
with 10% FCS (Cat. No. 10082-147; Invitrogen), 10 ml/L penicillin/ streptomycin (10,000 U penicillin and 10 mg streptomycin per 1 ml solution; Cat. No. P-4333; Sigma), 2 mM L-glutamine (Cat. No. G-7513; Sigma), and 2.5 µg/ml Fungizone (Cat. No. 15290-018; Invitrogen). Prior to experimentation, ESCs were transferred to medium that contained phenol-red free RPMI 1640 and charcoal-stripped FCS but otherwise supplemented as previously described for 48 h. FCS was charcoal stripped to remove endogenous steroids. Prior to decidualization ESCs were transferred to serum-depleted media (as above but 2% FCS) for 24 h. Decidualization was induced by adding decidualization media (RPMI 1640, 2% FCS, 0.1 mg/ml 8-Br-cAMP, and 1 µM progesterone) for up to 8 d. Control cells were incubated with vehicle (DMSO). Concentrations of IL-15 mRNA were determined using Taqman QRT-PCR.

Purification of immune cells from first-trimester decidual tissue. Cells were isolated from the decidua as described previously (24). Cells were then plated down on plastic for 2 h in RPMI 1640 medium plus 10% FCS. The nonadherent cells were stained for CD3 and CD56 and flow sorted on CD3+CD562 cells (T cells) and CD3−CD56+ cells (NK cells). Adherent cells were harvested by trypsin digestion (5 min, 37°C), stained for HLA-DR and CD10, and sorted on HLA-DR+CD10− cells (myeloid APCs) and HLA-DR−CD10+ cells (stroma) (23). Trophoblast cells were cultured overnight as described (24), harvested by trypsin digestion (5 min, 37°C), stained for HLA-G and CD14 (macrophage marker), and sorted on HLA-G+CD14− extravillous trophoblast (EVT) cells.

Sample analyses

Microarray analysis and data processing. Endometrial samples from placebo (n = 6), 10 mg asoprisnil (n = 11) and 25 mg asoprisnil (n = 10) treatments were hybridized to 27 microarrays. The array platform used was the Affymetrix Human Genome U133 plus 2.0 (www.affymetrix.com/support/technical/datasheets/human) whole human genome expression array. The microarray data were processed according to the following approaches. Between-array normalization followed a standard Robust Multi-array Average model, providing background-corrected, quantile-normalized, gene-level

![Color Key](https://www.jimmunol.org/)

Heatmap for hierarchical clustering of 245 significant genes (and >= 5 fold change)

FIGURE 2. Gene expression following administration of asoprisnil centers on IL-15. Heat map for hierarchical clustering of 245 statistically significant (adjusted \( p \leq 0.05 \)) and highly upregulated or downregulated (≥5-fold) genes in comparisons of placebo with 10 mg asoprisnil or placebo with 25 mg asoprisnil. Data were derived from endometrial samples. This heat map represents an overview of gene expression patterns contained among the set of significant genes; it is not an independent machine learning analysis of the complete data set. Blue represents low expression, and red represents high expression. Treatment group is shown in the top gray bar; light gray = placebo; dark gray = 10 mg asoprisnil; black = 25 mg asoprisnil. Hierarchical clustering was performed on genes, not on samples. Dendrogram branches partition genes into sets with similar gene expression across all samples in the study. Row labels are Affymetrix probe IDs followed by official gene symbol.
were incubated with CD56 Microbeads (1 ml of beads per 10^6 cells, 15 min, transmembrane). NK cells were then cultured for 5 d at 50,000–100,000 cells/well in 0.5 ng/ml recombinant human IL-15 (Peprotech) or 10 ng/ml IL-15Rα-Fc fusion protein (R&D Systems) or both. When both IL-15 and IL-15Rα-Fc were added, they were preincubated together for 2 h to allow complexes to form. At the end of the culture period, the cells were counted, excluding dead cells using trypan blue, and replated in 200 μL RPMI 1640 medium plus 10% FCS at 100,000 cells/well of a 96-well plate, with the addition of 0.037 MBq tritiated thymidine (Amersham Biosciences, Amersham, U.K.) and cultured for an additional 16 h. Thymidine incorporation was assessed by measuring counts per minute.

Immunohistochemistry: PR and CD56 immunolocalization. Immunohistochemistry was performed according to standard protocols with Ag retrieval in 0.01 M sodium citrate (27). The Bond-X Staining System (Leica, Milton Keynes, Bucks, U.K.) was used for further processing (27). mAbs to CD56 (1:200; Zymed Laboratories, San Francisco, CA) and to PR (1:400; Novocastra-Leica, Milton Keynes) together with negative control, isotope-matched mouse serum (MlgG1; Sigma-Aldrich, St. Louis, MO) at matched IgG concentrations. The automated staining process was completed according to the Bond Polymer Define Detection Kit (Leica Microsystems) 3h protocol per the manufacturer’s instructions. The intensity and frequency of positively immunostained UNK (CD56⁺) in the endometrial stroma was quantified.

CD56 immunostained tissue sections were observed under a Leitz DMRB research microscope under bright field illumination using a Q-imaging Fast-1394 digital camera and a Prior motorized stage. Media Cybernetics Image-Pro Plus software with a stereology module was used to tile areas of endometrium using a 4x objective. From the tilted overview image, a representative area of interest was delineated by the operator and 10 random fields were then acquired via software at original magnification ×40. CD56⁺ cells were counted over the 10 frames. Results were unblinded at the end of the count.

Histologic staining for a smooth muscle actin and collagen. To identify components of the arterial walls, an mAb to a smooth muscle actin (sMA) was used (1:5000; no. A-2547; Sigma-Aldrich) (28). Masson trichrome stain was used to identify collagen (29).

Statistical analyses
Each group of asoprisnil-treated subjects (10 and 25 mg) was compared with the subgroup of placebo-treated subjects who had undergone hysterectomy in the secretory phase of their menstrual cycle. These were determined by histologic examination (17) and correlated with patients’ menstrual diaries (14). Samples from the progesterone-dominated cycle phase were deemed most appropriate for comparison with samples following treatment with asoprisnil. A comparison of treatment groups as well as analysis of immunohistochemical protein expression was completed by applying the Wilcoxon rank sum test, with statistical significance determined at 0.05 using the Hochberg multiple comparison procedure.

For QRT-PCR of IL-15 and IL-15RA across phases of the menstrual cycle and cell types, significant differences were detected using the Kruskal–Wallis test. When differences were detected, pairwise comparisons were then made using the Mann–Whitney U test. For IL-15 trans-presentation assays, paired samples, cultured either with or without IL-15Ra, were compared using the Wilcoxon signed ranks test.

Results
Asoprisnil downregulates PR expression in endometrial stroma
Endometrial samples from 33 subjects were examined for immunolocalization of PR (10, 12, and 11 patients had received placebo, 10 mg asoprisnil, and 25 mg asoprisnil, respectively, as reported previously) (14). Control secretory phase endometrium (from women who received placebo) exhibited the characteristic strong expression of PR in the endometrial stroma with negligible expression in glandular epithelium (Fig. 1A). In contrast, exposure of endometrium to asoprisnil (25 mg) resulted in virtual complete loss of stromal PR expression. PR expression was also suppressed

Table I. Sequences for quantitative amplification by TaqMan QRT-PCR

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD56 ( Biosource International)</td>
<td>CTTCAACCTGACCACCTAT</td>
<td>TGGCTTGAAACACCACACT</td>
<td>CACATCGAGCGCCGCGG</td>
</tr>
<tr>
<td>IL-15 (Universal Probe Library)</td>
<td>CAAGATGGGCCAGCACTAAAAG</td>
<td>GCTGATGGCACAGGGTGT</td>
<td>ATGGGTC</td>
</tr>
</tbody>
</table>

Sequences used for quantitative amplification of CD56 and IL-15 by TaqMan QRT-PCR.

Table II. Gene expression in asoprisnil-treated endometrium, compared to placebo

<table>
<thead>
<tr>
<th></th>
<th>Asoprisnil 10 mg</th>
<th>Asoprisnil 25 mg</th>
<th>Significant for Both Doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;5-fold up from placebo</td>
<td>30</td>
<td>68</td>
<td>19</td>
</tr>
<tr>
<td>&gt;5-fold down from placebo</td>
<td>88</td>
<td>162</td>
<td>84</td>
</tr>
<tr>
<td>Total number of genes upregulated or downregulated</td>
<td>118</td>
<td>230</td>
<td>103</td>
</tr>
</tbody>
</table>

Number of endometrial genes with statistically significant 5-fold upregulation or downregulation compared with placebo using a p value level of 0.05. Details are available in Supplemental Table I.
in perivascular cells by both doses of asoprisnil compared with placebo (Fig. 1B). Conversely, PR expression was significantly upregulated in both surface and glandular epithelium in asoprisnil-exposed endometrium (Fig. 1B). To our knowledge, this dramatic switch in the distribution of PR expression from stroma to glandular epithelium in response to a PRM has not been reported previously.

Asoprisnil downregulates the expression of genes associated with endometrial immune cell function

To gain further insight into asoprisnil-mediated PR effects, genome-wide molecular profiling was used to provide an informative unbiased approach to identify the response of endometrial tissue to asoprisnil treatment. Microarray analysis of endometrial samples from the same 33 patients was performed (14). Twenty-seven of the 33 endometrial RNA samples (n = 6 placebo [secretory]; n = 11 asoprisnil 10 mg; n = 10 asoprisnil 25 mg) were of suitably high quality (RIN > 8.5; mean RIN = 9.35) for analysis. Two hundred forty-five genes were significantly (p < 0.05 after multiple testing correction) upregulated or downregulated 5-fold or greater in either or both treatment groups (10 and 25 mg asoprisnil) compared with placebo. Fig. 2 provides an overview of this gene set in the form of a heat map representing gene expression levels and their similarity across all samples and treatment groups.

The overall trend was a marked suppression of gene expression (166 genes), and gene ontology enrichment analysis of this set showed a significant overrepresentation of genes characteristic of NK cells, notably IL-15, IL-2RB, GZMB, NKG7, and GNLY. The downregulation of many genes was dose dependent (Table II and Supplemental Table 1), which is indicative of an association with drug action. Subsequently, 245 genes with significant differential change in expression were analyzed for association with biological functions using pathways and network analysis. Discrete biological networks of interacting genes can be integrated to test for connectivity and overlap. In this regard, integration of the networks associated with 166 downregulated genes uncovered IL-15 in the center of an immune mediator axis in the downregulated genes (Fig. 3).

Asoprisnil downregulates endometrial expression of IL-15 and dramatically reduces uNK numbers

To validate the microarray experiments, QRT-PCR was performed on endometrial tissue samples to evaluate the effects of asoprisnil on IL-15. Sequences for QRT-PCR are described in Table I. IL-15

FIGURE 3. Biological networks associated with immune cell function and apoptosis predominantly affected by asoprisnil. Most genes within networks are significantly downregulated (green); only few are upregulated (red). Central to the network and notably downregulated is IL-15.
mRNA expression was significantly downregulated by 25 mg asoprisnil compared with placebo (secretory) samples (Fig. 4A).

Because of the clear influence of IL-15 on NK cell development and function, we investigated how uNK numbers were affected by asoprisnil using immunohistochemistry to identify CD56+ cells. Fig. 1C shows the typical distribution of uNK in the normal secretory phase scattered throughout the stroma. In contrast, CD56+ uNK cells were absent or dramatically reduced following treatment with asoprisnil (25 or 10 mg) compared with placebo (Figs. 1D, 5). The three samples in the placebo group that had only small numbers of CD56+ uNK, were histologically dated as early secretory phase, which is before the major expansion of uNK (Fig. 5).

Asoprisnil alters endometrial vascular architecture

We examined the histological components of the endometrial spiral arteries (17); following asoprisnil administration, they exhibit an unusual appearance with abnormally thick muscular walls. αSMA is normally expressed only in the tunica media of arteries and arterioles, whereas collagen is localized in the tunica adventitia. Morphologic analysis of asoprisnil-treated endometrium showed that αSMA staining was increased within the tunica media and detected in the tunica adventitia (Fig. 1E). Within the clusters of thick-walled arterioles, increased collagen was found in both the tunica adventitia and tunica media following administration of asoprisnil (Fig. 1F). Neither effect on αSMA or collagen was dose-dependent. Figs. 1G and 1H illustrate the histologic appearance of normal endometrial spiral arteries in the secretory phase.

**Endometrial IL-15 expression correlates with progesterone levels**

To investigate whether IL-15 is induced by progesterone and to determine when IL-15 signaling to uNK cells mainly occurs, we measured the IL-15 transcript in endometrial samples taken throughout the normal menstrual cycle and the first trimester of pregnancy.
levels than in NK and T cells, but IL-15RA expression varied in stromal cells, IL-15 was also expressed at significantly higher number of samples was too small to make a statistical comparison. (Fig. 4C, 4D). IL-15 and IL-15RA were low in EVT, although the were significantly higher in decidual APCs than in NK or T cells were significantly increased in response to IL-15 mRNA expression were demonstrated (Fig. 4E). As a result, we can confirm that levels of IL-15 mRNA increase in response to progestrone, consistent with previous reports (30–32).

**uNK respond to IL-15 trans-presentation by stromal cells and APCs**

To investigate whether uNK respond to trans-presented IL-15, we used an assay first developed in mice and recently adapted for ex vivo use on human pNK (33, 34). In this assay, CD56+ NK cells (Fig. 6A, 6B) were cultured with recombinant human IL-15, which has been preincubated to form complexes with IL-15Rα–Fc fusion protein mimicking the effect of IL-15 trans-presentation. As a positive control, we first replicated experiments on the effect of the complexes on pNK from nonpregnant donors. After 5 d, there were significantly more cells and slightly more thymidine incorporation in the cultures with complexes than in those with IL-15 only (Fig. 6C, 6D). The 1.7-fold median expansion of cells observed in the trans-presentation condition, compared with soluble IL-15, is consistent with the 2-fold mean expansion previously reported (34). The results of the assay assessing cell culture growth in the presence of soluble IL-15 versus complexes with IL-15Rα–Fc fusion protein were similar for uNK and pNK. Significantly higher cell numbers and thymidine incorporation were present after culture with complexes than after culture with IL-15 alone (Fig. 6E, 6F). Therefore, we conclude that uNK respond to IL-15 trans-presentation.

To identify which cells are responsible for IL-15 trans-presentation, we examined transcription of IL-15 and IL-15RA by four different uterine cell subsets. Because we were unable to extract sufficient cell numbers from small endometrial biopsies, we isolated various decidual cell subsets. IL-15 and IL-15RA transcripts were significantly higher in decidual APCs than in NK or T cells (Fig. 4C, 4D). IL-15 and IL-15RA were low in EVT, although the number of samples was too small to make a statistical comparison. In stromal cells, IL-15 was also expressed at significantly higher levels than in NK and T cells, but IL-15RA expression varied considerably, such that it was neither significantly higher than in NK and T cells nor significantly lower than in APCs. Therefore, both IL-15 and IL-15RA transcripts are expressed in APCs and stromal cells at similar, high levels.

**Discussion**

Asoprisnil has been shown, with other PRMs, to be useful clinically because of its ability to reduce dramatically or stop endometrial bleeding (13, 14, 16). Although we have reported previously that thick-walled clusters of spiral arterioles within the endometrium are characteristic of asoprisnil treatment (17), the mechanism by which it mediates this effect is not understood. We now report the striking observations from asoprisnil-treated endometrium that expression of the progesterone receptor (PR) in stromal cells is negligible, there is gain of PR expression in uterine epithelium, and that uNK are absent. Stromal cell expression of PR and presence of uNK are both defining features of normal secretory endometrium. Our findings lead us to hypothesize that asoprisnil prevents endometrial bleeding by interfering with the complex interplay between endometrial stromal cells, uNK, and the spiral arteries. These novel data also complement the recent report that uNK play a role in the remodeling of spiral arterioles in the early stages of pregnancy (4).

Using whole genome expression arrays, we showed that asoprisnil downregulates a number of innate immune genes centered on the IL-15 signaling pathway, and this finding was confirmed by QRT-PCR. IL-15 mediates the differentiation of immature to mature uNK in humans (23, 35) and is essential for their development in mice (21). In this study, we have shown that IL-15 can also promote the proliferation of mature human uNK in the context of trans-presentation by IL-15Rα. Thus, the marked downregulation of IL-15 in asoprisnil-treated endometrium is likely to account for the remarkable absence of uNK in these samples. In agreement with others, we found that IL-15 variation over the course of the menstrual cycle and early pregnancy correlates with circulating progesterone levels (30–32, 36). In vitro modeling of secretory endometrium confirmed that IL-15 is upregulated during decidualization of endometrial stromal cells, a process that occurs in response to peak progesterone levels in vivo. Moreover, IL-15 expression decreases in response to treatment with the progesterone antagonist RU486 (unpublished observations), lending further support that progesterone signaling causes increased IL-15 signaling, rather than the two merely being correlated.

IL-15 expression in the endometrium is already well known to correlate with fluctuations in the number of uNK in mice (37). Thus, although uNK cells themselves do not express the PR, they are clearly influenced by progesterone levels (38). This suggests that progesterone-responsive cells in the endometrium, notably stromal cells, relay the signal to uNK via IL-15. Our finding that endometrial stromal cells and APCs express both IL-15 and IL-15RA, which are required to signal to uNK via trans-presentation (39), suggests that either or both of these cells are capable of supporting uNK differentiation and expansion. However, APCs and stromal cells are not equally responsive to progesterone, and by extension to asoprisnil. A major effect of asoprisnil administration was the observed downregulation of expression of PR in endometrial stromal cells. In contrast, APCs do not express PR (40, 41), a finding we confirmed in both asoprisnil-treated and control patients. Thus, because normally only stromal cells express PR, they are the likely candidates to promote uNK expansion in response to increased progesterone (20, 40–42), and to mediate the dramatic effects of asoprisnil on endometrial uNK numbers and in turn the structural changes in spiral arteries.

There are now several lines of evidence that uNK directly affect spiral arteries by modifying the structure of the tunica media. In humans, uNK are always preferentially located around the glands and arteries (43), and are thought to influence the loosening of the
smooth muscle of the media by production of a range of angiogenic factors (2, 4, 8–10). By staining for αSMA and collagen, we observe in this study that spiral arterioles in asoprisnil-treated endometrium have a thicker tunica media in the absence of uNK. There is a clear parallel between the endometrium of asoprisnil-treated women and the implantation site of uNK-deficient strains of mice. In addition to lacking uNK, both have arteries with elevated wall-to-lumen ratios and thickened walls (21). Thus, asoprisnil treatment results in a human endometrial phenotype resembling a uNK-null mouse (21). Furthermore, a recent study of decidual

FIGURE 6. Decidual NK cells respond to IL-15 trans-presentation. (A and B) Dot plots showing CD56 and CD3 staining before and after magnetic enrichment of CD56+ pNK cells (A) and CD56+ decidual leukocytes (dNK; B). Percentage of cells in each quadrant is shown. (C and D) Magnetically enriched pNK cells (n = 6) were cultured for 5 d either alone or in the presence of 0.5 ng/ml recombinant IL-15, 10 ng/ml (excess) IL-15Rα-Fc, or both. After 5 d, the cells were counted (C), replated at 100,000 cells/well and pulsed with tritiated thymidine (D). (E and F) The same procedure was performed on magnetically enriched dNK cells from unmatched donors (n = 7). Cell counts and thymidine incorporation in each set of experiments is presented relative to that when no additions were present. dNK, Decidual NK cells; pNK, peripheral blood NK cells.

FIGURE 7. Possible mechanisms of action of asoprisnil on progesterone-mediated effects on the endometrium. PR in endometrial stromal cells senses progesterone (1) and responds by increasing expression of IL-15 and IL-15Rα (2). Transpresented IL-15 facilitates the development of mature uNK from immature NK (iNK) cells (3), and promotes their expansion. Uterine NK cells then mediate vascular remodeling (4). Asoprisnil can affect arterial remodeling indirectly by blocking PR expression in endometrial stromal cells, resulting in loss of IL-15 trans-presentation, leading to no development of uNK or arterial remodeling (5). An additional direct mechanism of action of asoprisnil is to block PR expression in perivascular smooth muscle cells (6). VSM, Vascular smooth muscle cell.
Regulation of Endometrial Bleeding by uNK Cells

Spiral arteries after Ab depletion of uNK cells in rats also showed altered arterial development (44).

Data on endometrial IL-15 expression and uNK numbers are not available for PRMs other than asoprisnil, and none of the morphologic effects observed with asoprisnil occur in women treated with mifepristone. However, infrequent alterations in vascular morphology have been described with other PRMs, such as ulipristal acetate (CDB2914). Detailed histology from two large phase 3 clinical trials of more than 500 subjects revealed thick walled vessels similar to those seen in asoprisnil-treated endometrium, but only in ∼10% of subjects treated with ulipristal acetate (45). Experience with other PRMs is more limited. In a smaller clinical trial of telapristone acetate (Proellex; CDB4124), unusual patterns of endometrial vasculature (dilatation but no thickening of the walls) were found in only 4% of subjects (46).

To our knowledge, none of the morphologic effects observed with asoprisnil are found in women treated with progesterone antagonists such as mifepristone. Although uNK in normal endometrium are clearly regulated by progesterone, the effects of asoprisnil on stroma, uNK, and the stability of spiral arteries are likely to be due to the partial progesterone agonist activity of asoprisnil. A direct action of the PR ligand, asoprisnil itself, cannot be ruled out because PRs are expressed in the vessel wall of human and nonhuman primate endometrium (19). Menstrual bleeding disorders are a major health problem, and studies with asoprisnil have revealed a potentially useful class of drug and have highlighted the complex interplay between progesterone, the stroma, uNK, and spiral arteries in the endometrium, a pathway that is crucial for endometrial health and disease. In summary, we have shown that administration of asoprisnil inhibits endometrial expression of IL-15 and causes a profound reduction in the number of uNK. Furthermore, our findings that endometrial stromal cells express PR, that stromal cell PR expression is inhibited by asoprisnil, and that stromal cells are capable of trans-presenting IL-15 to uNK lead us to propose a model to account for the effects of asoprisnil on endometrial bleeding (Fig. 7). In normal nonpregnant endometrium, uNK proliferate and mature under the influence of progesterone that induces IL-15 trans-presentation from stromal cells. Along with the other predecidual changes that are a feature of midlate secretory phase endometrium, uNK modify the stromal cells. Along with the other predecidual changes that are a feature of midlate secretory phase endometrium, uNK modify the stromal cells. Along with the other predecidual changes that are a feature of midlate secretory phase endometrium, uNK modify the stromal cells.

As we have shown, the predecidual stromal cells are essential for spiral artery remodeling in early pregnancy. The effects of asoprisnil on endometrial bleeding (Fig. 7) are consistent with this model. The absence of uNK in asoprisnil-treated endometrium may be due to the lack of PR expression in the stromal cells, which would prevent efficient trans-presentation of IL-15 to uNK. This hypothesis is supported by the observation that uNK numbers are not reduced in asoprisnil-treated endometrium (46). The inability of asoprisnil to reduce uNK numbers may be due to the lack of PR expression in the stromal cells, which would prevent efficient trans-presentation of IL-15 to uNK. This hypothesis is supported by the observation that uNK numbers are not reduced in asoprisnil-treated endometrium (46).

Acknowledgments

We thank Pamela Cornes and Teresa Henderson for technical assistance with immunohistochemistry; Mary Ann Lumsden, Dharani Hapangama, Joan Kerr, and Sue Ingamells for assistance with several clinical aspects of the study; Nigel Miller and Lucy Gardner for technical assistance; Sheila Milne for secretarial assistance; Ronnie Grant for help with illustrations; Jeremy Brown and Mike Millar for assistance with analyses of CD56 immunodetection; Philippa Saunders for comments during manuscript preparation; and Fios Genomics for help with data analysis.

Disclosures

K.C. was previously employed by TAP Pharmaceutical Products. K.C. is a coinventor of several patent applications with asoprisnil. A.R.W.W. has consulted for TAP Pharmaceutical Products. H.O.D.C., I.T.C., and P.G. have received salary support for research staff and for laboratory consumables from TAP Pharmaceutical Products. P.G. and T.F. are founders of Fios Genomics. The other authors have no financial conflicts of interest.

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