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*J Immunol* published online 2 August 2013
http://www.jimmunol.org/content/early/2013/08/01/jimmunol.1300958
Uterine NK Cells Regulate Endometrial Bleeding in Women and Are Suppressed by the Progesterone Receptor Modulator Asoprisnil

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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, 191:000–000.
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 hysterectomy, 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. Samples were processed for RNA extraction and immunohistochemistry.

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/05/51/104/12; LREC/05/51/02/59).

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/Q01/23.

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...
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+CD56 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...
were incubated with CD56 Microbeads (1 ml of beads per 10^6 cells, 15 min, trans for each sample were normalized to 18S RNA. DNA present during the exponential phase of the reaction were determined using the TaqMan QRT-PCR.

Messenger RNAs encoding IL-15 and the uNK marker CD56 (NCAM1) were evaluated (Table I).

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD56 ( Biosource International)</td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>CTCCACCCCTACACCTCTAT</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>TGGCGTACACACACTACAT</td>
</tr>
<tr>
<td>Probe</td>
<td>CACATTGCCGACGCGCCG</td>
</tr>
<tr>
<td>IL-15 (Universal Probe Library)</td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>CAGATAGCCGCGGACACAGAGAG</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>GGCATTGCGCAGGTTT</td>
</tr>
<tr>
<td>Probe</td>
<td>ATGCCTGC</td>
</tr>
</tbody>
</table>

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

agglomerated and log2-transformed expression values. A non-specific filtering step was applied, removing probes that either fell below a threshold of detection or did not vary across samples. The corresponding filtering parameters were Affymetrix “present” or “marginal” calls in at least three arrays and variation greater than or equal to median Interquartile range estimate across all samples. After filtering, 26,111 of 54,675 probes on the chip were used as the analysis set. For statistical analysis of endometrial samples, a basic cell means model was fitted for each gene probe, with contrasts extracted for the analysis set. For statistical analysis of endometrial samples, a basic cell means model was fitted for each gene probe, with contrasts extracted for each sample were normalized to 18S RNA. DNA present during the exponential phase of the reaction were determined using the TaqMan QRT-PCR.

REGULATION OF ENDOMETRIAL BLEEDING BY uNK CELLS

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 by measuring counts per minute.

Histologic staining for a smooth muscle actin and collagen. To identify components of the arterial walls, an mAb to smooth muscle actin (sSMA) 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.

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...
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 25mg 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 GNYL. 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.
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.

**Figure 4.** (A) IL-15 mRNA is downregulated after treatment with asoprisnil. Endometrial IL-15 mRNA expression was measured by QRT-PCR in placebo-treated secretory phase endometrial samples and samples from women treated with 10 or 25 mg asoprisnil. IL-15 mRNA expression was significantly downregulated (p < 0.05) by 25 mg asoprisnil compared with placebo (secretory) phase samples. (B) IL-15 expression throughout the menstrual cycle and early pregnancy. Total RNA was extracted from endometrial or decidual samples. IL-15 expression levels were measured with QRT-PCR, relative to those of 18S RNA. Samples are arranged by menstrual cycle phase or first-trimester decidua. Menstrual, n = 7; proliferative, n = 11; secretory, n = 16; decidua, n = 6. (C and D) APCs and stromal cells are the main IL-15- and IL-15RA-expressing cells in first-trimester decidua. Total RNA was extracted from highly purified decidual NK cells, T cells, APCs, stromal cells and HLA-G⁺ EVT isolated by flow sorting. IL-15 (C) and IL-15RA (D) expression levels were measured by QRT-PCR, relative to those of 18S RNA. Medians and interquartile ranges are shown. Groups that are not significantly different from each other (p > 0.05) are indicated with the same lowercase letter. Trophoblast is not marked with a letter because the number of samples was insufficient for statistical testing. (E) In vitro modeling of the secretory endometrium reveals that IL-15 concentrations are significantly increased in response to decidualization. Human endometrial stromal cells were decidualized in vitro, and expression levels of IL-15 were measured with QRT-PCR, relative to those of 18S RNA. In decidualized ESCs (DEC), IL-15 is significantly increased compared with vehicle control (Ctrl) after 2 d (n = 4; p < 0.001), with 8-fold increase in expression. IL-15 is also increased after 1, 4, and 8 d decidualization (n = 4; p < 0.01, p < 0.001, and p < 0.01, respectively). AU, Arbitrary units.
IL-15 transcripts were low in the menstrual and proliferative phases, increased in the secretory phase, and higher still in first-trimester decidua (Fig. 4B). In addition, the nonpregnant endometrial secretory phase was modeled in vitro, and the response of primary human endometrial stromal cells decidualization media (progesterone plus cAMP) was examined for IL-15 production. Significant increases in IL-15 mRNA expression were demonstrated (Fig. 4E). As a result, we can confirm that levels of IL-15 mRNA increase in response to progesterone, 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-15RA-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 growth culture in the presence of soluble IL-15 versus complexes with IL-15RA–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 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-15Ra. 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 effectively 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.
spermatic 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 (CDP2914). 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 transiently 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 in the stromal cells. Along with the other predecidual changes that are a feature of midluteal secretory phase endometrium, uNK modify the smooth muscle media of the arteries and maintain arterial stability. If the corpus luteum is maintained by a pregnancy, these changes persist into early decidualia until the more dramatic arterial transformation mediated by trophoblast occurs in the decidua basalis (47). In a nonpregnant cycle, the corpus luteum involutes, and progesterone and IL-15 levels fall, resulting in the demise of uNK, vascular collapse, and the onset of menstruation. In miscarriage and failure of implantation following IVF, endometrial breakdown also occurs probably because of similar mechanisms. uNK have also been implicated in situations in which there are disturbances of hormonal regulation of the endometrium, such as perimenopausal bleeding and breakthrough bleeding on exogenous hormone therapy (48). We propose that uNK not only function in the regulation of placenta but also in the homeostasis of the cycling endometrium and in maintenance of the decidua. Compared with other mucosal surfaces, the uterine mucosa is uniquely dynamic and can change dramatically from total breakdown to decidualization in only a few weeks with uNK playing an integral part in this process.

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; Roni 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.

References


