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Steroid Receptor Expression in Uterine Natural Killer Cells

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The endometrium contains a unique subset of uterine-specific natural killer (uNK) cells, the proposed functions of which include a role in decidualization, menstruation, and implantation. These cells increase in number during the mid-late secretory phase of the menstrual cycle and are also present in large numbers in early pregnancy. The cyclical nature of uNK cell appearance suggests hormonal regulation of these cells. To date, it has not been possible to localize either estrogen receptors (ERs) or progesterone receptors (PRs) to uNK cells. In the present study, we have investigated the steroid receptor expression of uNK cells, including not only ERα and PR but also wild-type ERβ1, its variant form ERβcx/β2, and glucocorticoid receptor (GR) using specific monoclonal antibodies and real-time quantitative RT-PCR.

mRNA encoding ERα, PR, ERβcx/β2, ERβ1, and GR were identified in extracts of human endometrium across the menstrual cycle and in decidua. Quantitative real-time RT-PCR demonstrated an absence of ERα and PR mRNA in purified uNK cells. In contrast, mRNA for ERβcx/β2, ERβ1, and GR was present in uNK cells. ERα, PR, ERβcx/β2, ERβ1, and GR proteins were identified in endometrial and decidual biopsies. Colocalization using specific monoclonal antibodies confirmed that uNK cells were immunonegative for ERα and PR protein. These cells were also immunonegative for ERβcx/β2 but did express ERβ1 and GR proteins. These results raise the possibility that estrogens and glucocorticoids could be acting directly on uNK cells through ERβ and GR, respectively, to influence gene transcription in the endometrium and decidua. (*J Clin Endocrinol Metab* 88: 440–449, 2003)

THE HUMAN ENDOMETRIUM is a dynamic tissue that, to prepare for implantation, undergoes well defined cycles of proliferation, differentiation, and degradation in response to the prevailing steroid environment (1, 2). Leukocyte populations within the endometrial stroma also vary during the menstrual cycle and throughout pregnancy. Endometrial leukocytes include T and B cells, mast cells, macrophages, and neutrophils, but it is the phenotypically unique uterine natural killer (uNK) cells that make up the majority of the leukocyte population in the late secretory phase and early pregnancy (3).

These uNK cells have a unique phenotype ($CD56^{bright}$, $CD16^{-}$, $CD3^{-}$), which distinguishes them from peripheral blood NK cells ($CD56^{dim}$, $CD16^{bright}$, $CD3^{-}$). In the proliferative phase, few cells are apparent, but their numbers increase from day LH+3 and more dramatically in the mid-late secretory phase (day LH+11–13) where they are found in close contact with endometrial glands and spiral vessels (4, 5). It remains to be established whether the increase in cell number is solely the result of *in situ* proliferation or whether there is also *de novo* migration from the peripheral circulation. A precursor cell type might be selectively recruited into the endometrium where it differentiates to become the uterine-specific NK cell. In support of this theory is the existence of a subset of peripheral NK cells (around 1% of total circulating NK cells) that express a similar antigenic phenotype to uNK cells (6). However, proliferation of $CD56^{+}$ cells does occur in the endometrium because the proliferation marker Ki67 has been colocalized by immunohistochemistry (7, 8).

The increase in the number of uNK cells coincides with implantation and the early stages of placentation, and it has been suggested that this unique cell population may play a role in these processes (9). A role in endometrial breakdown and menstruation has also been proposed for uNK cells. In the nonpregnant cycle, King *et al.* (3) have observed changes suggestive of cell death of these cells on day LH+12–13, before any of the more accepted signs of menstruation such as neutrophil infiltration, clumping of stromal cells, and interstitial hemorrhage have occurred. The association of uNK cell demise and falling levels of progesterone as well as the cyclical nature of their appearance would seem to suggest hormonal regulation of these cells. However, to date, it has not been possible to localize either estrogen or progesterone receptors to these cells (10, 11), and therefore it has been proposed that estrogen and progesterone may exert their effects on uNK cells indirectly via cytokines such as IL-15 and prolactin (PRL) or other soluble factors (12–14).

Glucocorticoids have been shown to exert specific effects on endometrial cells (15–18), but their role in endometrial physiology is not well understood. Recently, Bamberger *et al.* (19) have briefly reported the immunoexpression of GR across the menstrual cycle. They found the receptor was almost exclusively expressed in the stromal compartment,

Abbreviations: ER, Estrogen receptor; GR, glucocorticoid receptor; HSD, hydroxysteroid dehydrogenase; NK, natural killer; NRS, normal rabbit serum; PR, progesterone receptor; PRL, prolactin; Q-RT-PCR, quantitative RT-PCR; RT, reverse transcribed; TBS, Tris-buffered saline; uNK, uterine NK; VEGF, vascular endothelial growth factor.

including endothelial and lymphoid cells. However, they did not investigate the type of lymphocytes that expressed the GR. It is therefore important to determine which leukocytes (and whether uNK cells, in particular) express the GR.

Two structurally related subtypes of estrogen receptor (ER), commonly known as α (ERα, NR3A1) and β (ERβ, NR3A2), have been identified in the human as well as in other mammals (20–22). Steroid receptors including ERα, ERβ, progesterone receptor (PR), and glucocorticoid receptor (GR) all act as ligand-activated transcription factors and share a common arrangement of structure/function domains with other members of the steroid receptor family (for review, see Ref. 23). *In vitro* studies have shown that homodimers (ERα-ERα or ERβ-ERβ) or heterodimers (ERα-ERβ) can be formed when both isoforms are expressed in the same cell (24, 25) and that the pattern and amount of expression of each isoform is likely to influence gene transcription within that cell. We have previously compared the spatial and temporal expression of ERα and ERβ in human endometrium and found that endothelial cells exclusively express ERβ (26). In the same samples, some immunopositive staining was also observed in cells that we tentatively identified as endometrial leukocytes. Recently, Stygar *et al.* (27) have reported that within the human cervix ERβ can be localized to cells that express leukocyte common antigen and macrophage markers.

In 1998, two papers reported that mRNAs encoding isoforms of human ERβ formed by alternative splicing of the last (eighth coding) exon were expressed in human tissues (28, 29). We have recently established that both the mRNA and protein corresponding to one of these splice variants (ERβcx/β2) are expressed in human endometrium (30). This splice variant lacks the ligand binding site and may act as a negative inhibitor of ERβ action (28). One objective of the current study was to establish whether human uNK cells express wild-type (ERβ1) and/or the variant ERβcx/β2 isoform.

The aim of this investigation was thus to determine the potential steroid responsiveness of uNK cells by using specific monoclonal antibodies and real-time quantitative RT-PCR (Q-RT-PCR) to establish whether selected steroid receptors (ERα, ERβ, GR, PR) are specifically expressed in this cell population.

Materials and Methods

Tissue collection

Endometrial tissue was collected from women undergoing hysterectomy or endometrial investigation for benign gynecological conditions (n = 42). First trimester decidual tissue samples (n = 8) were collected from patients undergoing surgical termination of pregnancy. Tissue was snap-frozen in liquid nitrogen before storing at −70 C for subsequent RNA extraction. Endometrial tissue was also fixed in 4% paraformaldehyde overnight at 4 C before routinely wax embedding using an 18-h cycle on a TP1050 machine (Leica Corp., Knowlhill, Milton Keynes, UK). In addition, decidua and full-thickness endometrial tissue including stratum functionalis, basalis, and myometrium were collected at hysterectomy or termination of pregnancy from representative patients for dual localization immunohistochemical investigations. All women described regular menstrual cycles and had not received exogenous hormones or used an intrauterine contraceptive device in the 3 months before inclusion in the study. Written informed consent was obtained from all subjects, and ethical approval was granted by the Lothian research ethics committee.

Endometrial biopsies were dated according to the criteria of Noyes *et al.* (1) and were found to be consistent with the patients' reported last menstrual period. In addition, all subjects had a serum sample collected at the time of biopsy for the determination of circulating estradiol and progesterone levels by RIA as previously outlined (31). Biopsies were classified as proliferative (n = 8), early (n = 8), mid (n = 8), or late secretory (n = 10), and a significant reduction in circulating progesterone levels was evident between biopsies in the mid and late secretory phases ($P < 0.01$; Table 1)

Isolation of uNK cells

$CD56^+$ decidual NK cells were isolated from first-trimester decidual tissue as previously described (12). Briefly, 1×10^8 decidual cells were suspended in 300 μl buffer (PBS/2 mM EDTA/1% human AB serum). After the addition of 0.5% human γ-globulins in PBS and 100 μl CD56 magnetic cell sorting microbeads (Miltenyl Biotech, Bergisch Gladbach, Germany), the suspension was incubated at 4 C for 20 min. The cells were washed, resuspended in buffer, and applied to a VarioMACS magnet (Miltenyl Biotech). The column was washed, and the $CD56^+$ cells were eluted and resuspended in RPMI/10% fetal calf serum. The purity of the decidual NK cells was greater than 97%, as confirmed by flow cytometry.

Analysis of mRNA by real-time Q-RT-PCR

Tissue samples and purified decidual NK cells were immersed in Trizol RNA isolation reagent (Invitrogen, Paisley, UK), homogenized, and RNA extracted according to the manufacturer's instructions. To remove genomic DNA, RNA was then subjected to DNAse treatment using DNAse I, Amp grade 1U/μg RNA in DNAse reaction buffer for 15 min at room temperature (Invitrogen). The reaction was stopped by the addition of EDTA (final concentration, 2.5 mM) followed by heating to 99 C for 5 min. Using random hexamers, 200 ng RNA was reverse transcribed (RT) in a buffered solution containing 5.5 mM $MgCl_2$, 2.5 μM random hexamers, 500 μM of each dNTP, 0.4U/μl Rnase inhibitor, and 1.25 U/μl multiscribe (all from PE Applied Biosystems, Cheshire, UK). Samples were RT by incubating for 60 min at 25 C, 45 min at 48 C, and 95 C for 5 min. Negative controls were included in every run. An RT-negative control had template RNA but no multiscribe enzyme included, and an RT H_2O had template RNA replaced by nuclease free water.

The primer/probe sets were designed using the Primer express program (PE Applied Biosystems) and, where possible, were chosen to span an intron to further reduce the chance of spurious readings due to genomic DNA contamination. The sequences of the primer/probe sets and their location within the specified cDNAs are given in Table 2. The 18S primers and probe were purchased from PE Applied Biosystems.

A Taqman real-time PCR mix was then prepared containing final concentrations of Taqman universal PCR master mix (1×), ribosomal 18S forward and reverse primers, and probe (50 nM; PE Applied Biosystems), forward and reverse primers (300 nM), and probe for sequence of interest (200 nM; PE Applied Biosystems). One microliter of cDNA was added per 25 μl of reaction volume, and each sample was done in triplicate. A no-template control (where water replaced cDNA) was included in every run, and the controls from the RT step were also run

TABLE 1. Circulating estradiol and progesterone levels at the time of collection of endometrial samples

Study group (histological stage of cycle)	Estradiol (pmol/liter)	Progesterone (nmol/liter)
Proliferative	400.39 (306–1731)	1.75 (1.2–12.17)
Early secretory	449 (206.96–481)	22.4 (2.17–56.2)
Mid secretory	480.50 (120.21–738)	47.27 (23.6–78.1)[a]
Late secretory	234 (129–507)	8.76 (3.9–22.4)[a]

Data are expressed as median (range).

[a] $P < 0.01$ significant difference between mid and late secretory groups for progesterone.

TABLE 2. Steroid receptor primer and probe sequences used for amplification by real time Q-RT-PCR

Primer/probe	Sequence	Position	Accession no.
ERβ1 forward	CCTGGCTAACCTCCTGATGCT	1459–1480	AB006590
ERβ1 reverse	CCACATTTTTGCACTTCATGTTG	1529–1552 (r)	AB006590
ERβ1 probe	AGATGTTCCATGCCCTTGTTACTCGCA	1499–1525 (r)	AB006590
ERβcx/β2 forward	ATCCATGCGCCTGGCTAAC	2628–2647	AB006589
ERβcx/β2 reverse	GAGTGTTTGAGAGGCCTTTTCTG	2684–2707 (r)	AB006589
ERβcx/β2 probe	TCCTGATGCTCCTGTCCCACGTCA	2648–2671	AB006589
ERα forward	TGATTGGTCTCGTCTGGCG	1523–1541	NM_000125
ERα reverse	CATGCCCTCTACACATTTTCCC	1602–1624 (r)	NM_000125
ERα probe	TGCTCCTAACTTGCTCTTGGACAGGAACC	1572–1600	NM_000125
PR forward	CAGTGGGCGTTCCAAATGA	2151–2170	NM_000926
PR reverse	TGGTGGAATCAACTGTATGTCTTGA	2209–2233 (r)	NM_000926
PR probe	AGCCAAGCCCTAAGCCAGAGATTCACTTT	2170–2199	NM_000926
GR forward	GCGATGGTCTCAGAAACCAAAC	4236–4257	NM_000176
GR reverse	GCAGAGGATAACTTCCTCTGTAATCTC	4332–4358	NM_000176
GR probe	TCAGAGCCTCAGCAACCTTCACTGCA	4300–4325 (r)	NM_000176

The positions of the sequences are given within the cDNA identified by the accession number; r denotes reverse strand.

TABLE 3. Steroid receptor intra-assay variability values for real time Q-RT-PCR

Primer/probe set	Intra-assay variability (%)
PR	0.8
ERα	4
ERβ1	2.1
ERβcx/β2	1.7
GR	4.5

at least once for each set of primers and probes. Wells were sealed with optical caps, and the PCR was run on the ABI Prism 7700 (PE Applied Biosystems) using standard conditions.

The linearity of the response of the primers and probe to specific cDNA was validated either by using serial dilution of a cDNA sample or by repeating experiments on a 1:5 dilution of all cDNA samples. Within-assay variation of the PCR measurement for each set of primer/probes in cDNA was calculated from six replicates (Table 3). Inter-assay variability for the samples was valued at 3.1% by running cDNA from one sample over five different Taqman RT-PCR experiments.

Significant difference was determined using one-way ANOVA, and individual differences were described using the least significant difference *post hoc* multiple comparison (SPSS, Inc., Chicago, IL).

Antibodies

Mouse monoclonal antihuman ERα was purchased from DAKO Corp. (Cambridge, UK; clone 1D5). ERβ proteins were detected using two isotype-specific mouse monoclonal antibodies directed against ERβ1 (peptide P7, IgG2a subtype) and ERβcx/β2 (peptide P8, IgG1 subtype). The monoclonals were prepared and validated as described in detail by Saunders *et al.* (32, 33). The specificity of all ER antibodies used was confirmed previously by Western blotting (32–34). Mouse monoclonal antibodies for PR and GR were supplied by Novocastra (Newcastle upon Tyne, UK; PR subtype IgG1, GR subtype IgG2a). Mouse monoclonal anti-CD56 antibody was supplied by Zymed Laboratories, Inc. (Cambridge, UK; subtype IgG1) and was used to label uNK cells.

Immunohistochemistry

All antibodies were tested individually at a range of dilutions and with different antigen retrieval conditions to determine the protocol that gave the least background and highest specific signal before additional optimization of double staining conditions. All sections were dual immunohistochemically stained with each of the steroid receptors and anti-CD56 antibody using 3,3-diaminobenzidine and fast blue (ERβ1/CD56 and GR/CD56; data not shown.) Additionally, ERβ1/CD56 and GR/CD56 coexpression was investigated using dual immunofluorescence.

Dual immunohistochemistry

Paraffin sections (5 μm) were dewaxed in Histoclear (National Diagnostics, Atlanta, GA) for 10 min before rehydrating in descending grades of alcohol to distilled H_2O. Sections were washed in Tris-buffered saline [TBS; 0.05 M Tris (pH 7.4), 0.85% saline] for 10 min before antigen retrieval by pressure cooking in 0.01 M sodium citrate (pH 6) for 5 min at setting 2/high (Tefal, Clipso, Nottingham, UK) for ERα and PR and 0.05 M glycine/0.01% EDTA (pH 8) for 7 min at setting 2 for ERβcx/β2. Sections were blocked for endogenous peroxidase in 3% hydrogen peroxide for 10 min before applying normal horse serum (Vector Laboratories, Inc., Peterborough, UK) for ERα and PR or a 1:5 dilution of normal rabbit serum (NRS; Diagnostics Scotland, Carluke, Lanark, UK) in TBS with 5% BSA (NRS/TBS/BSA) for ERβcx/β2. Sections were incubated overnight at 4 C in a 1:400 dilution of mouse anti-ERα, a 1:40 dilution of mouse anti-PR, or a 1:20 dilution of mouse anti-ERβcx/β2 antibody. Sections were incubated in biotinylated horse antimouse antibody for ERα and PR (prepared following manufacturer's instructions; Vector Laboratories, Inc.) or a 1:500 dilution of biotinylated rabbit antimouse antibody for ERβcx/β2 (DAKO Corp.) followed by an avidin biotin peroxidase complex (ABC Elite, Vector Laboratories, Inc., for ERα and PR; and ABC DAKO Corp., for ERβcx/β2) all for 60 min at room temperature. The sections were developed using 3,3-diaminobenzidine, before washing for 20 min in 0.05 M glycine/0.01% EDTA (pH 3). They were incubated sequentially in avidin, then biotin (Vector Laboratories, Inc.) for 15 min at room temperature, followed by normal horse serum, before applying monoclonal mouse anti-CD56 antibody at a 1:250 dilution overnight at 4 C. After washing, biotinylated horse antimouse antibody was applied, followed by an avidin biotin alkaline phosphatase complex (DAKO Corp.), both for 30 min at room temperature. The signal was developed using fast blue reagent (Sigma, Poole, Dorset, UK) before the sections were mounted in permafluor (Immunotech-Coulter, High Wycomb, Bucks, UK).

Negative controls were performed in which the primary antibodies were replaced with mouse IgG at a matched antibody concentration or blocking serum. Each antibody was also run separately to confirm the immunostaining pattern.

Dual immunofluorescence

Sections were dewaxed, and endogenous peroxidase was blocked as above. Sections were pressure cooked in 0.05 M glycine, 0.01% EDTA (pH 8) for 7 min at setting 2 for ERβ1 or 0.01 M sodium citrate (pH 6) for 5 min at setting 2 for GR before washing in TBS. They were then incubated in NRS/TBS/BSA for 30 min at room temperature before applying either a 1:20 dilution of mouse anti-ERβ1 antibody or a 1:10 dilution of mouse anti-GR antibody in NRS/TBS/BSA overnight at 4 C. Thereafter, sections were washed in TBS with Tween 20 (100 μl/liter) before the addition of a biotinylated rabbit antimouse antibody (DAKO Corp.) at a 1:500 dilution in NRS/TBS/BSA for 30 min at room temperature. After a further wash in TBS, followed by PBS [0.01 M PBS (pH 7.4), Sigma] the fluorochrome streptavidin 546 Alexafluor (Molecular Probes, Inc., Lei-

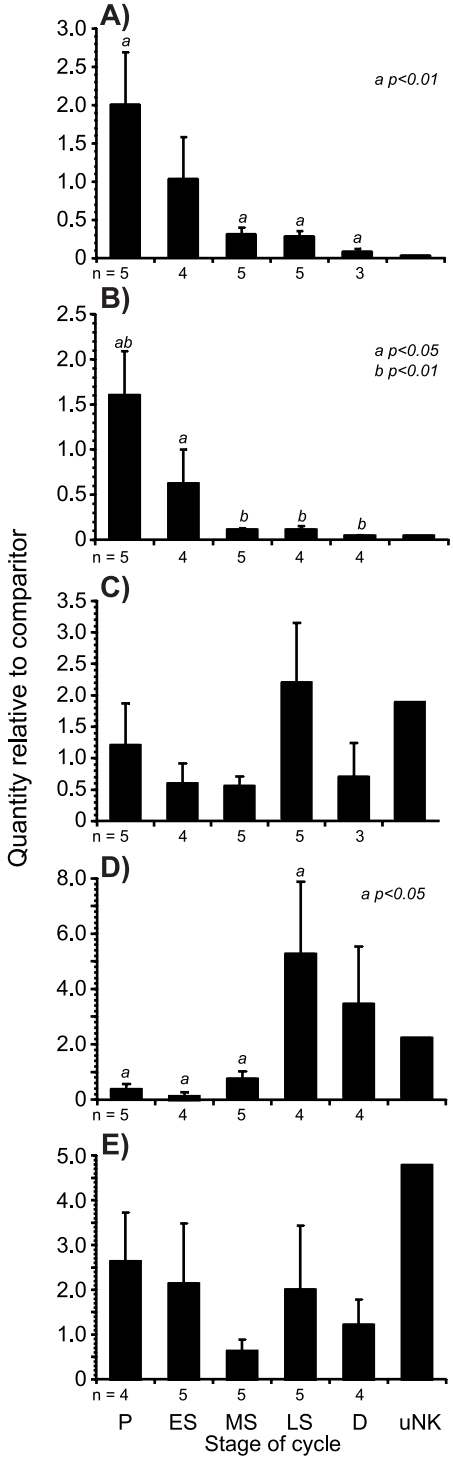

FIG. 1. Quantitative evaluation of steroid receptor mRNA by real-time Q-RT-PCR. All samples were compared with an internal control (comparator) obtained during the mid proliferative phase of the menstrual cycle. A, ERα across the menstrual cycle and in uNK cells. ERα mRNA levels fell significantly ($P < 0.01$) from the proliferative to the mid and late secretory phase endometrium and decidua. Levels were low in uNK cells. B, PR across the menstrual cycle and in uNK cells. PR mRNA levels fell from the proliferative to early ($P < 0.05$), mid, and late secretory phase endometrium and decidua ($P < 0.01$). Levels were again low in uNK cells. C, ERβcx/β2 across the menstrual cycle and in uNK cells. ERβcx/β2 message rose in late secretory phase endometrium, and mRNA was present in uNK cells. D, ERβ1 across the menstrual cycle and in uNK cells. ERβ1 mRNA was significantly increased in the late secretory phase ($P < 0.05$) and was high in uNK cells and decidua. E, GR across the menstrual cycle and in uNK cells. GR mRNA was present across the cycle and in decidua. High levels were present in uNK cells. P, Proliferative; ES, early secretory; MS, mid secretory; LS, late secretory; D, decidua.

den, The Netherlands) was added at a 1:200 dilution in PBS for 2 h at room temperature. Thereafter, the sections were incubated in 4 drops/ml biotin in TBS (Vector Laboratories, Inc.) for 15 min at room temperature. A 1:1000 dilution of mouse IgG (Vector Laboratories, Inc.) in NRS/TBS/BSA was then added at room temperature for 30 min. After blocking in NRS/TBS/BSA, a 1:250 dilution of CD56 antibody in NRS/TBS/BSA was added overnight at 4 C. Thereafter, the sections were incubated for 1 h at room temperature with a 1:100 dilution of rabbit-antimouse IgG1 subtype specific horseradish peroxidase-linked antibody (Zymed Laboratories, Inc.) made up in NRS/TBS/BSA. The sections were washed as before and incubated with Tyramide-Cyanine 5 fluorescent complex (kit NEL745, NEN Life Science Products, Boston, MA) at a 1:50 dilution for 10 min at room temperature. Sections were mounted in permafluor and left to dry in the dark.

Fluorescent images were taken on a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Thornwood, NY). The alexafluor 546 (ERβ1) was visualized using a helium/neon 1 laser with an excitation beam of 546 nm and detected using a band-pass filter from 560–615 nm. Cyanine 5 (CD56) was visualized using the helium/neon 2 laser with an excitation beam of 633 nm and detected using a long pass filter at 650 nm.

Results

mRNA expression quantified by real-time RT-PCR

All primer/probe sets were run on the same cDNA samples prepared from extracts of endometrium recovered at different stages of the cycle and from decidua. uNK cell RNA was extracted from purified $CD56^+$ cells from first-trimester decidua. All figures are given relative to a comparator used in all experiments that was a sample taken from the mid proliferative stage of the menstrual cycle.

Expression of ERα mRNA in human endometrium/decidua and uNK cells (Fig. 1A)

ERα mRNA levels in endometrial extracts were highest in the proliferative phase and fell (although not significantly) in the early secretory phase. Levels had fallen significantly by the mid and late secretory phases ($P < 0.01$) and were again further reduced in decidua ($P < 0.01$). uNK cell message levels were very low (0.03× levels found in the comparator).

Expression of PR mRNA in human endometrium/decidua and uNK cells (Fig. 1B)

The pattern of PR mRNA closely mirrored that of ERα. Levels were high in the proliferative phase of the cycle but fell significantly by the early secretory stage ($P < 0.05$). Levels were again reduced in the mid and late secretory phase and in the decidua samples compared with the proliferative stage ($P < 0.01$). uNK message levels were very low (0.02× levels found in comparator) when compared with the proliferative and early secretory phase, but they were similar to late secretory and decidual tissue levels.

Expression of ERβcx/β2 mRNA in human endometrium/decidua and uNK cells (Fig. 1C)

ERβcx/β2 showed moderate levels of mRNA in the proliferative, early, and mid secretory and decidual samples. An

increase in levels was apparent in the late secretory phase, following a similar pattern to our previous results (30) with similar levels present in the uNK cell mRNA (1.92× levels found in the comparator).

Expression of ERβ1 mRNA (wild-type receptor) in human endometrium/decidua and uNK cells (Fig. 1D)

ERβ1 mRNA levels were low in the proliferative and early secretory phases but showed a slight increase in the mid secretory phase of the menstrual cycle. A significant increase in mRNA levels was apparent by the late secretory phase of the cycle ($P < 0.05$), showing close agreement with previous observations (30). Levels were elevated compared with the early or mid secretory phase in the decidual samples and in the uNK cell mRNA (2.14× levels found in the comparator).

Expression of GR mRNA in human endometrium/decidua and uNK cells (Fig. 1E)

GR mRNA was present in all stages of the menstrual cycle and in decidua. Levels were high in the proliferative phase, maintained in the early and late secretory phases, but appeared to decrease (although not significantly) in the mid secretory phase. Decidua samples also showed significant levels of mRNA. High levels of mRNA (4.78× levels in comparator) were found in the purified uNK cells compared with all stages of the cycle and decidua.

A summary of the results given in Fig. 1 is presented in Table 4.

Protein expression as defined by double immunohistochemical labeling

Full-thickness endometrial biopsies from across the menstrual cycle and biopsies from first-trimester decidua were used for double immunohistochemical labeling.

ERα/CD56 colocalization

ERα was immunolocalized to the nuclei of stromal and glandular cells in the proliferative phase endometrium, but its expression declined in the mid and late secretory phase and in early decidua. As expected, the number of $CD56^+$ cells was greatest in the late secretory phase endometrium and decidua, and colocalization showed an absence of ERα in the $CD56^+$ cells (Fig. 2, a and b).

PR/CD56 colocalization

PR was immunolocalized to cell nuclei in endometrial glands and stroma of proliferative phase endometrium but was present only in the stromal compartment of mid and late secretory phase endometrium and decidua. The CD56 staining again followed a pattern similar to that seen using standard immunohistochemistry (data not shown) with strong cell surface localization. Dual immunohistochemistry revealed that PR immunoexpression was not present in the $CD56^+$ cells in endometrium (Fig. 2, c and d) or decidua.

TABLE 4. Summary of steroid receptor mRNA expression across the cycle, in uterine endometrium from the proliferative and the secretory phase, in decidua, and in uNK cells

mRNA expression	Proliferative	Secretory	Decidua	uNK cells
ERα	+	+/−	−	−
PR	+	+/−	−	−
ERβcx/β2	+	+	+	+
ERβ1	+/−	+	+	+
GR	+	+	+	+

ERβcx/β2/CD56 colocalization

ERβcx/β2 again showed specific nuclear localization to endometrial and decidual cells using both antibodies. Immunostaining was seen in the glands, stroma, and vessels. However, ERβcx/β2 did not colocalize to the $CD56^+$ cells (Fig. 2, e and f).

ERβ1/CD56 colocalization

ERβ1 showed specific nuclear localization in the glands, stroma, and endothelial cells of endometrium across the menstrual cycle (Fig. 3a, *inset*) and decidua. Again, CD56 immunostaining was specifically localized to the cell surface of a population of stromal cells. Dual immunofluorescence confirmed intense nuclear expression of ERβ1 by some, but not all, of the $CD56^+$ cells in endometrium (Fig. 3a) and decidua (data not shown).

GR/CD56 colocalization

The results show specific nuclear staining for GR in the stroma and endothelium of the endometrium (Fig. 3b, *inset*) and in the stroma, endothelium, and glandular epithelium of decidua. The endometrial glandular epithelium was negative for GR. CD56 was expressed on the cell surface of certain stromal cells. The dual immunofluorescence showed intense nuclear GR staining within cells stained for the CD56 cell surface marker in endometrium (Fig. 3b) and decidua (data not shown).

A summary of the results presented in Figs. 2 and 3 is given in Table 5.

Discussion

uNK cells are the major leukocyte population present in the endometrial stroma at the time when implantation, placentation, and decidualization occur. They have been proposed to have a function in all of these areas and might also be important in the initiation of menstruation. However, although their cyclical appearance indicates that they may be regulated by ovarian hormones, to date neither estrogen nor progesterone receptors have been localized to these cells.

This study has investigated the steroid receptor expression of uNK cells, including not only ERα and PR, but also wild-type ERβ, its variant form, ERβcx/β2, and GR. ERα and PR mRNA levels followed a similar pattern, showing significant amounts in the proliferative phase of the menstrual cycle, declining to low levels in the late secretory and decidual samples when uNK cells are most abundant. The expression of ERα and PR is tightly regulated by estrogen and progesterone, with estrogen inducing transcription and translation of both ERα and PR, whereas progesterone causes their down-regulation (35, 36). Therefore, the presence of high levels of ER and PR mRNA in the estrogen-dominated pro-

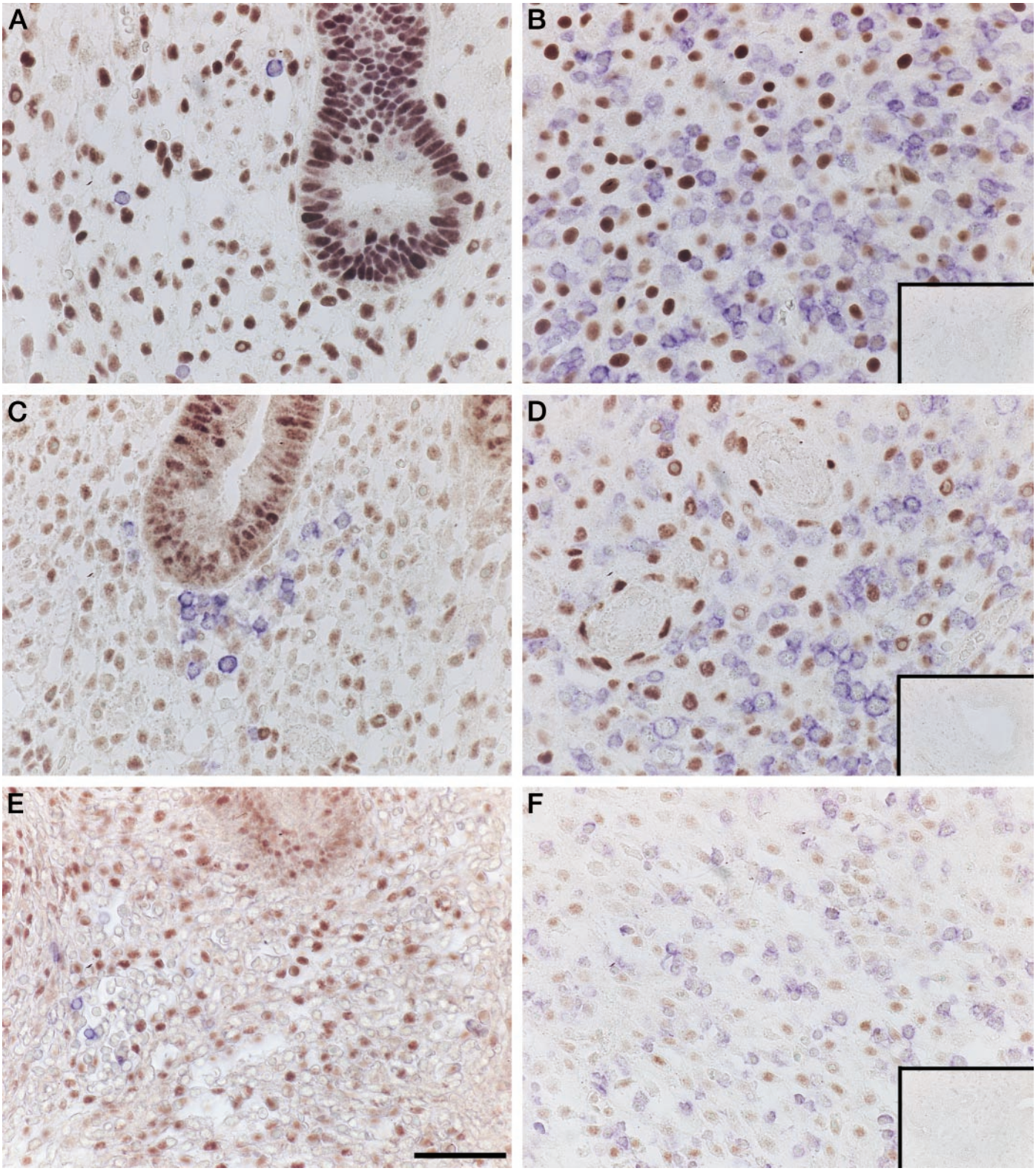

FIG. 2. Dual immunohistochemical localization of steroid receptors and uNK cells. Steroid receptor protein was expressed in the nuclei of endometrial cells (*brown staining*). uNK cells were visualized by staining for the cell surface marker CD56 (*blue staining*). A, ERα protein was present in the glands and stroma of proliferative phase endometrium. uNK cells showed no ERα immunoexpression. B, uNK cells were again immunonegative for ERα protein in the late secretory phase of the cycle. C, PR was expressed in the glands and stroma of proliferative phase endometrium but was absent from uNK cells. D, PR was again absent from uNK cells in the late secretory phase endometrium. E, ERβcx/β2 was present in the glands, stroma, and endothelium of proliferative phase endometrium but was absent from uNK cells. F, uNK cells were immunonegative for ERβcx/β2 in the late secretory phase. *Insets* are negative controls. Magnification, $\times$40; *scale bar*, 50 μm.

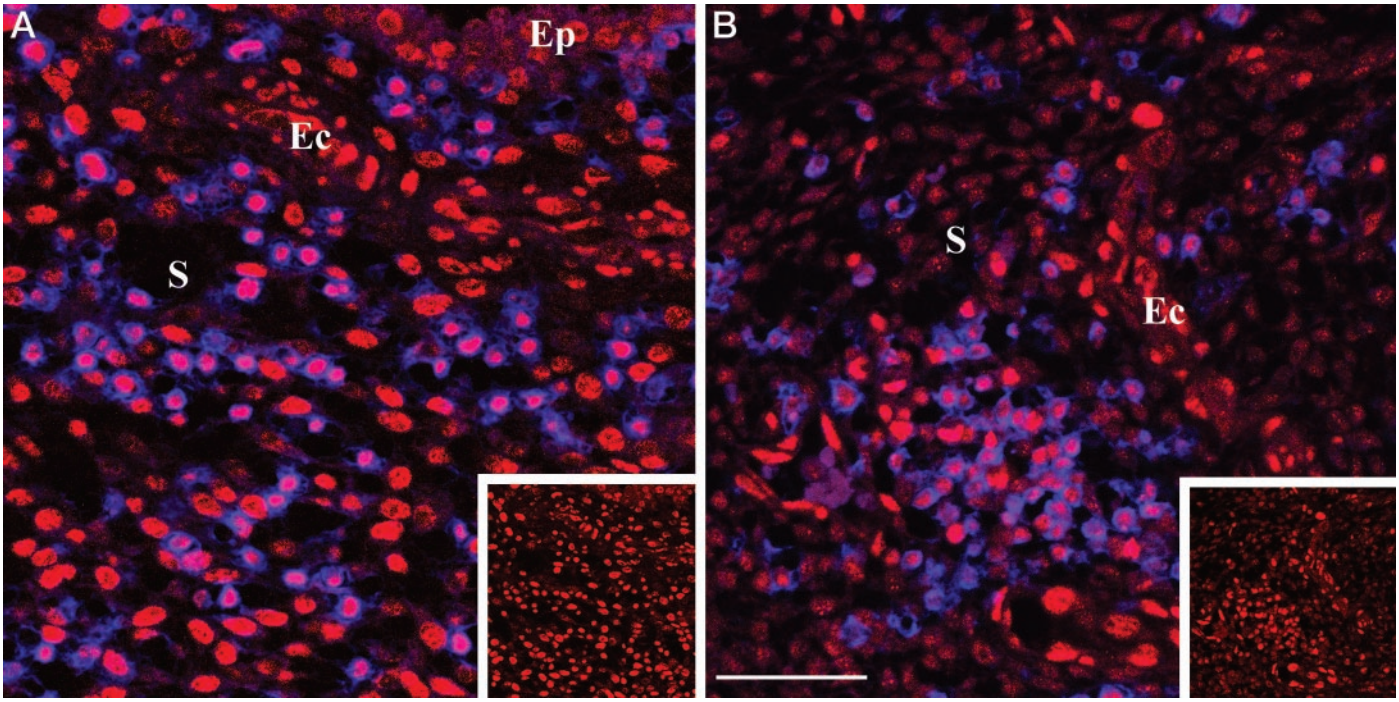

FIG. 3. Dual immunofluorescent localization of steroid receptors and uNK cells. Steroid receptor protein was expressed in the nuclei of endometrial cells (*red fluorescence*). uNK cells were visualized by staining for the cell surface marker CD56 (*blue fluorescence*). A (*inset*), ERβ1 was localized to the glands, stroma, and endothelium of late secretory phase endometrium. A, ERβ1 was colocalized to uNK cells. B (*inset*), GR was localized to the stroma and endothelium of mid secretory phase endometrium. B, GR was colocalized to uNK cells. Magnification, ×40; *scale bar*, 50 μm. Ec, Endothelial cells; Ep, epithelium; S, stroma.

TABLE 5. Summary of steroid receptor protein expression across the cycle, in uterine endometrium from the proliferative and the secretory phase, in decidua, and in uNK cells

Protein expression	Proliferative		Secretory		Decidua		uNK cells
	Glands	Stroma	Glands	Stroma	Glands	Stroma	
PR	+	+	−	+	−	+	−
ERα	+	+	+/−	+/−	−	+/−	−
ERβ1	+	+	+	+	+	+	+
ERβcx/β2	+	+	+/−	+	+	+	−
GR	−	+	−	+	+	+	+

liferative phase followed by a fall in their levels in the progesterone-regulated secretory phase is as expected. uNK cells exhibited very low levels of message for ERα and PR. Dual immunohistochemistry indicated that uNK cells were immunonegative for ERα and PR protein. These results confirm previously published data in which uNK cells have been reported to be negative for ERα and PR protein (10, 11).

Our results confirm that uNK cells do express GR mRNA and protein. GR, but not PR, mRNA and protein have been localized to normal human lymphocytes isolated from peripheral venous blood (37). Results published by Bamberger *et al.* (19) have described GR expression in endometrial leukocytes but did not specify which leukocyte subtypes were involved. The role of glucocorticoids in endometrial immune function has not been extensively studied, although in other systems their immunosuppressive effects have led to their wide application in the treatment of inflammatory states. Glucocorticoids have been shown to exert specific effects on endometrial cells by several groups. Suggested roles include effects on implantation (15), endometrial cellular proliferation (16), apoptosis (17), and endometrial remodeling (18). Glucocorticoids have also been shown to repress the decidual PRL promoter (38) and CRH promoter (39), both of which are markers of decidualization. This and the expression of GR in the endometrial stroma exclusively (19) mean that they may have a role in the process of decidualization. In this context, it is interesting that uNK cells, which strongly express GR, also have proposed roles in decidualization (5) and have recently been shown to express the PRL receptor (14). We have also found GR protein in the glandular epithelium of decidua, in contrast to the exclusively stromal expression pattern found in endometrium.

The effects of glucocorticoids on uNK cells are likely to be regulated not only by GR expression but also by the expression of steroid metabolizing enzymes. The 11β-hydroxysteroid dehydrogenase (HSD) family modulates the action of glucocorticoids by either converting cortisone to cortisol (11βHSD1) or cortisol to cortisone (11βHSD2). Smith *et al.* (40) reported that levels of the glucocorticoid-metabolizing enzyme 11βHSD2 are higher across the menstrual cycle than 11βHSD1. 11βHSD2 was present in the luminal and glandular epithelium, with raised levels in the secretory phase of the cycle. Smith *et al.* suggest that the expression of 11βHSD2 could facilitate trophoblast invasion by removing the glucocorticoid-mediated inhibition of matrix metalloproteinases. It is interesting, therefore, that GR-expressing uNK cells are found aggregated close to the glandular epithelium and also have proposed roles in controlling trophoblast invasion (40). Further investigations into the effects of glucocorticoids

in the endometrium are necessary to elucidate their role in uNK cell physiology.

Consistent with our previous data (30), ERβ1 and ERβcx/β2 mRNAs were detected in endometrial extracts from all stages of the menstrual cycle and showed an increase in levels in the late secretory phase. Significant levels of mRNA were also seen in decidual samples for ERβ1. A sample of purified $CD56^+$ uNK cells also showed high levels of mRNA for both ERβ1 and ERβcx/β2. These results were consistent with our previous findings in which we suggested that the increased levels of mRNA observed in total uterine extracts at the end of the secretory phase may be due to the influx of an undefined ERβ-positive cell subpopulation (30). In the present study, we have also confirmed that the protein for ERβ1 is expressed by some uNK cells using double immunostaining (data not shown) and dual immunofluorescence. Similar results have recently been published by Stygar *et al.* (27), describing the coexpression of ERβ with CD45 leukocyte common antigen and CD68 macrophage-specific antigen in lymphoid cells infiltrating the cervix during pregnancy. However, it appears from the present immunohistochemical studies that uNK cells do not express the protein for the variant isoform of ERβ, known as ERβcx/β2, suggesting that the mRNA for this receptor is not translated efficiently (29). Taken together, these results suggest that estrogens could act directly on uNK cells via ERβ1 receptor homodimers that have been shown previously to activate reporter gene transcription *in vitro* (24, 25, 41).

The response of a cell to estrogens depends not only on the estrogen receptor expression of that cell but also the availability of ligand able to bind to those receptors. ERα and ERβ exhibit different affinities for some ligands, notably genestein, raloxifene, and tamoxifen (42, 43), and novel ligands that act as selective estrogens or antiestrogens for ERα and ERβ have been identified (44). Estradiol is the physiologically important form of estrogen. Both estrone and estradiol can bind to ERα and ERβ, but estrone displays only around 1% of the biological potency of estradiol (45). The conversion of estrone to estradiol and vice versa is dependent on 17βHSDs, a family of enzymes of which there are at least eight members. The important isoforms present in the endometrium are 17βHSD type 1, which preferentially converts estrone to estradiol, and 17βHSD type 2, which primarily converts estradiol to estrone and testosterone to androstenedione. Human endometrial epithelial cells produce both 17βHSD1 and 17βHSD2, but 17βHSD2 is produced in much higher levels in the mid-late secretory phase. Its production has been demonstrated to be progesterone dependent (46). Therefore, at the time of uNK cell infiltration, the dominant estrogen at least in the surrounding tissue would seem to be estrone formed by oxidation from estradiol.

Estrogen is involved in the regulation of fundamental processes, including proliferation and vascularization. Importantly, uNK cells are present in large numbers in the endometrium at the time when implantation, placentation, and decidualization occur. Many studies have focused on the role of uNK cells in trophoblast invasion (9). Precise control of this process is vital to successful pregnancy, and under- or overinvasion of trophoblast can lead to a number of pregnancy-related health problems (preeclampsia, intrauterine growth retardation). It has been proposed that uNK cells may exert control over trophoblast invasion after interactions between NK cell receptors belonging to the CD94/NKG2, killer-Ig receptor, and Ig-like transcript families binding to human leukocyte antigen class 1 molecules expressed by extravillous trophoblast (9). Furthermore, the cyclic traffic of uNK cells in the nonpregnant uterus and their apparent death premenstrually implies a role in endometrial differentiation and menstruation (3).

Endometrial differentiation, menstruation, and placentation all involve the remodeling of endometrial vasculature. The angiogenic factor vascular endothelial growth factor (VEGF)-A plays an important role in new blood vessel formation inducing endothelial cell proliferation, migration, and differentiation in the endometrium and also affects vascular permeability. VEGF-A has been shown to be regulated by estradiol in isolated human endometrial cells, causing increased mRNA and protein levels (47). Interestingly, VEGF-A has also been localized to individual cells, thought to be leukocytes, scattered in the endometrial stroma. These cells have been identified as neutrophils through dual immunohistochemical staining by Mueller *et al.* (48). VEGF-A has also been reported in uterine macrophages in the secretory phase of the cycle (49). VEGF-C and other angiogenic factors, placenta growth factor, and angiopoietin 2 mRNA are expressed in uNK cells (50). VEGF-C was originally characterized as a growth factor for lymphatic vessels, but it can also stimulate endothelial cell proliferation and migration (51). This pattern of growth factor expression and the close spatial association of uNK with spiral arterioles is suggestive of a role for these cells in endometrial angiogenesis. In this context, it is interesting that both ERα and ERβ have been shown to induce luciferase expression when cotransfected into Ishikawa cells with a human VEGF-A promoter-luciferase reporter construct (52). ERα induced a 3.2-fold induction in reporter activity, whereas ERβ increased levels by 2.3 times. It is possible, therefore, that ERβ could induce the production of VEGF-A from endometrial cells. Little is known about steroid control of VEGF-C in the endometrium. Ruohola *et al.* (53) have demonstrated the regulation of VEGF-C by estrogen in human breast carcinoma cells. VEGF-C exhibited a decrease in mRNA after addition of estrogen. Further investigation into the effects of estrogen and ER subtype on uNK cell expression of angiogenic factors is required.

In summary, this study has demonstrated that uNK cells express both mRNA and protein for ERβ1 and GR. This expands previous published data (19) showing GR localization in endometrial lymphoid cells. In addition, we have demonstrated that ERβ1 is expressed by uNK cells. We therefore predict that any estrogen-regulated gene transcription in these cells will be mediated through ERβ1 homodimers.

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