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Recruitment of Uterine NK Cells: Induction of CXC Chemokine Ligands 10 and 11 in Human Endometrium by Estradiol and Progesterone[1]

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Uterine NK (uNK) cells express a unique set of markers compared with blood NK cells. However, recent studies suggest that uNK cells may be derived from the recruitment of blood NK cells into the endometrium. In this study, we used an in vitro organ culture system to demonstrate that estradiol induces expression of chemokines CXCL10 and/or CXCL11 within human endometrium in 85% of patient samples tested. The average increase in gene expression after 10^{-9} M estradiol treatment was 8.5-fold for CXCL10 and 7.7-fold for CXCL11 compared with medium alone. We observed that a specific estrogen receptor antagonist (ICI182780) was able to prevent chemokine gene induction, indicating that the effect of estradiol was receptor mediated. Moreover, our study showed that progesterone induced CXCL10 and CXCL11 expression in 83% of endometrial samples tested. We have also found that uNK cells and blood NK cells express the receptor for CXCL10 and CXCL11, CXCR3, with the highest expression found on uNK cells and CD56^{bright} blood NK cells. These data indicate that sex hormones induce specific chemokines in nonpregnant human endometrium that can activate NK cell migration, and suggest that this mechanism may account for the increased NK cell numbers in endometrium during the menstrual cycle. *The Journal of Immunology,* **2004, 173: 6760–6766.**

Natural killer cells play an important part in the innate immune system and were first defined functionally by their ability to kill certain tumors and virally infected cells without a requirement for MHC restriction or previous immunization (1). NK cells produce immunoregulatory cytokines that contribute to the early host defense against several types of viruses, bacteria, and parasites (2–4). In humans, ~10% of PBLs are NK cells (5). Human NK cells can be defined phenotypically by the expression of CD56 and the absence of CD3, and NK cells fall into two distinct subsets according to their surface density of CD56. The majority of NK cells in human blood have low CD56 expression (CD56^{dim}), express high levels of FcγRIII (CD16), and express CD57 (6). A small subset of blood NK cells (~10%) expresses high levels of CD56 (CD56^{bright}) and low or no CD16, and lack CD57 expression. Uterine NK (uNK)[3] cells account for a large percentage of leukocytes in the human endometrium (EM) and have similar expression of CD56, CD16, and CD57 as the CD56^{bright} blood NK cell subset (6, 7).

The human uterine EM is a complex mucosal tissue with a unique immune cell component that is regulated by sex hormones throughout the menstrual cycle (8, 9). The EM must be ready to respond to potential pathogen challenges, yet be able to control immune cell responses to allow the development of a semiallogeneic fetus. In the nonpregnant state, there is a tightly controlled influx, spatial compartmentalization, and regulation of immune cells (10, 11). In EM, macrophages, NK cells, T cells, B cells, and neutrophils interact with a variety of stromal and epithelial cells. The interplay between these different cell types and their roles in defense against pathogen invasion in this specialized tissue are poorly understood. Unlike in the murine uterus, uNK cells in the human uterus are found widely dispersed throughout the EM, and their numbers increase as the menstrual cycle progresses (12–14).

NK cells express receptors for specific chemokines and can be induced to migrate in response to several chemokines (15). Blood NK cells express CCR5, CCR7, CXCR3, and CXCR4, among other chemokine receptors. Ligands for these receptors (e.g., CXCL10 and CXCL11) have been shown to induce migration of blood NK cells in vitro (15–17). NK cells are present in large numbers in blood, spleen, lung, liver, and bone marrow, although NK cells can be found at other tissue sites, where the recruitment of NK cells to specific tissues is mediated by inflammation or other unknown factors. Studies in a murine system suggest that NK cells found in the decidua do not originate from uNK cells, but most likely arise from infiltration by blood or bone marrow NK cells (18). Recent data indicate that trophoblasts and placenta produce chemokines that may recruit blood NK cells into the decidua during pregnancy (17, 19).

The observation that NK cells appear in the EM in a regular manner during the menstrual cycle suggests that the recruitment and/or expansion of uNK cells may be regulated by sex hormones. NK cells do not express receptors for estradiol and progesterone, so the action of sex hormones on NK cell function or recruitment is most likely mediated via hormone action on other cells, such as fibroblasts or epithelial cells (20). If specific chemokines are involved in the recruitment of NK cells into human EM, then those chemokines should be expressed within the EM and may be regulated by sex hormones. In this study, we examined the regulation of chemokines in human EM by estradiol and progesterone. We present data that both hormones induce CXCL10 and CXCL11 expression in human EM and that uNK cells express high levels of receptors for these chemokines.

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[3] Abbreviations used in this paper: uNK, uterine NK; Ct, cycle time; EM, endometrium; SDF, stromal cell-derived factor.

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Materials and Methods

Isolation of human endometrial cells

Endometrial tissue specimens were obtained from women undergoing hysterectomy for various gynecological disorders. We used samples from 38 patients with an average age of 44 ± 11 years. Initial patient diagnosis included fibroids, pelvic pain, menorrhagia, and prolapse. The tissue samples that we used were distal to any pathological changes. We found no differences in experimental outcomes between those patients that had been given hormonal therapy presurgery and those that had not been given hormonal therapy. Tissue was directly snap frozen for examination of proteins or RNA in fresh tissue, or tissue was cut into small pieces for organ culture. For those experiments in which we used a single cell suspension, we used a cell dispersion method that used an enzyme mixture composed of pancreatin, hyaluronidase, and collagenase, followed by a 100-μm mesh screen to facilitate cell dispersion (21). Any RBC present were eliminated from the endometrial cells by treatment with lysis buffer (NH_4Cl/Tris-HCl) for 5–10 min at room temperature. Blood cell contamination of these endometrial tissue cells was <2% (21). Isolated cells were cultured or used for experimental treatments directly. All human studies were done with approval of the Dartmouth Institutional Review Board.

Treatment of endometrial samples with hormones

Endometrial tissue samples were divided into small sections (1 $mm^2 \times 100$ μm) and plated into 96-well flat-bottom plates containing various combinations of estradiol, progesterone, and/or ICI182780 (a specific estrogen receptor inhibitor) in DMEM-F12 complete medium (DMEM-F12 supplemented with 15% charcoal-dextran-stripped FCS (HyClone, Logan, UT), 10 mM HEPES, and penicillin/streptomycin). This culture medium was phenol red free. As previously reported, the tissue pieces in these cultures are viable for several days (22). Plates were incubated at 37°C for 48 h in 5% CO_2. At this point, medium was replaced with fresh medium and incubated for another 18 h. The tissue sections were removed, snap frozen, and stored at −80°C until processed for RNA or protein.

Isolation of RNA and production of cDNA

Total RNA was prepared from whole endometrial tissue using TRIzol reagent, as recommended by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA). DNA was removed from the RNA preparations using DNA-free kit from Ambion (Austin, TX), according to the manufacturer's protocol. After treatment, supernatants were transferred to new tubes and frozen at −20°C. The RNA concentration was determined by A^{260}. Synthesis of first strand cDNA was conducted using the First Strand cDNA Synthesis Kit from Fermentas (Hanover, MD), following the manufacturer's instructions. After cDNA synthesis, samples were diluted to obtain a cDNA concentration of 5 ng/ml, and samples were stored at −20°C.

Quantitative real-time PCR

Duplicate samples containing 25 ng of cDNA, Taqman mastermix (Applied Biosystems, Foster City, CA), real-time PCR primers, and specific minor group finder probes (Applied Biosystems) were mixed and analyzed on an ABI7700 thermal cycler (Applied Biosystems). To determine the amount of gene product present in the sample, cycle time (Ct) was determined. The average Ct value was calculated from duplicate wells for each sample with each primer set. Most duplicate samples varied by <0.5 Ct. No DNA template control samples were evaluated for each primer set. The relative gene expression for each individual cDNA sample was determined by calculating ΔCt values (ΔCt) by subtraction of the Ct value for GAPDH primers from the Ct value for each chemokine primer. This method removes potential errors due to loading or other pipetting errors for each sample. We have found no alteration in GAPDH expression due to hormone treatments in these samples (data not shown). The relative fold expression of each gene was determined compared with medium only treated samples in each experiment.

Isolation of endometrial protein

Endometrial tissue slices were placed in ice-cold PBS, and the tissue was homogenized using a PowerGen 125 homogenizer for 60 s. Large debris was removed by centrifugation at 10,000 × *g* for 5 min at 4°C. Supernatants were isolated and stored at −20°C until protein analysis.

ELISA

Human CXCL10 and CXCL11 were quantified by using duoset ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer's protocol. Total protein was determined using a Bradford protein assay (Bio-Rad, Hercules, CA). Data are presented as pg chemokine/mg total protein.

Isolation of PBMCs

PBMCs were isolated from healthy donors and from patients undergoing hysterectomy who had consented to donate blood. Cells were separated on Nycoprep or Lymphoprep gradients, according to protocols provided by the manufacturer (Axis Shield, Oslo, Norway).

Generation of uNK cell clones

Enzymatically isolated endometrial cells were cultured in 500 U of IL-2/ml for 2 or 3 days to allow for recovery of CD56 surface expression in complete medium (RPMI 1640 supplemented with 2-ME (50 μM), penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), and 5% human serum). Cells were harvested and stained for CD45, CD56, and CD3 surface Ags. uNK cells were then sorted using a FACStar cell sorter (BD Biosciences, San Jose, CA) by gating on the $CD45^+CD56^+CD3^-$ cells. Sorted uNK cells were cloned using standard NK cell cloning procedures (23). Briefly, sorted uNK cells were plated at 1–3 cells/well in U-bottom 96-well plates together with irradiated (100 Gy) feeder cells (10^5 allogeneic PBMCs together with either 10^4 RPMI 8866 cells or 10^4 DAUDI cells) supplemented with 1 μg/ml PHA. After 10 days, the wells were examined for growth of clones. Cells from positive wells were expanded further and maintained in complete medium containing 500 U/ml IL-2. These uNK cell clones have a similar phenotype and function as uNK cells (24).

Abs and reagents

Abs were obtained from Caltag Laboratories (Burlingame, CA): FITC-conjugated anti-CD45 (HI30), biotin-conjugated anti-CD3, allophycocyanin-conjugated anti-CD3, and fluorochrome-conjugated IgG isotype control Abs. R-PE-conjugated anti-CD56 (B159) and streptavidin PerCP were purchased from BD Biosciences. Streptavidin RED670 was obtained from Invitrogen Life Technologies. Abs against CCR5, CCR7, CXCR1, CXCR2, and CXCR3 were purchased from R&D Systems. Anti-CXCR4 mAb was obtained from eBioscience (San Diego, CA). Human FcR blocking reagent (Cohn's fraction) was obtained from Sigma-Aldrich (St. Louis, MO). FITC-conjugated goat anti-mouse $F(ab')_2$ sera were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Estradiol and progesterone were obtained from Calbiochem (San Diego, CA), and estrogen receptor antagonist ICI182780 was purchased from Tocris Cookson (Bristol, U.K.).

Flow cytometry

A FACSCalibur (BD Biosciences) was used for flow cytometric analysis of cell surface staining of all samples. Before mAb stainings, an FcR blocking reagent (Cohn's fraction) was added to reduce nonspecific staining. Endometrial cells were stained for CD3, CD45, and CD56 in addition to CXCR1, CXCR2, CXCR3, CXCR4, CCR5, CCR7, or IgG isotype controls. Single cell suspensions of endometrial cells and uNK cell clones were gated on $CD45^+CD56^+CD3^-$ cells and analyzed for surface expression of other cell surface molecules.

Statistics

Statistical comparisons were done using a Student's *t* test. Value of $p < 0.05$ was considered statistically significant.

Results

Estradiol induces CXCL10 and CXCL11 expression in human EM

The observation that NK cells appear throughout the EM and increase during the menstrual cycle suggests that the recruitment and/or expansion of uNK cells may be regulated by sex hormones. If specific chemokines are responsible for recruitment of NK cells into the human EM, then those chemokines should be expressed within the EM during the menstrual cycle and may be under control of sex hormones. We have tested this hypothesis and evaluated the ability of estradiol to regulate the expression of chemokines in endometrial tissue (Fig. 1). Human EM was processed into thin tissue slices before incubation with increasing concentrations of estradiol. Human EM is a heterogeneous tissue, so to ensure that each treatment group was representative of the entire sample, we used five to seven randomly selected tissue pieces for each treatment point. In each case, tissue pieces were cut to represent the entire depth of the tissue (luminal to myometrial). We have analyzed samples from 13 different patients and found 77% (10 of 13)

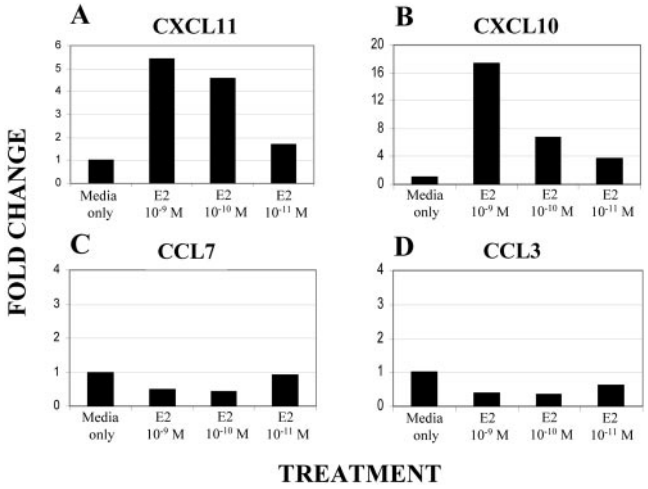

FIGURE 1. Induction of chemokines in EM after treatment with estradiol. Relative mRNA expression levels of CXCL11 (*A*), CXCL10 (*B*), CCL7 (*C*), and CCL3 (*D*) in endometrial tissue. Tissue sections were incubated with different doses of estradiol (E2), as indicated. Data are presented as relative expression compared with medium only (=1) for each chemokine. The data with estradiol dose response are representative of three experiments, and estrogen (10^{-9} M) stimulation has been tested on 13 samples.

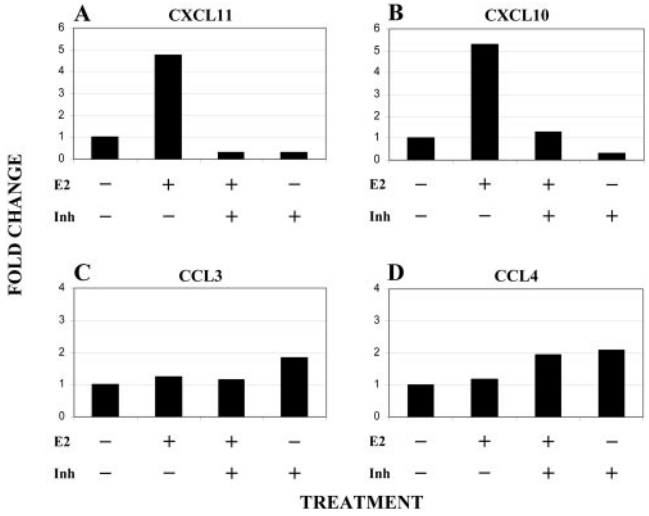

FIGURE 2. Chemokine induction is dependent upon estradiol receptor activity. Relative mRNA expression levels of CXCL11 (*A*), CXCL10 (*B*), CCL3 (*C*), and CCL4 (*D*) in endometrial tissue. Tissue sections were incubated with various combinations of estradiol (E2; 10^{-9} M) and/or ICI182780 (Inh (estrogen receptor antagonist); 10^{-7} M). Data are presented as relative expression compared with medium only (=1) for each chemokine. Data are representative of five experiments.

showed increased gene expression of CXCL10 and 77% showed increased gene expression of CXCL11 (Table I). Increased gene expression was defined as a greater than 2-fold increase compared with medium alone as assessed by real-time quantitative PCR. Most of the samples (85%) induced at least one of these two chemokines in response to estradiol. Of the tissues tested, only two failed to respond to estradiol with increased CXCL10 or CXCL11 expression. The maximal induction of these chemokine genes occurred at a concentration of 10^{-9} M estradiol. In these organ cultures, we observed a consistent up-regulation of both CXCL10 and CXCL11 RNA expression, with an average increase from the tested samples of 8.5- and 7.7-fold, respectively, compared with medium only controls. As part of these studies, we have observed an induction of CCL3 with estradiol (10^{-9} M) in 55% of samples (five of nine samples) with a 3.2-fold average increase compared with medium only treatment (Table I). The effect of estradiol was chemokine selective in that estradiol had no effect on expression of CCL4, CCL5, XCL1, CCL21, or CCL7 relative to medium controls.

To test whether up-regulation of CXCL10 and CXCL11 was estrogen receptor mediated, we examined gene induction in the presence and absence of a specific estrogen receptor antagonist, ICI182780 (Fig. 2). When tissues were cultured with estradiol (10^{-9} M) in the presence of an excess of ICI182780 (10^{-7} M), the stimulatory effect of estradiol on CXCL10 or CXCL11 expression was absent. In five of the six experiments, the antagonist completely inhibited estradiol induction of CXCL10 or CXCL11 expression (Table I). Increases in mRNA levels should lead to changes in the protein level, so we determined the total CXCL10 production after treatment of EM samples with estradiol (Fig. 3). After treatment with 10^{-9} M estradiol, 50% of patient samples (three of six) showed an increase in CXCL10 protein in human EM. Taken together, these organ culture data indicate that estradiol up-regulates the expression of CXCL10 and CXCL11 within human EM.

Progesterone induces CXCL10 and CXCL11 expression in human EM

Recognizing that progesterone levels in blood increase during the second half of the menstrual cycle (secretory phase), we tested whether progesterone can induce expression of chemokines in human EM (Fig. 4). Using our endometrial organ culture system, we observed an increase in CXCL10 and CXCL11 expression in response to progesterone treatment. Maximum induction of gene expression occurred at 10^{-9} to 10^{-8} M of progesterone in four dose response experiments. Higher concentrations of progesterone

Table I. *Hormonal induction of chemokine gene expression in human EM*

Mean Age	Experimental Stimulus[a]	Chemokine									
		CXCL10 and CXCL11[b]	CXCL10 or CXCL11[c]	CXCL10	CXCL11	CCL3	CCL4	CCL5	XCL1	CCL21	CCL7
42.2	Estradiol	9/13[d]	11/13	10/13	10/13	5/9	1/8	1/7	1/4	2/6	0/2
48.5	Progesterone	5/6		6/6	5/6	1/5	1/5	1/5	0/1	0/2	ND
48.6	Estradiol + ICI182780[e]		5/6	4/5	4/5						

[a] Endometrial tissue pieces were stimulated in medium containing estradiol (10^{-9} M) progesterone (10^{-8} M or 10^{-9} M), or ICI182780 (estradiol antagonist), as indicated.
[b] Fraction of samples that had increased expression of both CXCL10 and CXCL11.
[c] Fraction of samples that had increased expression of either CXCL10 or CXCL11.
[d] Number of samples with an increase in chemokine expression of the total number of samples tested. Induction indicates at least a 2-fold increase in gene expression compared with medium only
[e] Fraction of samples that did not induce expression of either CXCL10 or CXCL11 by estradiol in the presence of ICI182780.

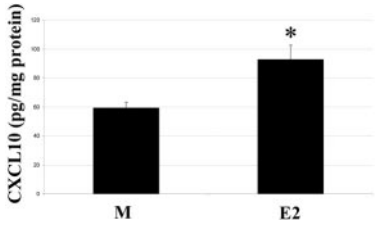

FIGURE 3. Estradiol and progesterone induce CXCL10 protein in EM. Concentrations of CXCL10 (pg/mg total protein) are shown after treatment with medium (no hormones) or 10^{-9} M estradiol. Data are representative of those patient samples in which an increase in CXCL10 was observed (three of six samples tested). *, Indicates $p < 0.05$ compared with medium treatment alone.

(10^{-6} M) had no effect on chemokine expression relative to that observed in controls. This may be due to nonspecific effects of progesterone, as has been reported by others (25). Overall, of the six patient tissues analyzed, all responded to progesterone with an increased expression of CXCL10 that averaged 8.6-fold greater than controls (Table I). Moreover, CXCL11 expression was increased in five of six samples (83%) with an average of 12.2-fold increase. In contrast, no consistent up-regulation of CCL3, CCL4, CCL5, or CCL21 expression was detected when endometrial samples were incubated with progesterone compared with medium only treatment. Taken together, these data indicate that progesterone as well as estradiol can induce CXCL10 and CXCL11 expression in human EM.

Chemokines are expressed in human EM

To determine whether chemokine expression varies with stage of the menstrual cycle, we analyzed the mRNA expression of CXCL11, CXCL10, and CCL4 in fresh human EM samples. As shown in Fig. 5, we found that all chemokines were present in freshly isolated EM. We found no significant differences between stages in the menstrual cycle, although there was a trend for higher CXCL10 and CXCL11 mRNA expression in samples from the secretory phase compared with the proliferative phase. We also examined CXCL10 and CXCL11 protein expression in fresh endometrial samples (Fig. 6). There were higher levels in the secretory phase compared with the proliferative phase for CXCL10 (125 pg/mg secretory and 37 pg/mg proliferative) and CXCL11 (38 pg/mg secretory and 25 pg/mg proliferative). These data indicate that both chemokines are found within the EM and that there is higher chemokine expression in the secretory phase. These data are in agreement with recent data that show increased levels of CXCL9 and CXCL10 in secretory EM (26).

uNK cells express chemokine receptors that mediate chemotaxis to CXCL10

To identify receptors that might account for the recruitment of NK cells to the EM, we analyzed the expression of different chemokine receptors on uNK cells (Fig. 7). We examined a series of uNK cell clones derived from different patients and found that all expressed high levels of CXCR3, no CXCR1 or CXCR2, and low levels of CXCR4. Most uNK cell clones expressed low levels of CCR7, whereas some clones expressed CCR5. Primary uNK cells expressed high levels of CXCR3 as well (Fig. 8*A*). These data indicated that uNK cells express chemokine receptors for CXCL10 and CXCL11.

$CD56^{bright}$ NK cells express higher levels of CXCR3 than $CD56^{dim}$ NK cells in human blood

One aspect of selective NK cell subset recruitment into EM may be due to differences in expression of chemokine receptors. We have examined blood NK cells for differences in chemokine receptors and found that $CD56^{bright}$ NK cells express higher levels of CXCR3 than do $CD56^{dim}$ NK cells (Fig. 8, *B* and *C*). This difference in CXCR3 expression was found on both fresh and activated blood NK cells. Thus, peripheral blood NK cells express receptors for chemokines induced by sex hormones in human EM.

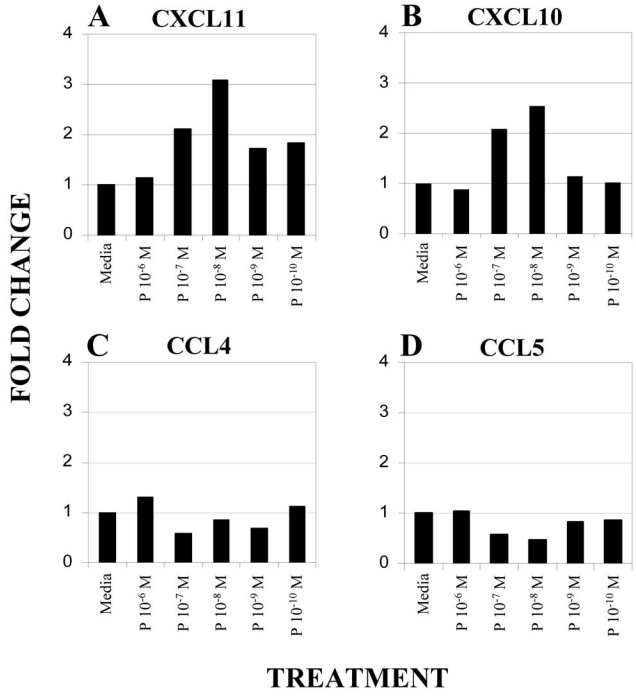

FIGURE 4. Induction of chemokines in EM after treatment with progesterone. Relative mRNA expression levels of CXCL11 (*A*), CXCL10 (*B*), CCL4 (*C*), and CCL5 (*D*) in endometrial tissue. Tissue sections were incubated with various amounts of progesterone (P), as indicated. Data are presented as relative expression compared with medium only (=1) for each chemokine. Data are representative of four different dose response experiments, and progesterone stimulation alone was done in six different samples.

Discussion

The biology of NK cells within the EM is poorly understood. uNK cells express a unique set of markers compared with blood NK cells (24). Increases in the number of NK cells during the menstrual cycle suggest that there may be specific recruitment of NK cells to the EM (27, 28). In this study, we demonstrate that estradiol or progesterone induces the expression of CXCL10 and/or CXCL11 in 85% of samples tested. This appears to be selective in that other chemokines (CCL4, CCL5, CCL7, CCL21, and XCL1) able to induce blood NK cell migration in vitro were not affected by sex hormones. We also found that uNK cells and blood NK cells expressed CXCR3, the receptor for these chemokines.

We have used an organ culture system to investigate the hormone-induced changes in gene expression in human EM. This approach allowed us to study the regulation within this complex tissue that might otherwise be absent or altered among single isolated cells. Because of patient-to-patient variation in the baseline expression of these chemokines, individual organ cultures allowed us to observe changes in gene expression after hormone treatment relative to each patient's own baseline expression.

What is the physiological significance of our findings that sex hormones induce CXCL10 and CXCL11 in human EM? NK cells appear during the proliferative phase and increase in cell numbers

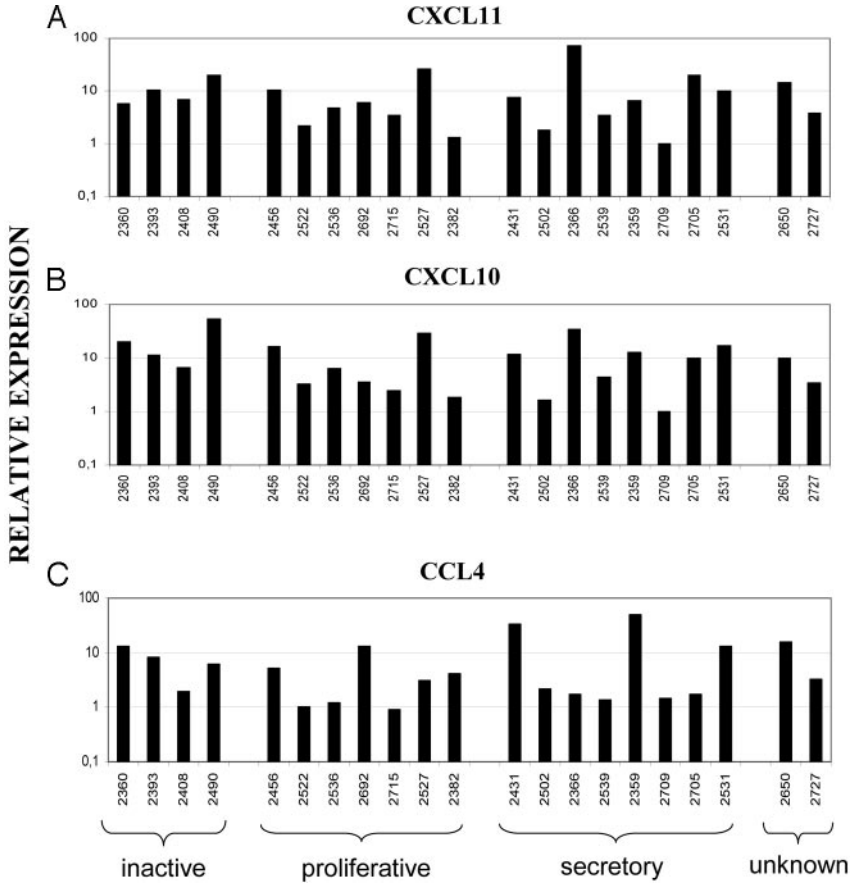

FIGURE 5. Chemokines are expressed in fresh human EM. Relative mRNA expression levels of CXCL11 (*A*), CXCL10 (*B*), and CCL4 (*C*) were determined in fresh endometrial tissue. Data from patients ($n = 21$) are grouped according to menstrual stage. Data are presented as relative expression (see *Materials and Methods*), and all samples are normalized to GAPDH for each sample.

during the secretory phase of the menstrual cycle (12–14). A portion of this expansion is most likely due to proliferation of NK cells, because some are found in late secretory phase that express the Ki-67 Ag indicative of cell proliferation (29). Animal studies indicate that uNK cells migrate from peripheral sites to the EM and do not originate within the uterus (18). This study suggests that in humans, NK cells are recruited into the EM and that infiltration may continue as the menstrual cycle progresses. A large number of chemokines can induce migration of NK cells in vitro (15–17). We have examined several of these and report in this study that CXCL10 and CXCL11 are induced after estradiol or progesterone treatment, with maximum gene induction at $\sim 10^{-9}$ M, well within the physiological range of these hormones. During a normal menstrual cycle, estradiol rises above 0.75 nM and progesterone levels are between 6 and 64 nM in plasma (30). CXCL10 and CXCL11 bind to CXCR3, which is found on both uNK cells and blood NK cells. These studies support the hypothesis that uNK cells are derived from the $CD56^{bright}$ subset of blood NK cells. We have observed that $CD56^{bright}$ NK cells express higher levels of CXCR3 than $CD56^{dim}$ NK cells. Moreover, it has been shown that a higher percentage of $CD56^{bright}$ NK cells migrate compared with $CD56^{dim}$ NK cells in response to CXCL10 and CXCL11 in vitro (15). Recent data have shown a positive correlation between CXCL9 and CXCL10 protein levels and numbers of NK cells in human EM (26). Our data support a model whereby $CD56^{bright}$ NK cells are recruited into the EM, in part due to the selective induction of CXCL10 and/or CXCL11 by sex hormones from EM stromal and epithelial cells.

Migration of cells into tissues is a complex process that involves many cell surface molecules, including selectins, adhesion molecules, and chemokine receptors. It is possible that sex hormones regulate specific adhesion molecules and selectins in the EM during the menstrual cycle. Chemokine biology appears to be redundant with several chemokines inducing apparently similar responses (31). Selective localization of NK cells may involve many chemokines, such that one chemokine may recruit cells into the tissue, while others help NK cells localize to specific tissue regions. The selective recruitment of NK cell subsets into the EM will most likely involve hormonal regulation of selectins, adhesion molecules, and chemokines. Our study suggests that at least two chemokines are involved in the recruitment of NK cells into the female reproductive tract. Moreover, our data suggest that this response is specific for CXCL10 and CXCL11 in that other chemokines were not affected by hormone treatment.

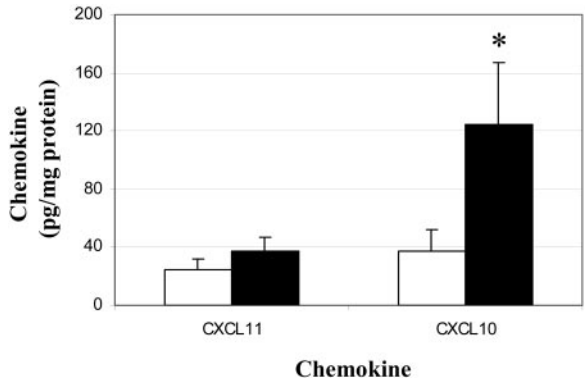

FIGURE 6. Expression of CXCL10 and CXCL11 proteins in fresh human EM. Concentrations of CXCL11 and CXCL10 for individual fresh EM samples are shown as pg/mg protein. The samples ($n = 19$) were classified according to menstrual stage: proliferative phase (□) and secretory phase (■). *, Indicates $p < 0.05$ between proliferative phase and secretory phase samples.

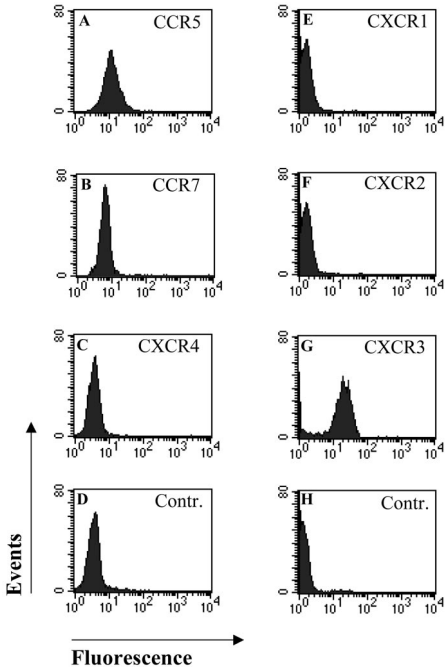

FIGURE 7. uNK cell clones express CXCR3. Clones of uNK cells were analyzed for surface expression of CCR5, CCR7, CXCR1, CXCR2, CXCR3, and CXCR4 by flow cytometry. Cells were labeled using an indirect (*A–D*) or direct (*E–H*) staining protocol. *D* and *H*, Show isotype control stainings. All uNK cell clones were $CD56^{+}CD3^{-}$. Results from clone 10.L are shown, and they are representative of data from 30 clones derived from four patients.

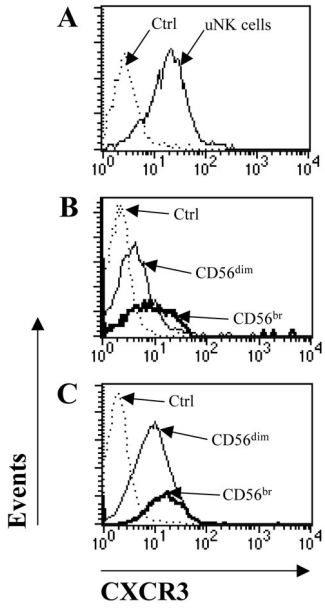

FIGURE 8. Uterine and blood $CD56^{bright}$ NK cells express high levels of CXCR3. Expression of CXCR3 on uNK cells (*A*) was determined after 3 days in culture with rIL-2. CXCR3 expression was determined on fresh blood NK cells (*B*) or after 3 days in culture with rIL-2 (*C*). Data representative of eight experiments (*A*, $n = 5$; *B* and *C*, $n = 3$).

A recent study suggests that trophoblastic cells produce stromal cell-derived factor (SDF)1-α to recruit NK cells to localize near the implantation site (17). We have not examined SDF1-α in our system, but the expression of its receptor, CXCR4, is low or absent on uNK cells in EM from nonpregnant women. The authors present data that SDF1-α is able to induce selective migration of $CD56^{bright}$ NK cells from peripheral blood, and therefore suggest that this chemokine is most likely important for NK cell recruitment into decidua during pregnancy. They also suggest that CXCR3 ligands may be important for maintaining cells within decidua. Our data suggest that a unique mechanism exists in human EM for NK cell recruitment. Whether estradiol and progesterone regulate NK cell recruitment during pregnancy remains to be determined. Because NK cells are present in the uterus at the time of implantation, it is possible that CXCL10, CXCL11, and CXCL12 (SDF1-α) may be involved in NK cell recruitment or localization. Once decidualization begins and trophoblasts invade the EM, CXCL12 or CCL3 (MIP1-α) produced by trophoblasts may be responsible for recruitment of NK cells to sites adjacent to the trophoblast (17, 19, 32).

CCL4 has also been reported to increase during the latter part (secretory phase) of the menstrual cycle (28, 33). The expression of CCL4 correlated with an increase in the number of NK cells, leading the authors to suggest that CCL4 may be important for recruitment of NK cells into the EM. Another interpretation of these findings is that cells that produce CCL4, including NK cells, increase in numbers during the menstrual cycle. We did not detect an increase in CCL4 expression after sex hormone treatment, which may suggest that CCL4 is not regulated by sex hormones. However, we cannot rule out that other experimental conditions or time points might show a hormonal regulation of CCL4. Even though CCL4 may not be regulated by sex hormones, CCL4 may be important to direct NK cells to particular sites within the EM.

Whether the effects of estradiol and progesterone on CXCL10 and CXCL11 expression are direct or indirect remains to be determined. Several cell types, including fibroblasts and epithelial cells, are known to produce CXCL10 and CXCL11 (34, 35). It is possible that several different cell types in the uterus may be responsible for the production of these chemokines. A recent study used immunohistochemistry to demonstrate that CXCL9 and CXCL10 are found in human EM in both epithelial and stromal cell layers (26). They also showed that these chemokines were found at higher levels as the menstrual cycle progressed. We do not believe that sex hormones act directly on NK cells, but rather act on stromal and epithelial cells to produce chemokines for NK cell recruitment.

Factors other than sex hormones can regulate these chemokines. For example, IFN-γ is known to induce the expression of CXCL9, CXCL10, and CXCL11. It is possible that sex hormone-induced IFN-γ may be responsible for the induction of these chemokines. All three of these chemokines are ligands for CXCR3, a receptor that is expressed on NK cells and activated T cells, and they can be induced by factors such as LPS, IL-1β, and TNF-α (35–38). Our findings that estradiol and progesterone regulate CXCL10 and CXCL11 expression in the EM demonstrate another level of control of NK cell function in the female reproductive tract. In contrast, a study of human keratinocytes suggests that 17-β estradiol inhibits CXCL10 production, while 17-α estradiol and progesterone have no effect (39). These data together with ours suggest that the regulation of CXCR3 ligands by sex hormones is complex, may be due to indirect effects, and is organ specific.

In summary, we have presented data indicating that sex hormones can regulate the expression of two chemokines, CXCL10 and CXCL11, in human EM. We have also shown that both uNK cells and blood NK cells express high levels of specific receptors for these chemokines. Our data suggest that sex hormone regulation of CXCL10 and CXCL11 may mediate NK cell recruitment into the EM during the menstrual cycle.

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