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Coordinate Regulation of Lymphocyte-Endothelial Interactions by Pregnancy-Associated Hormones

Srirak Chantakru, Wan-Chao Wang, Marianne van den Heuvel, Siamak Bashar, Amanda Simpson, Qing Chen, B. Anne Croy, and Sharon S. Evans

Precursors of uterine NK cells home to the uterus during early pregnancy from multiple lymphohemopoietic sources. In mouse uterine tissue, pregnancy markedly up-regulates both L-selectin- and α4 integrin-dependent adhesion pathways for circulating human CD56bright cells, the phenotype of human uterine NK cells. Based on roles for these adhesion molecules in lymphocyte homing, we examined effects of pregnancy or the steroid hormones 17β-estradiol or progesterone on lymphocyte-endothelial interactions in secondary lymphoid tissues and in uterus. From preimplantation gestation day 3, specialized high endothelial venules in peripheral lymph nodes and Peyer’s patches supported elevated L-selectin and α4β7 integrin-dependent lymphocyte adhesion under shear throughout pregnancy, as compared with high endothelial venules of virgin or postpartum donors. Squamous endothelium from nonlymphoid tissue was not affected. Pregnancy-equivalent endothelial responses were observed in lymph nodes and Peyer’s patches from ovariectomized mice receiving 17β-estradiol and/or progesterone replacement therapy. Adhesion of human CD56bright cells to uteri from pregnant or hormone-treated ovariectomized mice was enhanced through L-selectin and α4 integrin-dependent mechanisms and involved multiple vascular adhesion molecules including mucosal addressin cell adhesion molecule-1, VCAM-1, and peripheral lymph node addressin. Analysis of Tie2-green fluorescence protein transgenic mice demonstrated that CD56bright cells adhered primarily to vascular endothelium within the decidua basalis. Microdomain localization of adhesion involving large clusters of lymphocytes was induced on uteri from natural matings, but not pseudopregnancy. Steroid hormones also had independent effects on L-selectin function in splenic lymphocytes that mimicked physiological stimulation induced by pregnancy or fever-range temperatures. These results provide the first evidence for coordinated, organ-specific, steroid hormone-induced changes in lymphocyte homing mechanisms that could contribute to local and systemic immune responses during pregnancy. The Journal of Immunology, 2003, 171: 4011–4019.

Abbreviations used in this paper: E2, 17β-estradiol; P4, progesterone; uNK, uterine NK; gd, gestation day; MLAp, mesometrial lymphoid aggregates of pregnancy; DB, decidua basalis; LN, lymph node; PLN, peripheral LN; PP, Peyer’s patch; PNA, PLN addressin; MAdCAM-1, mucosal addressin cell adhesion molecule-1; HEV, high endothelial venule; Ovx, ovariectomized; FN, fibronectin; FN40, 40-kDa cysmotropic fragment of human FN; GFP, green fluorescence protein; HPF, high-power field.
ICAM-1-associated firm adhesion and subsequent transendothelial migration (11). In inflamed sites, cytokine-inducible molecules on endothelial cells such as VCAM-1, E-selectin, and ICAM-1 contribute to lymphocyte tethering and vessel egress (11).

Molecules known to orchestrate lymphocyte trafficking to PLN and PP may also contribute to trafficking to the uterus during decidualization and pregnancy. Kruse et al. (4, 12) identified α4 integrinLFA-1⁺ NK-like cells in mouse central DB in close proximity to vessels expressing VCAM-1 at gd 8 (using copulation plug as gd 0). In humans, α4 integrin and LFA-1 are expressed on CD56⁺ decidual uNK cells, whereas VCAM-1 is expressed on decidual vessels (13–15). Recently, we reported pregnancy-induced changes in mouse uterus that preferentially supported gains in adhesion of the human peripheral blood CD56⁺L-selectin⁺α4 integrinLFA-1⁺ NK cell subset under shear forces (10, 16). Adhesion at gd 3, 6, or 10 depended on L-selectin and α4 integrin and was localized to DB (10). These data suggest gestational development of microenvironments in the uterus specialized for pre-uNK cell recruitment.

Mammalian ovulation is triggered by an abrupt surge in luteinizing hormone. Following ovulation, levels of E2 drop transiently while plasma concentrations of P4 increase, and then plateau (17), promoting both local and systemic effects. In rodent uterus, E2 priming followed by high P4 is required for decidualization, which normally accompanies blastocyst implantation on gd 4 (2, 3). In women, decidualization occurs at 5–8 days post-luteinizing hormone before conception (1). In vitro, E2 inhibits endothelial cell apoptosis and stimulates proliferative responses to vascular growth factors, whereas P4 inhibits endothelial cell proliferation (18, 19). Both E2 and P4 stabilize expression of VCAM-1, ICAM-1, and E-selectin in primary vascular endothelial cell cultures (20–22).

In our original study, HEV in PLN from pregnant mice were compared with those in virgin mice at a single time, gd 6 (10). Gestation promoted L-selectin-dependent adhesion, which suggested that pregnancy might cause egress of lymphocytes from the circulation into organs other than the uterus. The present study was undertaken to define the temporal effects of pregnancy on HEV adhesion in the secondary lymphoid tissues, PLN and PP, and on squamous endothelium in nonlymphoid tissue. To address the potential mechanisms promoting adhesion, we further investigated roles for E2 and P4 in modification of endothelial adhesion in multiple tissue sites (PLN, PP, pancreas, and uterus) from ovariectomized (Ovx) mice. An important goal was to discriminate contributions of vascular addressins (i.e., PNAd, MAdCAM-1, and VCAM-1) or extracellular matrix proteins (i.e., fibronectin (FN)) in mediating adhesion of CD56⁺ NK cells to uterine tissues under shear. To determine whether lymphocyte function was also targeted by pregnancy, splenocytes from pregnant or hormone-treated Ovx mice were assessed for interactions with HEV in virgin PLN. The changes in adhesion induced by normal pregnancy were compared with the known physiological activation induced by febrile temperatures (23, 24).

**Materials and Methods**

**Mice and tissue dissections**

C57BL/6 (B6), homozygous B6-Tie2-GFP (The Jackson Laboratory, Bar Harbor, ME), and BALB/c (Taconic Farms, Germantown, NY) mice aged 7–10 wk were studied. Some females were used for timed matings, with the morning of the copulation plug designated as gd 0. Other females (n = 32) were surgically ovariectomized under Avertin anesthesia (25). Females were rested 6 days and then received daily s.c. injections of either hormone dissolved in sesame oil or sesame oil alone between 9:00 and 11:00 a.m. as indicated in Fig. 1 (26–29). Some of the Ovx, hormone-treated mice also had 10 μl of sterile sesame oil injected into a uterine horn to induce decidualization 48 h before euthanasia (26, 28). Ovx mice were euthanized 24 h after receiving their last injection, and vaginal smears were collected and stained with Wright’s Giemsa to confirm treatment success. Nonpregnant controls were virgin females that had never been paired with males.

Blood was collected from virgin mice (n = 2), Ovx-oil mice (n = 2), Ovx-E2 mice (n = 2), and one pregnant mouse to measure serum E2 using a commercial ELISA kit (DRG Instruments, Biophase Diagnostic Laboratories, Mississauga, Ontario, Canada). All procedures were performed under approved animal use protocols (Animal Care Committee, University of Guelph).

Cryosections (12-μm thick) were prepared from the following tissues embedded in OCT (Miles Laboratories, Elkhart, IN): 1) a pool of 10–12 PLN from s.c. and intermuscular sites, 2) a pool of 10–12 intestinal PP, 3) the entire pancreas, and 4) the uterus. For some experiments, the median iliac LN that drain the pelvic organs, including the uterus, were also dissected. Nondecidualized uteri were trimmed at the ovarian and cervical ends, and then incised longitudinally along one side on an axis midway between the mesometrial and antimesometrial aspects. The mesometrium (the mesentery suspending the uterus, also known as the broad ligament) was not removed and served as a positional landmark during analyses. For embedding, samples were oriented to produce full-thickness longitudinal sections that included both antimesometrial and mesometrial sides. Uteri from gd 3 were flushed for blastocysts to confirm pregnancy.
Assay of cell adhesion under shear to frozen tissue sections

Three types of cell suspensions were evaluated for adhesive interactions with endothelium. These were human PBL isolated from random (female and male), normal-donor buffy coat leukocyte concentrates (American Red Cross, Rochester, NY) (23, 24); splenocytes from virgin, pregnant, hormone- or placebo-treated Ovx mice, and TK-1 cells (an α6 integrin+/β1 L-selectin+/CD34+/CD44+/CD90+ cell line) (24, 30). In selected experiments, human PBL were cultured 6 h at 37 or 40°C before being applied to tissue sections, as previously described (24). Lymphocytes were incubated with function-blocking mAb specific for human L-selectin (31) (10 μg/ml; DREG-56; American Type Culture Collection (ATCC), Manassas, VA), mouse L-selectin (32) (MEL-14; ATCC) (hybridoma-conditioned medium diluted to 1/4), human α6 integrin (33) (10 μg/ml; HP2/1; Coulter Immunology, Hialeah, FL), mouse αββ integrin (34) (10 μg/ml; DATK-32; Coulter Immunology), or with a 40-kDa chymotryptic fragment of human FN (100 μg/ml; FN40; Chemicon International, Temecula, CA). Alternatively, tissue cryosections were pretreated with mAb specific for MadCAM-1 (35) (MECA-367; ATCC) (1/4 dilution of hybridoma-conditioned medium), PNA (36) (ATCC), or VCAM-1 (37) (20 μg/ml; clone 429; BD PharMingen, San Diego, CA). To identify adherent human NK cells, PBL aliquots were prelabeled with anti-CD56 mAb (NKG1; clone 429; BD PharMingen, San Diego, CA). To identify adherent human lymphocytes bound per vessel, and are based on triplicate counting of 300–500 HEV/PLN or PP specimens or equivalent numbers of small vessels in pancreatic tissue. When CD56-prelabeled cells were used, tissues were fixed in 3% formaldehyde, and adherent fluorescent cells were quantified in uterine tissues in 10 high-power fields (HPF; 1 HPF = 5 mm²) at ×200 magnification using an Olympus BZ-FLRL fluorescence microscope (Olympus Optical, Tokyo, Japan) (10, 16). In LN and PP, the number of CD56-labeled cells bound per HEV was quantified. All enumerations were done in triplicate.

Analysis of adhesive substrate in B6 Tie2-GFP mice

Implantation sites from Tie2-GFP mice at day 8 of pregnancy were used in adhesion assays as described above, except that the human NK cells were prelabeled with anti-CD56-PE-Cy5 mAb (NKH-1; Immunotech/Beckman-Coulter, Mississauga, Ontario, Canada) (diluted 1/100). After washing in PBS, tissues were fixed in 4% paraformaldehyde/PBS for 30 min, rinsed in fresh PBS, and mounted with AquaPolymount (Polysciences, Washington, PA). Sections were visualized using a Leica TCS SP2 scanning confocal microscope (Leica Microsystems, Richmond Hill, Ontario, Canada). Projections of optical section in the z-axis were generated using Leica confocal software. Wavelengths in the 500- to 550-nm range to detect green fluorescence protein (GFP) (peak emission at 509 nm) and 660- to 700-nm overlaid on 12-rhodamine isothiocynate Ab as described previously (10, 16). Control aliquots were treated with the secondary reagent alone. Lymphocytes were overlaid on 12-μm cryosections of murine tissues and rotated (112 rpm for PBL and splenocytes; 95 rpm for TK-1 cells) at 4°C for 30 min. Following removal of nonadherent cells, glutaraldehyde-fixed specimens were stained with 0.5% toluidine blue, and adhesion was scored on 300–500 HEV/PLN or PP specimens or equivalent numbers of small vessels in pancreatic tissue. When CD56-prelabeled cells were used, tissues were fixed in 3% formaldehyde, and adherent fluorescent cells were quantified in uterine tissues in 10 high-power fields (HPF; 1 HPF = 5 mm²) at ×200 magnification using an Olympus BZ-FLRL fluorescence microscope (Olympus Optical, Tokyo, Japan) (10, 16). In LN and PP, the number of CD56-labeled cells bound per HEV was quantified. All enumerations were done in triplicate.

Statistical analysis

A two-way ANOVA was used to evaluate differences over the time course of pregnancy on LN, PP, and pancreatic tissues. Student’s t test was used to determine significant differences between control and experimental conditions (i.e., hormone treatment groups or pregnancy at a single time point) in the absence or presence of adhesion-blocking Abs or FN40.

Results

Pregnancy promotes organ-specific adhesive function in endothelial cells

To evaluate the effects of pregnancy on endothelial cell interactions in peripheral organs, adhesion of human PBL to endothelium was assessed in PLN, PP, and pancreas from virgin, pregnant, and postpartum mice (Fig. 2). Onset of pregnancy, before blastocyst implantation (gd 3), significantly elevated adhesion to HEV in PLN above that of PLN from virgin mice. Peak levels of adhesion were achieved early postimplantation (gd 6–8), and adherent cells were uniformly small in size (6.8 ± 0.4 μm). An equivalent gain in adhesion was demonstrated at gd 6 in LN that provide pelvic organ drainage (not shown). Once peak levels of adhesion were achieved, these levels were sustained throughout the remainder of gestation (i.e., to gd 18 with birth at gd 19). In PLN of mice analyzed 5 and 9 days postpartum (times selected to represent completion of the first and second postpartum estrous cycles), adhesion of PBL dropped to levels found in PLN from virgin mice. Pregnancy-induced adhesion of PBL to HEV in PLN was L-selectin-dependent, as shown using the L-selectin function-blocking mAb DREG-56 (Fig. 2A).

Pregnancy also increased lymphocyte adhesion to HEV in PP (Fig. 2B). However, in contrast to LN HEV, adhesion in PP HEV declined to baseline during late pregnancy. Adhesion to PP HEV was blocked by MECA-367, a mAb that functionally inhibits both L-selectin and α6 integrin-mediated binding to MadCAM-1 (35) (Fig. 2B). In sharp contrast to the gains in lymphocyte-endothelial cell adhesion detected in HEV of LN and PP, adhesion was not altered in the nondifferentiated squamous endothelium lining small

FIGURE 2. Time course of human PBL bound per HEV of PLN (A), PP (B), or endothelial cells of small blood vessels of pancreas (C) over pregnancy and postpartum periods. Data shown are from one experiment using PBL from one anonymous human donor and the same mouse as donor for all tissues. Similar outcomes were obtained in replicate (n = 3) experiments that examined the full gestational time course with peak adherence detected between gd 6 and 8. Data are the mean number (±SD) of lymphocytes bound per vessel, and are based on triplicate counting of 300–500 HEV or small vessels. Function-blocking mAb specific for L-selectin (DREG-56) or MadCAM-1 (MECA-367) significantly reduced adhesion compared with that of untreated controls (p < 0.001 using Student’s t test). * Indicates a significantly different mean (p < 0.05) to that in the same tissue from virgin females (V) as analyzed by two-way ANOVA (SAS 6.12; SAS Institute, Cary, NC).
vessels of the pancreas at any gestational time point (Fig. 2C). These data suggest that adhesion is functionally up-regulated in response to pregnancy in a restricted subset of vessels, which could confine lymphocyte egress to specific tissues.

**Ovarian steroid hormone replacement therapy mimics effects of pregnancy on adhesion**

Receptors for E2 and P4 are expressed by endothelial cells and may contribute to the observed pregnancy-induced changes in lymphocyte-endothelial cell interactions. To address these potential mechanisms, Ovx mice receiving hormone replacement (100 ng of E2 (26), 1 mg of P4 (26, 27), or combined E2 plus P4, as shown in Fig. 1) were compared with virgin mice, Ovx oil-treated control mice, and pregnant mice (gd 6). ELISA results confirmed that serum E2 concentrations in treated mice were four to five times that of virgin or oil-treated control mice and were within the range achieved during normal pregnancy (i.e., 16.6–20.5 pg/ml for E2-treated compared with 20.5 pg/ml for a gd 6 mouse, and 2.7–5.4 pg/ml for virgin controls). E2 could not be detected in Ovx oil-treated controls. In addition, because decidualization is an important feature of mouse implantation and is not induced by hormone treatment alone, additional E2- plus P4-treated Ovx animals were studied, in which decidualization was induced artificially by injection of sesame oil into a uterine horn (26, 28). Human PBL that are L-selectin⁺ and α4β2 integrin⁺ were used to assess adhesiveness of PLN and pancreatic vessels in frozen-section adhesion assays under mechanical shear. In addition, CD56-labeled human PBL were applied to uteri or lymphoid tissues to assess steroid hormone-mediated effects on adhesion for the CD56brightL-selectin⁻/α4β2 integrin⁻ NK cell subset. The murine TK-1 indicator cell line (α4β2 integrin⁺, L-selectinlow) (24, 30, 38) was used to identify the effects of hormone treatment on α4 integrin/MAdCAM-1-dependent, L-selectin/MAdCAM-1-independent adhesion events in PP HEV.

All hormone treatments promoted statistically significant gains in L-selectin-dependent adhesion of human PBL indicator cells to PLN HEV (Fig. 3A). The gains were comparable to the peak levels stimulated by pregnancy at gd 6. No difference was detected in the level of HEV adhesion induced by E2 alone, P4 alone, or in response to E2 plus P4. Similar increases in TK-1 cell adhesion to PP HEV occurred in hormone-treated pseudopregnant (induced decidual) Ovx mice through a mechanism that could be blocked by the α4β2 integrin-specific DATK-32 mAb (Fig. 4A). In contrast, there was no change in L-selectin or α4β2 integrin-dependent adhesion of lymphocytes to pancreatic vessels following hormone treatment (Fig. 3B), consistent with the finding that pregnancy did not affect adhesion in nonactivated vessels of pancreatic tissues (Figs. 2C and 3B).

Functional adhesive changes, similar to the levels stimulated by pregnancy, were also achieved in the uterus by hormone replacement therapy in Ovx mice (Fig. 3A). In this regard, a marked increase in CD56bright NK cell adhesion to uterine tissues was observed as compared with the basal levels of adhesion observed in either placebo-treated (oil) or virgin control mice. The presence of decidualized stroma had no independent effect on intrauterine adhesion. Moreover, identical increases in adhesion occurred when therapeutic (1-μg) doses of E2 (29) were administered to the Ovx mice (not shown). Adhesion of CD56bright cells to uterine tissues was blocked using either DREG-56 or HP2/1 mAbs to human L-selectin and α4 integrin, respectively (Fig. 3A). Following hormone replacement, adhering CD56bright cells were randomly distributed as single cells and were heterogeneous in size, i.e., the majority were 6.8 ± 0.4 μm in diameter, while a minor population of larger cells (8.01–9.01 μm) was also detected. Quantification of the number of CD56bright cells relative to the total number of lymphocytes bound indicated that significant enrichment of this NK cell subset occurred in the adherent population in uteri of both

![FIGURE 3.](http://www.jimmunol.org/)
Evidence that CD56bright NK cell adhesion to uterine tissues under shear depended on L-selectin and α₄ integrin function strongly suggested that pregnancy-associated hormones regulate adhesion in intrauterine vessels. However, because the structural integrity of vessels within the DB was not fully maintained in tissue cryosections, it was difficult to morphologically discriminate the substrate underlying CD56bright adherent cells (i.e., endothelial vascular wall or associated stroma and extracellular matrix components). The nature of the substrate is important in light of reports that α₄ integrins can mediate uNK cell binding to purified FN and to isolated decidual stroma cells (15). Blockade of CD56bright NK cell adhesion to L-selectin- and α₄ integrin-specific mAb did not resolve this issue, because these reagents inhibit lymphocyte adhesion to multiple vascular and nonvascular substrates. In this regard, L-selectin mediates adhesion to sulfated isoforms of sialyl Lewis-X that decorate the vascular addressins CD34 (PNAd) and MADCAM-1 as well as leukocyte-leukocyte adhesion via interactions with P-selectin glycoprotein ligand-1 (11, 39). Moreover, α₄ integrin adhesion to vascular substrates, MadCAM-1 and VCAM-1, as well as to the extracellular matrix protein FN is blocked by the HP2/1 mAb (40). Two approaches were taken to establish the uterine substrate of adhesion.

The first approach involved the use of blocking agents to competitively inhibit adhesion mediated by vascular addressins or FN (Fig. 5A). Uterine tissues from Ovx-oil control, E2, or pregnant mice (gd 7) were pretreated with blocking mAb specific for the vascular addressins, MadCAM-1, PNAAd, or VCAM-1, before analysis of CD56bright cell adhesion. Alternatively, CD56-labeled cells were preincubated with the C-terminal FN40 that contains the binding site for α₄ integrins (41). FN40 competitively blocks the FN binding domain of α₄ integrin without impeding interactions with vascular addressins including VCAM-1 (40) or MadCAM-1 (not shown). In both E2-treated and pregnant mice, mAb blockade of PNAAd, MadCAM-1, or VCAM-1 reduced adhesion to the basal levels observed in the presence of blocking mAb for L-selectin.

**FIGURE 4.** Row A depicts low-power histological images of uterus and implantation sites in B6 mice stained with H&E, to provide orientation. The mesometrial triangle (M), where development of the MLAp occurs, is uppermost in all panels. The antimesometrial (AM) side of the uterus is at the bottom of all images. At gd 3, decidualization has yet to commence. At gd 6, maternal deciduial tissue (D) fills the uterus, with the embryo at the primitive streak stage occupying the embryonic crypt (EC). By gd 10, there is a fully developed implantation site. The microdomains of the MLAp and DB are maternal in origin, whereas that of the placental implantation site. The microdomains of the MLAp and DB are maternal in origin, whereas that of the placental implantation site. The microdomains of the MLAp and DB are maternal in origin, whereas that of the placental implantation site. The microdomains of the MLAp and DB are maternal in origin, whereas that of the placental implantation site.
Hormonal regulation of lymphocyte-endothelial cell interactions

Pregnancy and hormone therapy increase adhesive properties of splenic lymphocytes

Having established that both pregnancy and pregnancy-associated hormones stimulate endothelial cell adhesion, we extended the functional analysis of these physiologic mediators to lymphocytes. Splenocytes from all groups of Ovx mice (Fig. 1) were assessed for L-selectin-dependent adhesion to HEV of PLN from virgin mice. As shown in Fig. 7A, all steroid hormone treatments enhanced L-selectin-dependent adhesion of splenic lymphocytes under shear compared with levels in the placebo treatment group. Moreover, these increases in L-selectin binding function were comparable to those induced by pregnancy as well as to those induced by in vitro lymphocyte stimulation using physiologic fever-range temperature (Fig. 7B). Adhesion induced in this assay by fever-range hyperthermia was previously equated to a 4- to 5-fold increase in lymphocyte homing potential to PLN in vivo (24).

Discussion

The exquisite microdomain compartmentalization of the decidualized mouse uterus of early pregnancy is due to the transient development of highly differentiated vessels expressing known vascular adhesion molecules in nonoverlapping patterns (4, 12). The biological function of this compartmentalization is postulated to be recruitment and localization of specialized, distinct leukocyte subsets. Precursors for the dominant lymphocyte subset of early pregnancy, the uNK cells, do not self-renew in the uterus but are recruited during the first trimester (10). Transplantable uNK precursor cells have been found in BM, neonatal and adult thymus, spleen, LN, and liver with PLN and spleens from pregnant donors being the richest sources (10). For pre-uNK or uNK cells to leave the circulation and move into the uterus, interactions with endothelial cells are required. We previously observed a pregnancy-induced gain in adhesive function of HEV using human PBL as

![FIGURE 6.](http://www.jimmunol.org/)

FIGURE 6. CD56<sup>bright</sup> cells adhere to weakly expressing Tie2-GFP<sup>+</sup> vascular endothelium in the central DB. Confocal images of 12-μm cryosections of uterine tissue from Tie2-GFP transgenic mice at gd 8 are presented. Human lymphocytes prelabeled with anti-CD56-PE-Cy5 were applied to frozen tissue sections under mechanical shear. In the left panel, vessels in the central DB demonstrate weaker expression of Tie2-GFP but support adhesion of CD56<sup>bright</sup> cells (indicated by arrow); arrowheads demark perimeter of vessels. Bars, 50 μm.

transgenic mice (n = 10 mice). GFP, under the direction of the endothelial-specific receptor tyrosine kinase (tek)/Tie2 promoter is expressed only in endothelial cells (42). Using confocal laser-scanning microscopy, Tie-2-GFP was found to be weakly expressed in vessels within the DB, whereas there was strong expression of Tie-2/GFP in vessels lateral to the DB (Fig. 6) and in the developing embryo (not shown). Adherent PE-Cy5-tagged CD56<sup>bright</sup> fluorescent cells were found exclusively in the DB in association with Tie2-GFP<sup>+</sup> vascular endothelium (Fig. 6).

The second approach examined interactions of CD56-PE-Cy5-labeled NK cells with vessels within the DB of pregnant Tie2-GFP

(DREG-56) (Fig. 5A) or α<sub>ε</sub> integrin (HP2/1) (not shown). These data strongly implicate vascular addressins in supporting CD56<sup>bright</sup> cell adhesion under shear in uterine tissues. Evidence that FN40 had no affect on CD56<sup>bright</sup> cell binding to uterine tissues further suggests that adhesion of this NK cell subset in frozen-tissue section assays does not reflect interactions between α<sub>ε</sub> integrin and FN present in the extracellular matrix of uterine tissue stroma. Interestingly, the effects of hormone or pregnancy on CD56<sup>bright</sup> cell adhesion were not restricted to the uterus, because both states markedly increased L-selectin-dependent adhesion of CD56<sup>bright</sup> cells to vascular addressins on HEV of LN and PP (Fig. 5B). However, although there was enrichment of CD56<sup>bright</sup> cells in the adherent population in uteri (i.e., from 1–2% in original PBL population to 60–80% of the adherent population detected in uteri during pregnancy or hormone replacement therapy), no enrichment was observed in LN or PP (i.e., CD56<sup>bright</sup> cells comprise ~1–2% of PBL bound to HEV of peripheral lymphoid tissues). FN40 was further shown to have no effect on CD56<sup>bright</sup> cell adherence to LN or PP HEV (Fig. 5B).

The second approach examined interactions of CD56-PE-Cy5-labeled NK cells with vessels within the DB of pregnant Tie2-GFP...
results in increased expression of the adhesion molecule ICAM-1 (20), and increased mRNA for the adhesion molecules ICAM-1, VCAM-1, and E-selectin (46), it is possible that ligands for L-selectin (PNAd) and $\alpha_4\beta_1$ integrin (MAdCAM) are similarly influenced by hormonal stimulation, although that has not been reported to date. VCAM-1 has been found to be the most dramatically up-regulated endothelial cell adhesion molecule in mouse DB (12), but platelet endothelial cell adhesion molecule-1 and $\alpha_4$ integrin were strongly up-regulated on human uterine myometrial endothelium when cells were cultured with E2 and P4 (47).

Gain in uterine adhesiveness for CD56$^{bright}$ cells was induced equally by all steroid hormone treatments. This likely suggests that maximal functional changes are induced using single agents and points to redundancy in the mechanisms used by the pregnant uterus to promote homing of specific lymphocyte subsets. Blockage studies indicate that known endothelial adhesion pathways (11) were promoted by the steroid hormones in the uterus. In this regard, L-selectin- and $\alpha_4$ integrin-dependent adhesion of CD56$^{bright}$ NK cells to uterine vessels of pregnant (gd 7) or hormonally treated mice was inhibited by mAb directed against vascular adresses, i.e., PNAd, MAdCAM-1, and VCAM-1. Moreover, CD56$^{bright}$ cells were shown to bind to vessels in the DB during pregnancy. These data support a model in which vascular adresses direct recruitment of blood-borne uNK cell precursors across specialized decidual vessels. Although contributions of $\alpha_4$ integrin binding to FN under shear were not revealed in frozen-tissue section adhesion assays, these results do not formally exclude the involvement of FN or other extracellular matrix proteins in maintaining uNK cells in restricted microdomains during the physiological conditions of pregnancy. An interesting finding was that the CD56$^{bright}$ NK cell subset was significantly enriched among cells adhering to the uterus, whereas no enrichment occurred in cells adhering to LN or PP HEV. One possible explanation is that multiple vascular ligands (PNAd, VCAM-1, MAdCAM-1) collaborate in uterine tissue to support L-selectin- and $\alpha_4$ integrin-dependent adhesion, whereas a single ligand is operative in stabilizing lymphocyte adhesion under shear in LN (i.e., PNAd) or PP (i.e., MAdCAM-1). Thus, there may be a greater opportunity for synergistic cooperativity between overlapping adhesion pathways in uterine vessels.

A role for $\alpha_4$ integrin/VCAM-1 in NK cell trafficking is consistent with previous reports demonstrating VCAM-1 expression on decidual vessels in humans or in mice at gd 8 (4, 13, 14). However, neither MAdCAM-1 nor PNAd-1 expression was detected on decidual vessels by immunohistochemical analysis in a prior study in mice at gd 8 (4). Temporal studies are required to fully address the kinetics of expression of selected vascular adresses in response to pregnancy or hormonal stimulation. Based on the collective evidence that neutralizing mAb specific for L-selectin or its ligands (PNAd, MAdCAM-1) fully block pregnancy-induced adhesion to DB in uterine tissues at various times throughout pregnancy (gd 3–10) (Ref. 10 and the present study), it is likely that vascular adresses are functionally displayed on decidual vessels at levels below detection by immunohistochemical techniques. Notably, administration of E2 or P4 to mice increases the enzymatic activity in the uterus of glycoproteinases and sialyltransferases (48), which are known to modify L-selectin ligands (49). We have also identified $\alpha$ (1.3) fucosyltransferase and amine N-sulfotransferase expression in the MLAp of gd 6 and 10 B6 mice in a cDNA microarray analysis (our unpublished observations).

The decidualized, pregnant uterus induced an aggregating/clustering behavior in the viable adherent lymphocytes, which was
restricted to DB microdomains. This is in contrast to E2/P4 replacement therapy, which stimulated adhesion of single cells to uterine tissues without microenvironmental restriction. Moreover, artificial induction of endometrial stroma cell decidualization (28) did not modify the levels of adhesion beyond that seen following administration of hormones only. Although artificially induced decidua in normal mice lacks compartmentalization, this tissue recruits uNK cells (2). It is noteworthy that lymphocytes adhering to artificially induced decidua were always dispersed and never found as aggregates. Thus, NK cell recruitment appears to be hormone mediated, whereas localization appears to be independent of either E2 or P4. Broadly, these data define limits in the widely used model of deciduomata induction in rodents and demonstrate that this model is not fully representative of decidua induced by blastocyst implantation. Clustering behavior has been documented in memory T cells by confocal microscopy in LN organ bath cultures where both swarming, nonstable but enlarging clusters as well as stable cluster formation are triggered by immature dendritic cells (50). Thus, the observed differences in lymphocyte interactions with artificially induced vs conceptus-induced decidua could relate to activation of APCs by trophoblast. Alternatively, fetally derived trophoblast cells or activated endothelium may underlie the clusters. The enlargement of cluster size with advancing gestation could reflect expansion of the underlying targeted stroma cells or spreading of an activation epitope between stromal cells. The lymphocytes may also undergo alterations induced by hormone-mediated events that promote homotypic cell clustering.

Pregnancy and steroid hormones appeared to coordinately change the adhesion potential of lymphocytes as well as endothelium. Splenocytes from pregnant or hormone-treated mice were more adhesive to HEV in PLN from virgin mice than were splenocytes from virgin mice, and the difference was L-selectin mediated. This observation is supported by a study that describes reduced shedding of soluble L-selectin from lymphocytes (thereby increasing their adheriveness) under the influence of E2, but not P4, in patients receiving controlled ovarian stimulation for in vitro fertilization (51). The expression of the receptors for estrogen (ER) and P4 (PR) on lymphocytes is somewhat controversial (52–57). Recently, ERβ message was demonstrated in human uNK cells (58), although neither ERα nor ERβ are expressed by mouse uNK cells (59). These results imply possible direct as well as indirect steroid hormone influences on lymphocyte behavior and merit further study. Nongenomic effects must also be considered such as the P4-mediated changes in human T cells related to inhibition of potassium ion channels and Ca2+ signaling (60).

Findings presented here and in recent reports (10, 61–63) support an emerging view of the uterus as a unique extralymphoid site for microenvironmental control of L-selectin and α4 integrin-dependent adhesion events. Of particular interest are recent data showing that L-selectin on human trophoblasts can support adhesion under shear to PNAd displayed on the uterine epithelial lining during pregnancy (61). This adhesive interaction is proposed to play a major role in successful blastocyst implantation. Thus, pregnancy appears to exert tight, microdomain control on L-selectin/ PNAd adhesion in vascular beds as well as extravascular systems within uterine tissues. The coordinated effects of pregnancy on endothelium, lymphocytes, and their interactions are reminiscent of findings in other models of normal physiological change such as inflammation and fever. Amplification by fever-range thermal stress of lymphocyte/endothelial interactions preferentially in specialized HEV of selected tissues (LN, PP) has been proposed as a mechanism to focus the immune response to these sites, thus preventing an unproductive exodus to less relevant tissues (24). The physiological relevance of increased homing to lymphoid tissues during pregnancy remains to be determined. It is tempting to speculate that this targeted migration developed evolutionarily to result in heightened innate immune surveillance to protect both the maternal host and future offspring from infection. Alternatively, homing of NK cells to LN and PP may prime these cells for maturation or functional activation en route to the uterus. This is supported by evidence that LN from pregnant mice are a significant source of NK cells in adoptive transfer studies (10). Because pregnancy mimics many aspects of inflammation and mild temperature elevation (23, 24, 64), it is not unreasonable to suspect that the mechanisms defined in fever and inflammation models will be useful in explaining the mechanisms for the pregnancy and steroid hormone effects we have observed. Defining the steroid-regulated pathways for lymphocyte recruitment to the uterus has potential therapeutic importance for promoting lymphocyte localization to uteri of patients at risk for implantation failure or pre-eclampsia and to nonuterine tissues in patients with other diseases. Such information may also help to explain inefficiencies in combination therapies that involve steroid hormone replacement.

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