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Contributions from Self-Renewal and Trafficking to the Uterine NK Cell Population of Early Pregnancy[1]

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Uterine NK (uNK) cells are abundant in human and murine uteri during decidualization. It is unclear whether precursors of uNK (pre-uNK) cells self-renew or are recruited from other sites. To assess self-renewal of pre-uNK cells, uterine segments from NK cell-competent mice were grafted orthotopically into NK/uNK cell-deficient or wild-type mice. Only in wild-type recipients did decidualized grafts contain uNK cells, indicating that pre-uNK cells do not self-renew in uterus. To identify pre-uNK cell sources, thymus, bone marrow, lymph node, or spleen cells were grafted from virgin or pregnant NK cell-competent donors into mated NK/uNK cell-deficient recipients. Cells from secondary lymphoid tissues of pregnant donors gave high level uNK cell reconstitution, which was independent of chemokine receptors CCR2 or CCR5. Pregnancy-induced changes to lymphocyte-endothelial cell interactions were documented using adhesion of human lymphocytes to frozen mouse tissue sections under shear. A dynamic increase was observed in L-selectin- and α_4 integrin-dependent adhesion of CD56^{bright} NK cells to decidualizing uterus and in human PBL adhesion to lymph node endothelium. These data support a model that attributes the dramatic increases in human and murine uNK cells during decidualization to precursor cell recruitment. ***The Journal of Immunology*, 2002, 168: 22–28.**

Natural killer cells are the transiently dominant lymphocyte population in decidualizing human and murine uteri (1, 2). Decidualization is a process that transforms uterine stromal cells into large, endocrinologically competent, decidual cells. In mice, primary decidualization is triggered by the implantation of hatched blastocysts, and is followed by secondary decidualization that envelops each invading conceptus. A decidualization response (deciduoma) can also be artificially induced in hormone-primed uterus by introduction of inert objects (such as thread) into the lumen (3). Activation and terminal differentiation of uterine NK (uNK)[3] cells happen only in a deciduoma or on the mesometrial side of the naturally pregnant uterus, in which decidua basalis (DB) develops between the placenta and the myometrial wall. Differentiating uNK cells divide, enlarge dramatically (~45 μm diameter), acquire cytoplasmic granules containing lytic proteins, and initiate IFN-γ synthesis. In mouse pregnancy, uNK cells are found in two specific microdomains, DB and mesometrial lymphoid aggregate of pregnancy (MLAp, also termed metrial gland), a zone found in the uterine wall at implantation sites from gestation day 8 (gd8) (1, 2). The MLAp and DB are traversed by branches of the uterine artery that will supply the placenta beginning at gd9–10. Data from pregnancies in NK/uNK cell-deficient mice (tgϵ26 and RAG-2$^{-/-}$/common γ-chain (γ_c)$^{-/-}$) indicate that DB and its spiral arteries are major targets of uNK cell actions, mediated via release of IFN-γ (4–7). An unresolved question is whether adult uterus self-renews uNK cells or recruits precursors of uNK cells (pre-uNK) from other tissues.

Generation and maintenance of specialized lymphocyte subsets occur in some adult, nonlymphoid abdominal tissues, but have not been studied in uterus (8, 9). To establish the site of self-renewal for intestinal intraepithelial T lymphocytes, intestinal segment grafting into T cell-deficient hosts was used (10). Availability of tgϵ26 and RAG-2$^{-/-}$/$\gamma_c^{-/-}$ mice which have phenotypically similar anomalous implantation sites suggests that a uterine segment transplant approach would be possible. However, poor outcomes have been reported following grafting of uterine and oviductal tissues in rats, guinea pigs, rabbits, and primates (11–14). Inflammation, necrosis, and fibrosis limited the ability of these grafts to support implantation.

NK cell progenitors (pro-NK) occur in fetal liver (15, 16) and fetal/neonatal thymus (17). In adults, bone marrow (BM) is the major source of pro-NK cells (18). Transplantable pro-NK cells are also found in spleen (19). Progenitors of uNK cells (pro-uNK) are in BM as transgene-marked adult BM injected into fetuses generates uNK cells following birth, adulthood, and pregnancy in the recipients (20). The more differentiated pre-uNK cell also occurs in BM, as pseudopregnant, irradiated mice given male rat BM differentiated some uNK cells with rat morphology (21). However, the origins of pre-uNK cells have not been studied during normal pregnancies.

Lymphocyte mobilization is induced by pregnancy; primary lymphoid organs (BM and thymus) involute (22–24), while secondary lymphoid organs, such as spleen and some lymph node (LN), hypertrophy (25–27). Chemokine signaling directs cell

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[3] Abbreviations used in this paper: uNK, uterine NK; BM, bone marrow; DB, decidua basalis; gd, gestation day; HEV, high endothelial venule; LN, lymph node; MAdCAM-1, mucosal addressin cell adhesion molecule-1; MLAp, mesometrial lymphoid aggregate of pregnancy; MLN, pools of mesenteric and iliosacral LN; MT, mesometrial triangle; PAS, periodic acid Schiff's; PLN, pools of s.c. LN; PP, Peyer's patch; pre-NK, precursors of NK cell; pro-NK/uNK, progenitors of NK/uNK cell; RITC, rhodamine isothiocyanate; SC, spleen cell; γ_c, common γ-chain.

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movement toward tissues, while egress of circulating cells from vessels requires lymphocyte/endothelial cell interactions. Receptors for CC chemokines are found on NK cells (CCR2 for monocyte chemoattractant protein-1, CCR5 for macrophage-inflammatory protein-1α and RANTES) (28, 29), and these chemokines are up-regulated in pregnant uteri (30, 31). L-selectin and α_4 integrin are critical molecules involved in slowing circulating leukocytes and facilitating rolling, tethering, and docking for vessel egress in secondary lymphoid tissues and at extralymphoid sites of inflammation (32). In humans, functionally active L-selectin is expressed at extremely high levels by the numerically minor, circulating $CD56^{bright}$ NK cell subset, compared with the more prevalent $CD16^+$, $CD56^{dim}$ subset (33). The α_4 integrin is expressed equivalently by $CD56^{bright}$ and $CD56^{dim}$ NK cells (33). Human uNK cells are $CD56^{bright}$ (2), suggesting a potential for preferential recruitment of L-$selectin^{bright}$, α_4 $integrin^+$, $CD56^{bright}$ cells into the uterus. Goals of this study were 1) to determine whether uterus self-renews uNK cells; 2) to examine lymphoid tissues as sources for pre-uNK cells; and 3) to explore underlying chemokine- and adhesion-related mechanisms that contribute to mobilization of cells into decidualizing uterus.

Materials and Methods

Animals

Immunocompetent mice (random bred CD1; Charles River Laboratories, St. Constant, Quebec, Canada), C57BL/6J (B6), and C57BL/6 × 129/J F_1 (F_1; The Jackson Laboratory, Bar Harbor, ME)) were housed under standard husbandry in the Central Animal Facility, University of Guelph. Immunodeficient mice (random bred ICR-*scid/scid* (SCID; NK^+, T^-, B^-; Taconic Farms, Germantown, NY), tgϵ26 ($H\text{-}2^{k/b}$; NK^-, T^-, B^+), and RAG-$2^{-/-}/\gamma_c{}^{-/-}$ ($H\text{-}2^b$; NK^-, T^-, B^-) were housed in the University of Guelph's barrier-husbandry facility. Both tgϵ26 and RAG-$2^{-/-}/\gamma_c{}^{-/-}$ lack uNK cells and are referred to as NK/uNK cell deficient (5). Mice ablated for CCR2 ($H\text{-}2^{k/b}$) and CCR5 ($H\text{-}2^{k/b}$) (34, 35) were bred at University of Texas (Austin, TX) and shipped to Guelph. Virgin females were used at 8 wk of age, unless different ages are stated and, if bred, were mated to males of the same strain. gd0 was the morning of copulation plug detection. Euthanasia was by CO_2, followed by cervical dislocation.

Uterine segment transplantation

Uterine segments (10 or 5 mm), trimmed of mesentery and vessels, were grafted from virgin donors to virgin recipients in an orthotopic manner meant to preserve both cranial-caudal and mesometrial-antimesometrial orientations. For autotransplantation ($n = 2$), CD1 females were anesthetized (0.35 ml xylaxine (20 mg/ml) and ketamine (100 mg/ml)), and the donor horn was reanastomosed with simple interrupted 8-0 Vicryl (Polysorb, Norwalk, CT) sutures. For all other grafting, donors (CD1 or SCID) were euthanized, while recipients (tgϵ26 or CD1) were anesthetized as above. Recipient horns were cut at their midpoint, and donor tissue was inserted and anastomosed. No vascular anastomosis was attempted. The abdomen was closed surgically. After 7 days, recipients were paired for breeding and euthanized at gd10.

Thymic engraftment

Thymuses were dissected from nonpregnant or pregnant (gd3 or 5) adult, or neonatal (48 h) B6 mice and grafted under the renal capsule of anesthetized gd0 RAG-$2^{-/-}/\gamma_c{}^{-/-}$.

Adoptive transfer of BM, LN, or splenocytes

BM and spleen cell (SC) donors were nonpregnant or pregnant SCID mice, while LN donors were B6 (gd3, 5, or 7). As pregnancy changes cellularity of these organs, one donor tissue equivalent dose was used for each recipient, pooling donors if several mated recipients were available on the same day. Uteri from all gd3 donors were flushed to confirm pregnancy by detection of preimplantation blastocysts. BM was flushed from femurs and tibias of each donor. Twelve s.c. LN (PLN), three iliosacral LN, and the mesenteric LN chain were harvested from each donor. The mesenteric and iliosacral LN were pooled (MLN). LN and spleens were dissociated mechanically. PBS (400 μl with/without cells) was infused via tail veins into gd0 tgϵ26 or RAG-$2^{-/-}/\gamma_c{}^{-/-}$ recipients, who were sacrificed on the recipients' gd10.

Morphometric analyses

Abdominal contents were examined grossly, then uteri were dissected, fixed in Bouin's solution, processed routinely for paraffin embedding, serially sectioned at 7 μm (transversely for normal uteri and longitudinally for surgically manipulated uteri), and stained with H&E for routine histopathology or periodic acid Schiff's (PAS) for uNK cell enumeration. Eleven central tissue sections from each implantation site were scored as previously described (3). One square millimeter per section was analyzed in each mesometrial microdomain, DB, and MLAp. Circular smooth muscle was used as the boundary between these. When the MLAp was absent or rudimentary, mesometrial triangle (MT) is the term used to describe the scored region, rather than MLAp. For longitudinally sectioned uteri, those containing a conceptus were scored as above. Uteri containing a deciduoma were scored in two independent 1-mm^2 areas of 10 of the serial sections separated by 42 μm to avoid duplicate counting of uNK cells, which may reach 45 μm by gd10. Diameter and granularity were estimated from a total of 30 cells from 10 sections at 1000×. Means and SDs of uNK cells/mm (2) diameters, granularity, p values, and Student-Newman-Keul test for ANOVA were conducted using PC-SAS 6.12 for Windows (SAS Institute, Cary, NC).

Lymphocyte adhesion to frozen mouse tissues under shear

Human PBL were isolated from buffy coat leukocyte concentrates of anonymous donors (American Red Cross, Buffalo, NY) by Ficoll/Hypaque centrifugation and adherent cell depletion (36). Information regarding donor sex and hormonal status was not available; thus, equal numbers of nonpregnant female and male donors are probable. Uteri and PLN from nonpregnant and pregnant (gd3, 6, or 10) B6 mice were cryopreserved immediately upon collection. PBL were either unstained or stained with anti-CD56 PE mAb (1/100; Coulter Immunology, Hialeah, FL) and goat anti-mouse IgG-rhodamine isothiocyanate (RITC) Ab to permit analysis of NK cell subset adhesion to high endothelial venules (HEV) by fluorescence microscopy, as described previously (36). Before assay, PBL were incubated with or without blocking mAb (10 μg/ml) specific for L-selectin (DREG-56; American Type Culture Collection, Manassas, VA) or α_4 integrin (HP2/1; Coulter). Treated PBL (5×10^6) were overlaid on 12-μm cryosections of mouse tissues and rotated at 112 rpm (Labline Instruments, Melrose Park, IL) for 30 min at 4°C. After removal of nonadherent cells, specimens were fixed vertically in 3% formaldehyde in PBS for 1 h. For unlabeled PBL, glutaraldehyde-fixed specimens were permeabilized in 70% ethanol and stained with 0.5% toluidine blue in absolute ethanol. Adhesion of PBL was scored in 300–500 HEV per specimen. All enumeration was done in triplicate. If CD56-prelabeled cells were used, specimens were washed in PBS and fluorescent cells were quantified in double-blind analysis of 10 high powered fields (equivalent to 5 mm^2) at ×200 magnification, while total cells were quantified in the same section by bright field using an Olympus BH2-RFL fluorescence microscope (Olympus Optical, Tokyo, Japan). As reported previously (33), the accuracy of using fluorescence microscopy to visually discriminate $CD56^{bright}$ and $CD56^{dim}$ cells in human PBL suspensions and adherent cell populations was verified by flow cytometry and confocal fluorescence microscopy, respectively, to quantify the fluorescence intensity of individual cells. Fluorescent-labeled cells were not detected in specimens labeled with isotype-matched negative control murine IgG1 Ab (Coulter).

Results

Assessment of uNK cells in uterine segment transplants

Feasibility of orthotopic uterine grafting was assessed in autologously grafted CD1 mice using grafts of 10 mm ($n = 3$) and 5 mm ($n = 4$). The longer grafts showed gross and histological full-length necrosis, while the shorter grafts were viable. All recipients of the 5-mm grafts were pregnant, with three grafts containing implantation sites and the fourth a deciduoma (Table I). Typical uNK cells (16–45 μm diameter with 9–25, PAS-reactive cytoplasmic granules/cell) were found in these decidualized grafts (Fig. 1, *A* and *B*). Thus, orthotopically grafted mouse uterus supports uNK cell differentiation. Next, 5-mm uterine segments were grafted from uNK cell-competent CD1 or SCID donors into NK/uNK cell-deficient tgϵ26 recipients ($n = 7$). Two females mated, but were not pregnant at euthanasia, despite grossly and histologically normal grafts. Five recipients were pregnant, and each grafted segment contained a large deciduoma indicative of viable,

Table I. *Results of uterine segment transplantation*

Donor→Host	Animal Identity Number	Grafted Horn: No. of fetuses in graft:host segments	Grafted Horn: Maternal tissues in graft	Density of uNK Cells at Graft Sites[a]	Nongrafted Horn: No. of fetuses
CD1→CD1[b]	1	1:3	MLAp, DB	+++	0
	2	0:0	Deciduoma	+	7
CD1→CD1[c]	3	1:1	MLAp, DB	+++	4
	4	1:3	MLAp, DB	+++	6
CD1→tgε26	5	0:0	Deciduoma	−	6
	6	0:0	Deciduoma	−	1
	7	0:1	Deciduoma	−	3
	8	0:0	Normal uterus	−	0
SCID→tgε26	9	0:0	Deciduoma	−	5
	10	0:0	Normal uterus	−	0
	11	0:0	Deciduoma	−	1

[a] +++, More than 50 uNK cells/mm^2; +, 5 or fewer uNK cells/mm^2; −, no uNK cells.
[b] Autografted, nongrafted horn was reanastomosed.
[c] Donor and recipient were different CD1 mice.

hormonally responsive graft tissue. By serial section analysis, neither the deciduomata in donor tissues nor implantation sites in host tissues contained uNK cells (Fig. 1, *C* and *D*). Thus, the donor segments did not contain self-renewing pro/pre-uNK cells that could differentiate in situ or migrate to adjacent implantation sites.

Development of uNK cells from thymus, BM, LN, and SC

At gd10 in normal and SCID mice, the range in uNK cell frequency is 27–53 cells/mm^2 in DB and 72–129 cells/mm^2 in MLAp (Table II). Uterine segment transplantation suggested that migration of pre-uNK cells accounts for filling of these microdomains. Lymphoid tissues were assessed for pre-uNK cells by grafting to mated, NK/uNK cell-deficient mice. Thymic engraftment generated limited numbers of uNK cells at gd10 (Table II). There were no statistical differences in reconstitution of DB or MT by thymuses of different ages or from different donor pregnancy states ($p > 0.05$). BM from nonpregnant donors or donors at three early times of pregnancy also gave low uNK cell reconstitution in all recipients (Table II). No significant differences were found in uNK cells/mm^2 in DB or MT between the BM donor groups ($p > 0.05$). MLN gave low uNK cell restoration, while implantation sites in recipients of PLN showed MLAp development. Both MLAp and DB of PLN-grafted mice contained mature uNK cells. Implantation sites in NK/uNK cell-deficient mice receiving SC from pregnant donors also showed development of MLAp and high levels of uNK cells in both MLAp and DB. However, if the SC donors were not pregnant, uNK cell reconstitution was much lower ($p < 0.001$) in both areas. As shown in Fig. 1, *E* and *F*, levels of engraftment resulting from inoculation of SC from pregnant donors were sufficient to modify the decidual spiral arteries. In sharp contrast, host arterial vasculopathy persisted in recipients of SC from nonpregnant donors. For all SC donors, uNK cells were present at higher frequencies in MLAp than in DB ($p < 0.01$), the typical gd10 pattern in normal mice. Morphological assessment of graft-derived uNK cells showed that uNK cells derived from thymus, BM, LN, and SC were similar in diameter (means ± SD of 14.5 ± 4, 15.6 ± 4.5, 18.7 ± 7.5, and 13.9 ± 4.7 μm, respectively) and in numbers of granules/cell (12.8 ± 5.8, 15.6 ± 5.5, 20.5 ± 11.9, and 17.8 ± 7.7 granules/cell, respectively). These properties are identical with uNK cells in unmanipulated B6 (14.3 ± 2.9 μm diameter and 18 ± 8.2 granules/cell) and SCID (15.9 ± 4 μm diameter, 19.4 ± 9 granules/cell) mice, implying equivalent maturity.

To test the role of specific chemokines expressed by the pregnant uterus on pre-uNK cell recruitment, mated RAG-2$^{-/-}$/$\gamma_c^{-/-}$ females were infused with SC from pregnant (gd3 or 5) CCR2$^{-/-}$ or CCR5$^{-/-}$ mice. High levels of uNK cells were found in all recipients that did not differ numerically (Table II) or morphometrically from uNK cells in gd-matched, F_1 controls. These data

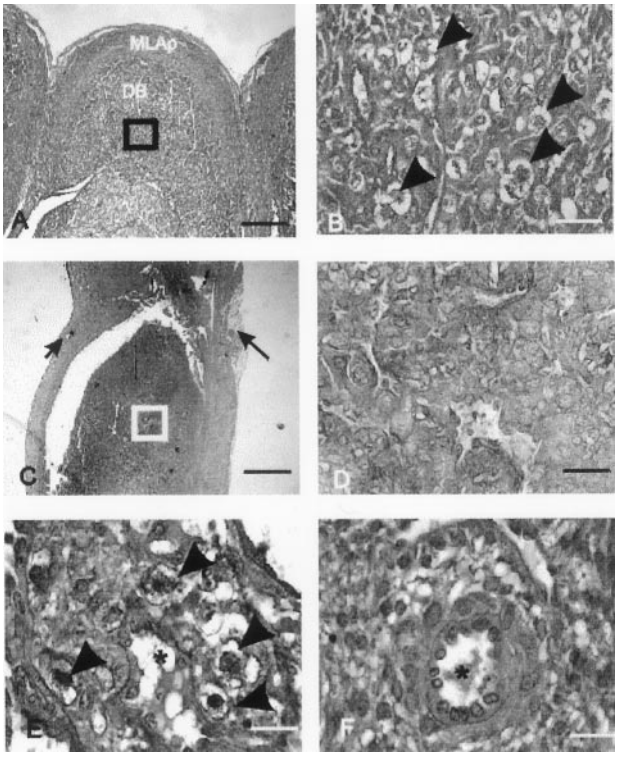

FIGURE 1. Photomicrographs of grafted uterine segments at gd10. Boxed regions in *A*, *C*, and *E* are enlarged in *B*, *D*, and *F*. *A* and *B*, Lower and higher power images of a CD1 graft site in a CD1 recipient with a normally developed implantation site including the MLAp, DB, and placenta (pl). *B*, Shows numerous, mature uNK cells (arrowheads). *C* and *D*, Lower and higher power images of a SCID graft segment in a tgε26 recipient revealing development of a deciduoma (DC). Image D is representative of serial sections of deciduoma that contained no uNK cells. *E*, uNK (arrowheads) cells established within the DB by SC from gd5 SCID donors were mature granulated cells. *F*, SC from nonpregnant SCID donors did not generate uNK cells during the 10-day assay protocol. The blood vessel (asterisk) is an unmodified decidual spiral artery. pl, Placenta. *A–F*, Stained with PAS; bars in *A* and *C*, 400 μm; bars in *B*, 40 μm; and bars in *D–F*, 25 μm.

Table II. *Mean density of uNK cells on gd10 in mesometrial tissues of NK/uNK-deficient mice transplanted on gd0 and controls*

Genotype	Graft	Cells Inoculated/ Recipient $\times 10^7$	Dam/Fetuses	Means (cells/mm^2 ± SD) DB	MT
Control					
tgε26	PBS	–	3/9	0	0
RAG-2$^{-/-}$/$\gamma_c^{-/-}$	PBS	–	3/9	0	0
SCID	PBS	–	2/8	48.5 ± 14.3	129.1 ± 26.0
B6	PBS	–	2/4	26.5 ± 3.3	74.7 ± 4.8
F1	PBS	–	1/3	53.3 ± 8.3	72.1 ± 5.1
Transplanted					
RAG-2$^{-/-}$/$\gamma_c^{-/-}$	NP B6 adult thymus	NA[a]	2/6	1.6 ± 2.2*†	0.6 ± 1.3*†
RAG-2$^{-/-}$/$\gamma_c^{-/-}$	gd3 B6 thymus	NA	2/6	2.5 ± 1.1*†	5.2 ± 2.7*†
RAG-2$^{-/-}$/$\gamma_c^{-/-}$	gd5 B6 thymus	NA	3/9	9.6 ± 5.2*†	7.0 ± 5.0*†
RAG-2$^{-/-}$/$\gamma_c^{-/-}$	Neonatal B6 thymus	NA	2/6	1.7 ± 0.8*†	3.7 ± 1.0*†
tgε26	NP adult SCID BM	1.0	3/9	3.8 ± 3.3*†	2.7 ± 2.1*†
tgε26	gd3 SCID BM	0.6	3/9	5.9 ± 3.8*†	4.9 ± 4.8*†
tgε26	gd5 SCID BM	0.5	3/9	4.7 ± 1.4*†	4.3 ± 0.8*†
tgε26	gd7 SCID BM	0.4	3/9	4.9 ± 1.8*†	7.8 ± 4.3*†
RAG-2$^{-/-}$/$\gamma_c^{-/-}$	NP B6 MLN	0.5	2/6	1.2 ± 1.4*†	1.0 ± 0.7*†
RAG-2$^{-/-}$/$\gamma_c^{-/-}$	gd3 B6 MLN	0.6	2/6	0.5 ± 0.6*†	0.1 ± 0.2*†
RAG-2$^{-/-}$/$\gamma_c^{-/-}$	gd5 B6 MLN	0.5	1/3	0*	0*
RAG-2$^{-/-}$/$\gamma_c^{-/-}$	gd7 B6 MLN	0.6	1/3	0.2 ± 0.3*†	0.9 ± 0.5*†
RAG-2$^{-/-}$/$\gamma_c^{-/-}$	NP B6 PLN	1.0–1.2	3/9	14.6 ± 6.0*†‡	5.9 ± 4.7*†
RAG-2$^{-/-}$/$\gamma_c^{-/-}$	gd3 B6 PLN	1.0–1.2	2/6	14.3 ± 3.7*†	22.5 ± 15.6*†§
RAG-2$^{-/-}$/$\gamma_c^{-/-}$	gd5 B6 PLN	0.5	2/6	8.0 ± 5.5*†	7.2 ± 6.9*†
RAG-2$^{-/-}$/$\gamma_c^{-/-}$	gd7 B6 PLN	1.5	2/6	10.1 ± 8.6*†‡	0.7 ± 0.4*†
tgε26	NP adult SCID SC	0.76–1.0	3/9	2.9 ± 1.3*†¶	1.2 ± 1.6*†
tgε26	gd3 SCID SC	1.6–2.3	3/9	16.6 ± 0.4*†¶‖**	47.4 ± 0.9*†**
tgε26	gd5 SCID SC	2.0–2.8	3/9	21.8 ± 1.7*†¶‖**	56.7 ± 10.9*†**
tgε26	gd7 SCID SC	2.0–2.8	3/9	11.2 ± 5.9*†¶‖	24.4 ± 11.2*†
RAG-2$^{-/-}$/$\gamma_c^{-/-}$	gd3 CCR2$^{-/-}$SC	2.0–2.2	2/4	32.8 ± 4.7	57.5 ± 6.7
RAG-2$^{-/-}$/$\gamma_c^{-/-}$	gd5 CCR2$^{-/-}$SC	2.1–2.5	2/4	32.7 ± 11.9	54.4 ± 23.1
RAG-2$^{-/-}$/$\gamma_c^{-/-}$	gd3 CCR5$^{-/-}$SC	2.0–2.3	3/6	49.3 ± 11.0	70.3 ± 15.2
RAG-2$^{-/-}$/$\gamma_c^{-/-}$	gd5 CCR5$^{-/-}$SC	2.3–2.5	3/6	30.8 ± 6.2	32.6 ± 6.3*

[a] Intact thymus was transplanted.
*, $p < 0.05$; significantly different from uNK$^+$ controls.
†, $p < 0.05$; significantly different from uNK$^-$ controls.
‡, $p < 0.05$; significantly different from uNK cell density/mm^2 in the MT/MLAp of the members of the same treatment group.
§, $p < 0.05$; significantly different uNK cell density/mm^2 in PLN recipients from gd3 B6 donors than in other recipients from NP, gd5, or gd7 donors.
¶, $p < 0.01$; significantly different from uNK cell density/mm^2 in the MT/MLAp of the members of the same treatment group.
‖, $p < 0.001$; spleen cell recipients with significantly different uNK cell numbers than tgε26 receiving spleen cells from NP SCID donors.
**, $p < 0.01$; spleen cell recipients with significantly different uNK cell numbers from tgε26 receiving spleen cells from gd7 SCID donors.

indicate that the chemokines macrophage-inflammatory protein-1α, monocyte chemoattractant protein-1, and RANTES are not essential for uterine recruitment of pre-uNK cells.

Human PBL adhesion under shear to murine tissue sections

Human anti-CD56 mAb-labeled cells were assessed for adhesion under shear to frozen uterine sections from nonpregnant or pregnant B6 mice (gd3, 6, 10). Consistent with previous reports (33, 47), the proportion of labeled cells in test PBL suspensions was 10–12% CD56$^+$, with <1.5% being CD56^{bright}. Prelabeling with anti-CD56 (murine IgG1) did not affect the total number of cells bound to uterine tissues at any of the time points (data not shown). CD56$^+$ cells adhered to nonpregnant endometrial stroma in a randomly dispersed, low frequency manner (Fig. 2). Numbers of CD56$^+$ cells adhering to nondecidualized uterine stroma markedly increased in gd3 tissue, and the adhering cells remained randomly distributed. At gd6 and 10, cell adhesion was significantly elevated, compared with nonpregnant uterus ($p < 0.001$), and the cells were restricted to the mesometrial side of the uterus and localized exclusively to DB. The adhering CD56$^+$ cells were predominantly small sized (6.8 ± 0.4 μm, measured from 12 adherent cells). A minor adhering population (1–10%) of larger, CD56$^+$ cells (8.01–9.01 μm, measured from three adherent cells) was also detected (Fig. 2*B*). In comparison with the numbers of CD56^{bright} cells in the initial overlay cell suspension, adhesion to uterine tissue significantly enriched the CD56^{bright} cells (Fig. 2). Pregnancy-induced adhesion was dependent on both L-selectin and α_4 integrin homing receptors (Fig. 2) because it was inhibited by specific function-blocking Abs, i.e., DREG-56 and HP2/1, respectively, but not by an IgG1 isotype-matched control Ab. Pregnancy induced dramatic and unexpected gains in lymphocyte adhesion in HEV of PLN from gd6 in C56/BL6 mice (Fig. 2). As predicted from earlier reports, lymphocyte adhesion to LN HEV from nonpregnant and pregnant mice was L-selectin dependent and did not involve α_4 integrin (32). Only uniformly sized small lymphocytes bound to PLN HEV.

Discussion

This is the first comprehensive study, in any species, to address the source of the immediate precursors of uNK cells in a pregnant adult. Availability of NK/uNK cell-deficient mice that reliably carried pregnancies was central to the study's success. Following transplantation of uterine segments from NK$^+$ mice into NK/uNK cell-deficient mice, no uNK cells were found in decidual tissue within the grafts, or at any of the implantation sites in host tissue. The latter observation excluded migration of pro/pre-uNK cells from the graft segments into host tissue and established that mouse uterus does not contain self-renewing pro/pre-uNK cells. Uterine and oviductal grafts used in published studies (11–14) may have scarred and died due to problems of excessive length, inadequate

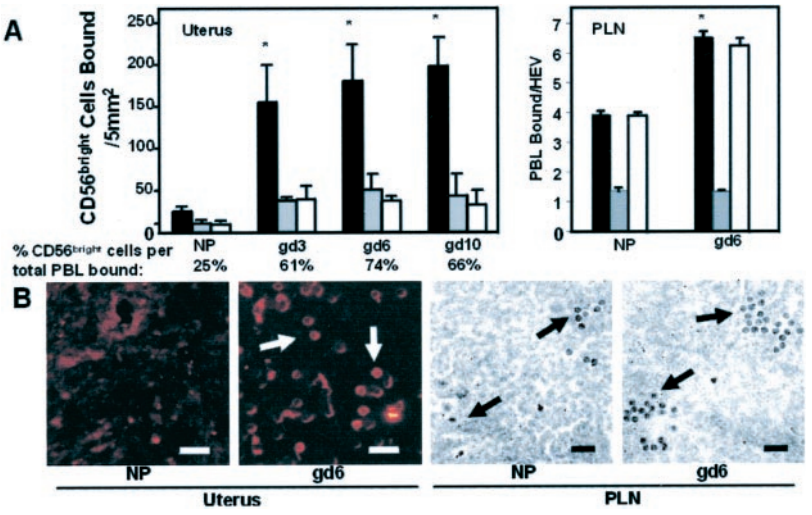

FIGURE 2. Human lymphocyte adhesion in uterine and peripheral LN tissues. *A*, Uterine or PLN tissues were isolated from nonpregnant (NP) and pregnant mice at various times of gestation, cryopreserved, and used in adhesion assays. Before assay, lymphocytes were incubated for 30 min with function-inhibiting mAb specific for L-selectin (DREG-56; ▨), α_4 integrin (HP2/1; □), or an isotype-matched control Ab (mouse IgG1; ■). To permit discrimination of NK cells bound to uterine tissues, PBL were initially stained with anti-CD56 mAb and goat anti-mouse IgG-RITC secondary Ab. The initial PBL sample was comprised of 11% $CD56^+$ cells and <1.5% $CD56^{bright}$ cells (not shown). The relative proportion of $CD56^{bright}$ cells within the total lymphocyte adherent populations was determined by direct analysis of 10 high powered fields (5-mm^2 area) under fluorescence and light microscopy. ∗, Denotes statistical differences calculated using an unpaired two-tailed Student's *t* test for the increase in adhesion detected in response to pregnancy, $p < 0.001$. Data are the mean ± SD of triplicate specimens prepared using a single lymphocyte pool. Results are representative of three independent studies, each using different human donors and a different series of gestationally timed murine tissues. *B*, Photomicrographs showing adhesion of $CD56^{bright}$ cells to uterine tissues (*left panels*) or toluidine-stained PBL bound to PLN HEV (*right panels*). Fluorescently labeled lymphocytes were not detected in uterine tissues when PBL were stained with isotype-matched control Ab and goat anti-mouse IgG-RITC secondary Ab (not shown). Bar, 25 μm.

perfusion, and/or immune rejection. Our choice of an immune-deficient host eliminated host vs graft rejection. Early graft vs host disease was not a problem, as allografts from CD1 were as equally viable and hormone responsive as T cell-deficient SCID allografts. Duration of the transplantation experiments was shorter (17 days) than mouse gestation (19–20 days), permitting the conclusion that uterine recruitment most likely occurs during gestations. Previous grafting of mated, immunocompetent mice with virgin uterine tissue in sealed diffusion chambers showed that uterus has some pre-uNK cells with a 12-day maximum survival time (37).

To explain the dramatic rise in human uNK cells during decidualization, some authors suggest that uNK cells self-renew in the uterus (38), while others suggest direct trafficking from BM is the major supply mechanism (39). Our studies indicate that both ideas may be incorrect. In our adoptive transfers, BM made only a minor contribution to uNK cell numbers during early to mid gestation. Despite the known and observed pregnancy-induced involution of BM (Table II), there was no loss in BM-derived progenitors able to populate uterus with uNK cells, during the first trimester of pregnancy. Levels of uNK cell generation from thymus were also low and independent of donor age or pregnancy status. However, our results show for the first time that thymus retains its capacity for NK cell generation into adulthood.

LN was shown to be a source of pre-uNK cells, but not all LN had transplantable pre-uNK. Hypertrophy was anticipated in the iliosacral LN draining the uterus (26, 27), but was not measurable when these smaller nodes were pooled with the much larger mesenteric LN chain. This pool, which includes the LN draining the pelvic region, hind limb, and uterus, had limited pre-uNK cells. Transplantable pre-uNK cells were present in PLN. However, further study would be necessary to precisely define which LN in the PLN pool contained transplantable pre-uNK. Development of the LN in the PLN that were pooled is heterogeneously regulated with some nodes (i.e., cervical) differentiating analogously to MLN (40). These nodes would perhaps lack transplantable pre-uNK cells.

Spleen contained pre-uNK cells that were mobilized to the uterus by pregnancy. In comparison with nonpregnant donors, SC numbers doubled in pregnant donors, while numbers of uNK cell progeny increased 4–47 times (Table II). This suggests that numerical alterations are not the sole pregnancy-induced changes in SC accounting for uNK cell reconstitution. The developmental stages of hemopoietic cells that move into the uterus are not yet known. Because uNK cells differentiating from thymus, BM, LN, and SC are identical morphologically and morphometrically and match those in gd10 unmanipulated, normal mice, the cells that moved into the uterus from these tissues were probably at relatively similar stages of differentiation. Alternatively, uNK cells may differentiate rapidly, and cells at various pro/pre-uNK stages may have had sufficient time to complete differentiation under our experimental conditions. The heterogeneity in size of human lymphocytes adhering to murine uterus suggests that circulating cells at more than one stage of differentiation/activation may have uterine homing potential. Despite the fact that splenic NK cells express the genes for chemokine receptors CCR2 and CCR5 (Refs. 28 and 29; W. A. Kuziel, unpublished data) and that pregnant mouse and human uteri express high levels of the ligands for these receptors (30, 31), we found that the targeted absence of either CCR2 or CCR5 on pre-uNK cells did not reduce homing from spleen to uterus. These observations raise the possibility that the CCR2 and CCR5 chemokine receptors are functionally redundant, or that pre-uNK cells use alternative receptors for their recruitment.

Functional assays of $CD56^+$ lymphocyte (male or female) adhesion to frozen tissue sections under shear showed that all uteri (nonpregnant and gestational) bound labeled cells, including a rare population of larger cells. Adherent cell localization was dramatically altered by development of decidua, changing from random to mesometrial indistribution. At gd6, adhering cells were absent

over fetal trophoblasts and maternal MT/MLAp regions and localized exclusively over DB. In gd10 mice, ~10% of the large, mature uNK cell population is intravascular in DB, while intravascular uNK cells are rare in MLAp, a domain containing uNK cells with less mature morphology (41). Thus, adherence of $CD56^+$ cells in DB could indicate a specialized endothelium participating in pre-uNK cell recruitment and/or serving as a target of uNK cell functions within implantation sites.

Selective recruitment of NK cells to uterus most likely involves dual control of adhesion molecules on both trafficking NK cells and target uterine vascular endothelial cells. $CD56^+$ cell adhesion to uterine tissues was dependent on both L-selectin and α_4 integrins. Coordinate expression of these molecules has been previously documented on circulating $CD56^{bright}$ cells, with L-selectin expressed at very high density (33). Both L-selectin and α_4 integrins (i.e., $\alpha_4\beta_7$ and $\alpha_4\beta_1$) function as gatekeepers, controlling lymphocyte extravasation through their ability to bind to vascular addressins under shear (32, 43). Thus, it is tempting to speculate that pregnancy induces differential display of vascular adhesion molecules on uterine microvessels. The almost complete blocking of $CD56^{bright}$ cell adhesion by mAb to both L-selectin and α_4 integrin suggested that these adhesion molecules are used by the same $CD56^{bright}$ subset. The data are consistent with the coutilization of a common endothelial ligand for both L-selectin and α_4 integrin, analogous to the situation in Peyer's patches (PP) in which trafficking of naive lymphocytes is mediated by cooperative interactions of L-selectin and $\alpha_4\beta_7$ integrins with spatially distinct domains within the mucosal addressin cell adhesion molecule (MAdCAM-1) (44, 45). Blockade of L-selectin and α_4 integrins under the same conditions as used in the present study results in complete inhibition of human PBL binding to PP HEV (46). Alternatively, if independent ligands are involved in mediating $CD56^+$ NK cell adhesion to uterine tissues, partial inhibition by L-selectin and α_4 integrin-blocking mAb might be expected in frozen-section adhesion assays, as reported for mesenteric LN that dually express peripheral LN addressins (PNAd; an L-selectin ligand) and MAdCAM-1 (a ligand for α_4 integrins and L-selectin) (44–45).

The ligands for α_4 integrins and L-selectin in uterine stroma remain to be defined. Recent studies by Kruse et al. (42) showed VCAM-1 is restricted to endothelium of the central DB in gd9 mice. While this could account for the α_4 integrin-mediated binding observed in the present study, caution must be used in this interpretation because extracellular matrix is also exposed in the assayed tissue and α_4 integrins bind to fibronectin, a molecule prevalent in mesometrial decidua (47). High level MAdCAM-1 expression has also been reported on mesometrial endothelium in the vascular zone, lateral to the DB in gd9 mice (42). This counterreceptor for L-selectin and $\alpha_4\beta_7$ in PP (32) may have the same function in decidualized uterus. Message for glycosylation-dependent cell adhesion molecule-1, an L-selectin ligand (48), is upregulated mesometrially between gd6 and 10 in B6 uterus, while P-selectin glycoprotein ligand-1, a P-selectin ligand (49) used by NK cells, is constitutively expressed at these times (S. Chantakru and B. A. Croy, unpublished data). Regardless of the mechanisms involved, exposure of indicator PBL to pregnant uterine tissue results in preferential adhesion and the enrichment of the $CD56^{bright}$ NK cell subset. Further studies are required to determine whether pregnancy alters human lymphocyte function in the binding assay. This outcome is anticipated because pregnancy conferred increased potential for reconstitution of uNK cells in the splenocyte adoptive transfer experiments and from recent studies in our laboratories showing that pregnancy promotes mouse splenocyte adhesion to HEV in LN from virgin mice (S. Chantakru, W. C. Wang, B. A. Croy, and S. S. Evans, manuscript in preparation).

Pregnancy, unexpectedly, altered lymphocyte/endothelial interactions in nonuterine sites. Thus, elevated adhesion to LN HEV was observed by gd3, whereas maximal adhesion occurred by gd6 that was sustained throughout pregnancy (shown in this study, S. Chantakru, W. C. Wang, B. A. Croy, and S. S. Evans, manuscript in preparation). The physiological roles of these changes in endothelial cells in peripheral lymphoid tissues are undefined, but they may contribute to cell recruitment to the uterus or in immune protection by surveillance for fetal Ag-sensitized cells. This study has shown that pregnancy induces functional changes in lymphocytes in vivo and adhesion molecules on endothelial cells, and it provides novel transplantation and adhesion assays that will facilitate further studies on the mechanisms regulating pre-uNK cell recruitment to the uterus in both mice and women.

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