Interferon γ Contributes to Initiation of Uterine Vascular Modification, Decidual Integrity, and Uterine Natural Killer Cell Maturation during Normal Murine Pregnancy

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Abstract

The dominant lymphocytes in human and murine implantation sites are transient, pregnancy-associated uterine natural killer (uNK) cells. These cells are a major source of interferon (IFN)-γ. Implantation sites in mice lacking uNK cells (alymphoid recombinase activating gene [RAG]-$2^{-/-}$ common cytokine receptor chain γ [γ_c]$^{-/-}$) or IFN-γ signaling (IFN-$\gamma^{-/-}$ or IFN-γR$\alpha^{-/-}$) fail to initiate normal pregnancy-induced modification of decidual arteries and display hypocellularity or necrosis of decidua. To investigate the functions of uNK cell–derived IFN-γ during pregnancy, RAG-$2^{-/-}\gamma_c^{-/-}$ females were engrafted with bone marrow from IFN-$\gamma^{-/-}$ mice, IFN-γ signal-disrupted mice (IFN-γR$\alpha^{-/-}$ or signal transducer and activator of transcription [Stat]-$1^{-/-}$), or from mice able to establish normal uNK cells (severe combined immunodeficient [SCID] or C57BL/6). Mated recipients were analyzed at midgestation. All grafts established uNK cells. Grafts from IFN-$\gamma^{-/-}$ mice did not reverse host vascular or decidual pathology. Grafts from all other donors promoted modification of decidual arteries and decidual cellularity. Grafts from IFN-γR$\alpha^{-/-}$ or Stat-$1^{-/-}$ mice overproduced uNK cells, all of which were immature. Grafts from IFN-$\gamma^{-/-}$, SCID, or C57BL/6 mice produced normal, mature uNK cells. Administration of murine recombinant IFN-γ to pregnant RAG-$2^{-/-}\gamma_c^{-/-}$ mice initiated decidual vessel modification and promoted decidual cellularity in the absence of uNK cells. These in vivo findings strongly suggest that uNK cell–derived IFN-γ modifies the expression of genes in the uterine vasculature and stroma, which initiates vessel instability and facilitates pregnancy-induced remodeling of decidual arteries.

Key words: interferon γ signaling • uterine lymphocytes • decidual spiral arteries • bone marrow transplantation • tumor necrosis factor α

Introduction

IFN-γ, a cytokine secreted predominantly by activated NK cells and T cells, has important regulatory effects on many cell types (1, 2). During early pregnancy in humans and rodents, an NK cell subset, known as uterine (u)NK[1] cells, is the most abundant maternal lymphocyte population in the uterus (3, 4). IFN-γ mRNA has been documented in healthy human and murine implantation sites (5, 6). uNK cells are among the cells expressing IFN-γ mRNA and are positive for IFN-γ protein (7, 8). In normal mice and T and B cell–deficient SCID mice, concentrations of IFN-γ have been measured in the mesometrial triangle where uNK cells aggregate. Local IFN-γ concentrations increased from baseline values found in nonpregnant uteri to peak amounts of ~10 U per implantation site on gestation day (gd) 10 (9). In tgε26 females (uNK$^-$NK$^-$T$^-$), pregnancy induced a 10-fold lower elevation in mesometrial IFN-γ, suggesting that uNK cells are the major source of IFN-γ in the pregnant mouse uterus. At midgestation, histologic anomalies are found within implantation sites of mice genetically ablated in IFN-γ or IFN-γRα. Three striking features are consistent. First, there are excessive numbers of uNK cells, which are uncharacteristically small and have limited numbers of cytoplasmic granules (9). Second, the major decidual arteries, analogous to the uterine spiral arteries of women, do not undergo normal gestation-induced remodeling. Third, decidualization is initiated, but cellular-

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[1]*Abbreviations used in this paper:* BM, bone marrow; BV, blood vessels; gd, gestation day; MLAp, mesometrial lymphoid aggregate of pregnancy; PAS, periodic acid-Schiff; RAG, recombinase activating gene; Stat, signal transducer and activator of transcription; u, uterine.

ity of the decidua is not maintained. In contrast to the hypocellularity of decidua in uNK cell–deficient mice, decidua in IFN-$\gamma^{-/-}$ and IFN-$\gamma R\alpha^{-/-}$ mice progress to overt necrosis during the second half of gestation. These findings led us to hypothesize that uNK cell–derived IFN-γ contributes to initiation of pregnancy-induced uterine vascular modification, maintenance of decidual integrity, and regulation of maturation and senescence of the uNK cell population.

During pregnancy, distinctive changes occur in uterine tissues including endometrial stromal cells, leukocytes, and blood vessels (BV). In primates and rodents with hemochorial placentae, decidualization of the uterus is hormonally regulated (10, 11). Decidualization is triggered late in each menstrual cycle in women and by implantation in mice (10, 12). During decidualization, activation of small agranular lymphocytes, believed to be precursors of uNK cells, is thought to occur and lead to the appearance of granulated uNK cells that rapidly proliferate within the uterus. In mice, mature uNK cells become localized to the mesometrial side of each implantation site. Some are found within arterial vessels and tissue of the decidua basalis, but more form a lymphocyte-rich structure known as either the mesometrial lymphoid aggregate of pregnancy (MLAp) or the metrial gland (13). uNK cells reach peak numbers and IFN-γ production at about the middle of the 19-d mouse gestation (9). Beginning at gd 12, uNK cells undergo progressive nuclear fragmentation and decline in number (14). In mice, the major decidual arteries undergo gestationally induced modification beginning about gd 9 (15). This process involves thinning of the muscular coat, increasing lumen diameter, and vessel elongation (16).

tgϵ26 females transplanted with bone marrow (BM) from SCID mice have high numbers of uNK cells, decidual vessel modification, and normal decidual cellularity during pregnancy (17). This suggests that the highly specific local-

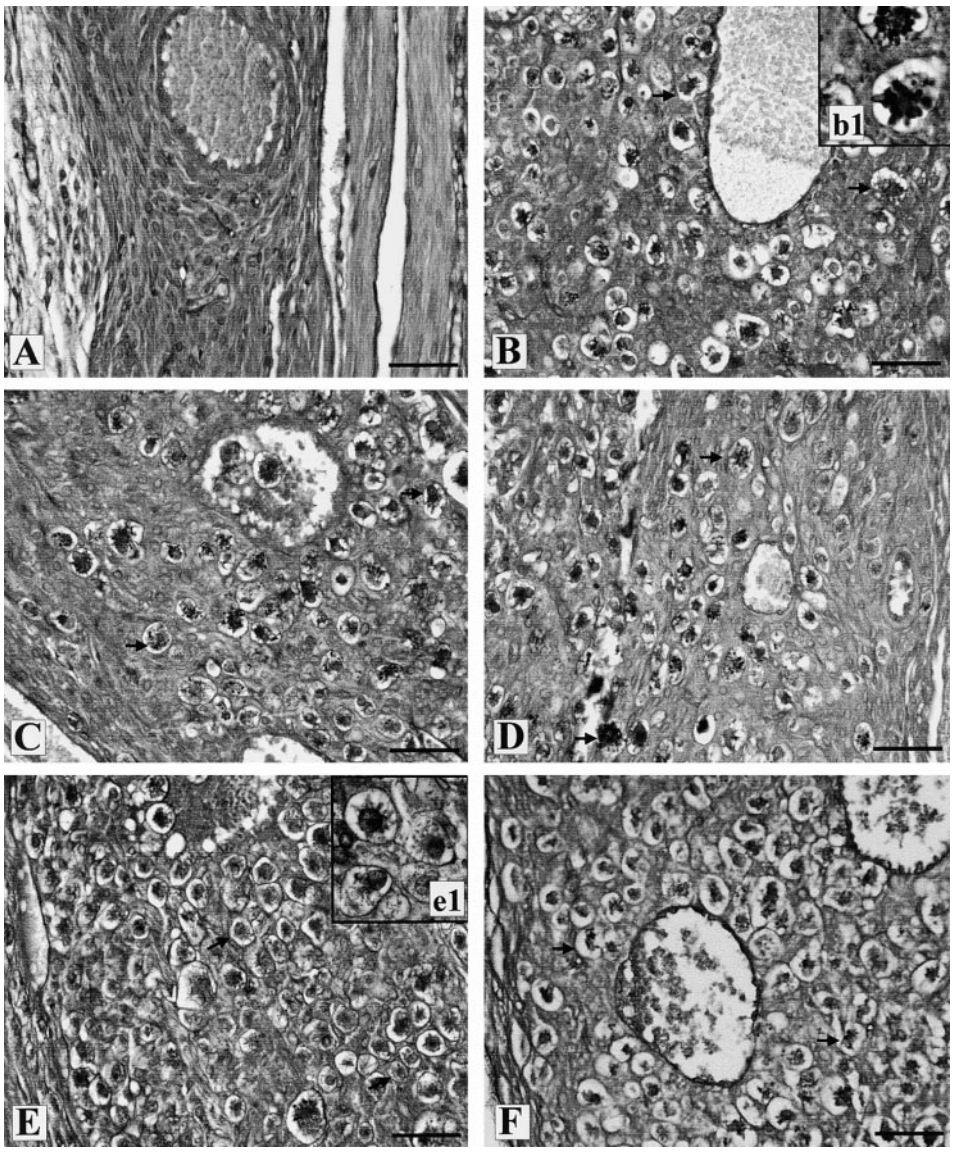

Figure 1. Photomicrographs of uNK cells in MLAp cross-sections from BM-reconstituted RAG-$2^{-/-}$ $\gamma_c^{-/-}$ mice (B–F) and nonmanipulated RAG-$2^{-/-}\gamma_c^{-/-}$ mice (A) at gd 12. Excessive numbers of uNK cells (arrows) with immature appearance and poor granule development were present in MLAp of RAG-$2^{-/-}\gamma_c^{-/-}$ mice receiving BM from IFN-$\gamma R\alpha^{-/-}$ or Stat-$1^{-/-}$ mice (E and F) compared with those receiving control B6 BM (B). e1, representative hypogranular uNK cells from E and F at higher magnification. Normal frequencies of mature uNK cells were observed in MLAp of RAG-$2^{-/-}\gamma_c^{-/-}$ mice engrafted with BM from IFN-$\gamma^{-/-}$ or SCID mice (C and D). b1, representative uNK cells with normal granularity from B–D at higher magnification. No uNK cells were present in nonmanipulated RAG-$2^{-/-}\gamma_c^{-/-}$ mice (A). A–F were stained with PAS. Bars, 50 μm.

ization of uNK cells may provide short range cytokine signaling that could influence gene expression in target tissues including BV walls, endothelium, and the uterine stroma (18). IFN-γ regulates the expression of >0.5% of the mouse genome, including genes involved in smooth muscle cell proliferation, cell adhesion, regulation of MHC gene expression, apoptosis, and processing pathways for protein synthesis and packaging (2, 19). Specific examples of IFN-γ–regulated genes that could be important in implantation sites include inducible nitric oxide synthase (iNOS), endothelial (e)NOS, and endothelin-1, all major regulators of vascular contractility (2), and α_2-macroglobulin, a regulator of proteases and cytokines that is the major known product of mesometrial decidua (reference 20 and our unpublished data).

The studies presented here were undertaken to assess the role of IFN-γ derived from uNK cells in decidual vascular remodeling, maintenance of decidual integrity, and maturation of uNK cells in vivo. In this work, recombinase activating gene (RAG)-$2^{-/-}$ common cytokine receptor chain γ $(\gamma_c)^{-/-}$ mice were used as transplant recipients. Due to combined deficiencies of products from RAG-2 and γ_c, these females lack all lymphocyte subsets but are reliable breeders. RAG-$2^{-/-}\gamma_c^{-/-}$ are superior to tgε26 females for these studies because they have an absolute deficiency in uNK cells rather than the greatly reduced frequency observed in tgε26 mice (1% of normal; reference 21). Histologically, the anomalies of the decidual vessels and decidua are identical in both strains, and pregnancy in RAG-$2^{-/-}\gamma_c^{-/-}$ mice induces mesometrial IFN-γ at the same low concentrations as in tgε26 mice (9, 15, 21). Different patterns of morphological changes were documented, without pregnancy loss, in the decidual arteries, decidua, and MLAp of syngeneically mated RAG-$2^{-/-}\gamma_c^{-/-}$ females that received BM from cytokine or cytokine signal gene–disrupted donors or repeated injections of murine recombinant (mr)IFN-γ. This shows that IFN-γ plays an important role in healthy implantation sites and that the RAG-$2^{-/-}\gamma_c^{-/-}$ mouse is a new and informative model with which to address the biological significance of IFN-γ in the pregnant mammalian uterus.

Materials and Methods

Mice. IFN-$\gamma^{-/-}$, IFN-γRα$^{-/-}$, TNF-$\alpha^{-/-}$, TNF-R1$^{-/-}$, and C57BL/6J (B6) mice were obtained from The Jackson Laboratory. Signal transducer and activator of transcription (Stat)-$1^{-/-}$ mice and breeding pairs of C.B-17 *scid/scid* (SCID) mice were purchased from Taconic Farms Inc. B6 congenic RAG-$2^{-/-}\gamma_c^{-/-}$ mice (22) were bred under barrier husbandry at the University of Guelph (OMAFRA Isolation Unit). Female RAG-$2^{-/-}\gamma_c^{-/-}$, Stat-$1^{-/-}$, or B6 mice were mated by overnight cohabitation with syngeneic males. The morning a vaginal plug was observed was considered gd 0. At gd 12, pregnant mice were killed by CO_2 inhalation, which was followed by cervical dislocation. Pregnant uteri were dissected and viable implantation sites were enumerated and then processed for study. No significant fetal loss was observed after any of the matings.

Tissue Acquisition and ELISA Quantification of IFN-γ. To quantify mesometrial concentrations of IFN-γ, mesometrial tissues were dissected from nonpregnant RAG-$2^{-/-}\gamma_c^{-/-}$ mice or from implantation sites on gd 6–8, 10–13, or 16. Dissected tissue was immediately homogenized, and supernatants were collected and assayed for IFN-γ using a standard ELISA as described previously (9).

BM Transplantation. 6–8-wk-old RAG-$2^{-/-}\gamma_c^{-/-}$ females were used as recipients of BM. Each recipient was pretreated with a single i.p. injection of 150 mg/kg of 5-fluorouracil (5-FU) 48 h before BM infusion (23). 6–8-wk-old IFN-$\gamma^{-/-}$, IFN-γRα$^{-/-}$, Stat-$1^{-/-}$, SCID, B6, TNF-$\alpha^{-/-}$, or TNF-R1$^{-/-}$ mice were used as BM donors. Donor cells were depleted of RBCs using hypotonic lysis. When donor cells were histoincompatible, pretreatment with anti–Thy-1.2 and complement was also used as

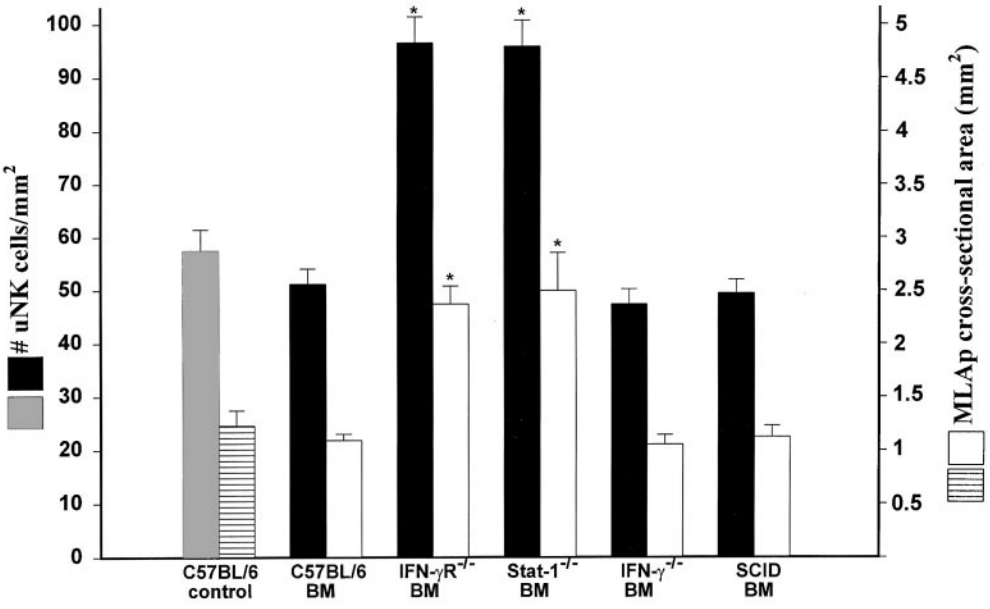

Figure 2. Density of uNK cells in MLAp and cross-sectional area morphometry of the MLAp in engrafted RAG-$2^{-/-}\gamma_c^{-/-}$ mice and control B6 mice at gd 12. The left y-axis indicates number of uNK cells per square millimeter in MLAp, and the right y-axis indicates MLAp cross-sectional area. RAG-$2^{-/-}\gamma_c^{-/-}$ recipients of IFN-γRα$^{-/-}$ or Stat-$1^{-/-}$ BM cells had larger MLAp areas and higher uNK cell density than B6 BM–engrafted controls (*$P <$ 0.01). No significant differences were found in uNK cell density and area of MLAp between RAG-$2^{-/-}\gamma_c^{-/-}$ recipients of IFN-$\gamma^{-/-}$ BM or SCID BM and B6 BM–engrafted controls. Values were obtained from 11 central cross-sections per implantation site, two to three implantation sites per dam. Four to five dams were used in each experimental group.

previously described (23, 17). A total of 2×10^7 viable BM cells was given i.v. to each 5-FU–treated RAG-$2^{-/-}\gamma_c^{-/-}$ female. 3 wk later, recipients were paired with RAG-$2^{-/-}\gamma_c^{-/-}$ males for mating and killed at gd 12.

Assay of mrIFN-γ and Treatment of Mice with mrIFN-γ. Homozygously mated RAG-$2^{-/-}\gamma_c^{-/-}$ mice were treated once per day for 6 d with mrIFN-γ (Sigma-Aldrich) or placebo (PBS) i.v. or i.p., beginning on gd 6, and killed at gd 12. Doses were 0 (n = 4), 100 (n = 2), 300 (n = 2), or 1,000 U (n = 5) in 0.2 ml of PBS. Viability of implantation sites was recorded, and viable implants were processed for study. Bioactivity of IFN-γ was confirmed in two assays: WEHI 279 cell proliferation inhibition (24) and viral inhibition using L-929 cells infected with encephalomyocarditis virus (25).

Histological Analysis. Implantation sites were fixed in Bouin's fixative (Fisher Scientific) and processed into paraffin using standard methodology. Two or three implantation sites from each pregnant mouse (four to five pregnant females per study group except in the mrIFN-γ treatment study) were serially sectioned (7 μm) and stained with hematoxylin and eosin or periodic acid-Schiff (PAS) before microscopic examination. The number of uNK cells per square millimeter was measured on 11 sections from the center of each implantation site and averaged as previously described (9). Cross-sectional areas of the MLAp and ratios for vessel/lumen diameters of the main decidual arteries, cut in cross-section, were measured on the same slides that were used for uNK cell numeration using OPTIMAS™ image analysis software (version 6.2; Optimas Corp.). The means of experimental groups were compared using two-factor ANOVA (analysis of variance). The variances between mice and implantation sites were considered as analytic factors ($P < 0.05$). Groups with significantly different means were identified using Tukey's test ($P < 0.05$).

Results

Mesometrial Concentrations of IFN-γ in RAG-$2^{-/-}\gamma_c^{-/-}$ Mice. Supernatants from homogenates of mesometrial tissues from cycling RAG-$2^{-/-}\gamma_c^{-/-}$ mice contained very low concentrations of IFN-γ (<0.1 U per uterus; data not shown). This is consistent with previously reported results

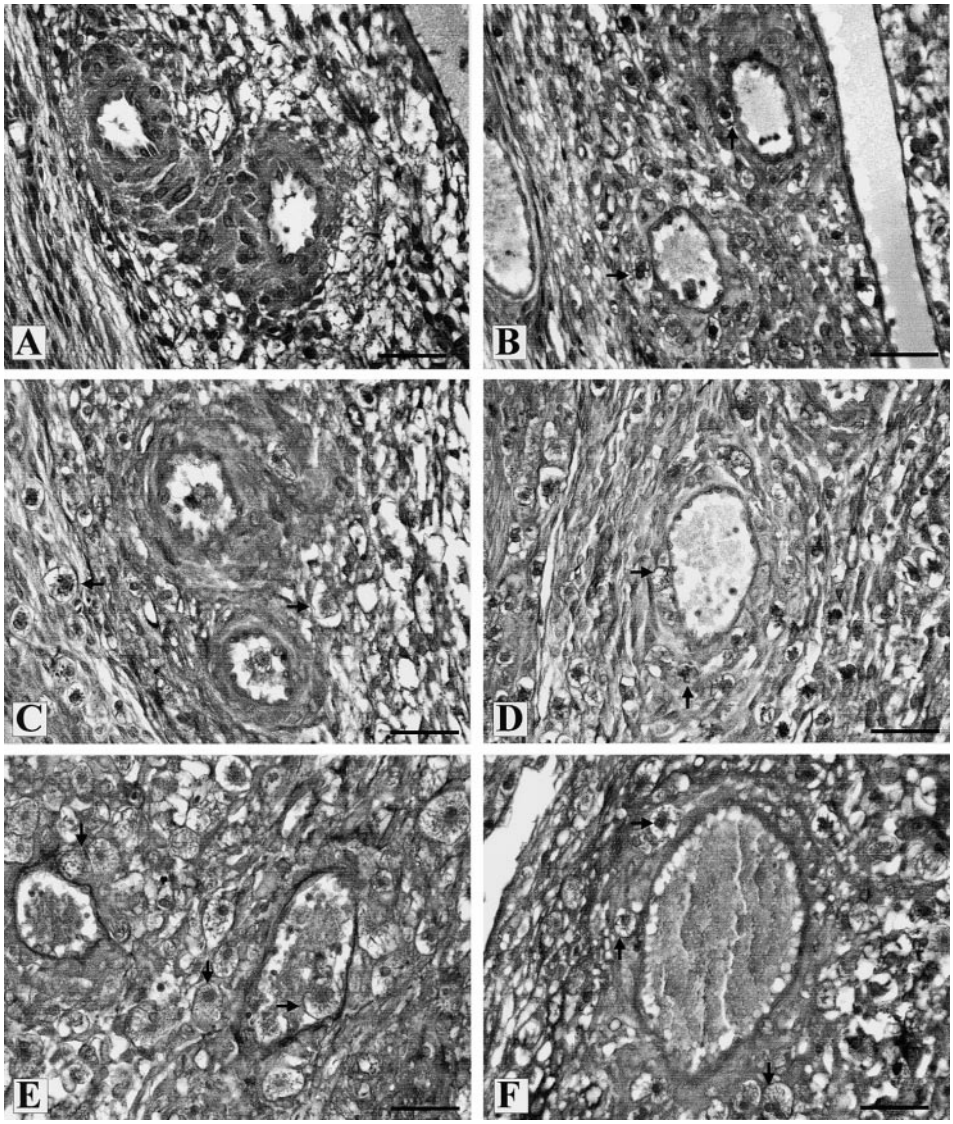

Figure 3. Comparison of decidual artery remodeling at implantation sites from nonmanipulated and BM-engrafted RAG-$2^{-/-}\gamma_c^{-/-}$ mice on gd 12. A is a typical cross-section of unmodified artery from an unmanipulated RAG-$2^{-/-}\gamma_c^{-/-}$ mouse. C is a typical cross-section of an unmodified decidual artery in a RAG-$2^{-/-}\gamma_c^{-/-}$ mouse engrafted with IFN-$\gamma^{-/-}$ BM. B–F show normal, pregnancy-induced remodeling of the spiral artery in RAG-$2^{-/-}\gamma_c^{-/-}$ mice infused with BM from B6, SCID, IFN-γR$\alpha^{-/-}$, or Stat-$1^{-/-}$ mice, respectively. Arrows point to vessel-associated uNK cells. A–F were stained with PAS. Bars, 50 μm.

in cycling normal and immune-deficient mice (9). During pregnancy (gd 6–8, 10–13, and 16), the concentration of IFN-γ was stable at 1 U per implantation site. This is similar to results previously reported in $T^-NK^-uNK^-B^+$ tgε26 mice and is different from the results in uNK^+ mice. The measured values were above background (data not shown), since the assay did not detect IFN-γ in mesometrial uterine homogenates from IFN-$\gamma^{-/-}$ females (9).

Assessment of Implantation Sites in RAG-$2^{-/-}\gamma_c^{-/-}$ Females Engrafted with Normal or IFN-γ Signal-disrupted BM Cells. Pregnancy in NK cell–deficient RAG-$2^{-/-}\gamma_c^{-/-}$ mice is associated with an absolute absence of uNK cells, absence of MLAp, hypocellularity of decidua, and unmodified decidual arteries, despite the low amounts of IFN-γ from nonlymphoid sources. To investigate the actions of IFN-γ derived from uNK cells, implantation sites were studied from RAG-$2^{-/-}\gamma_c^{-/-}$ females engrafted with BM cells unable to produce IFN-γ but able to respond to host IFN-γ (BM from IFN-$\gamma^{-/-}$ mice). These females were compared with RAG-$2^{-/-}\gamma_c^{-/-}$ females engrafted with BM cells able to produce IFN-γ (from B6 or SCID mice). To establish values for normal density and granularity of uNK cells and their effects on decidua and decidual arteries, in an engrafted RAG-$2^{-/-}\gamma_c^{-/-}$ female, implantation sites from RAG-$2^{-/-}\gamma_c^{-/-}$ recipients of B6 BM were compared with implantation sites from gd-matched unmanipulated congenic B6 mice. In comparison with unmanipulated B6 controls, transplantation of 2×10^7 BM cells from B6 to RAG-$2^{-/-}\gamma_c^{-/-}$ females established similar numbers of uNK cells with morphology typical for the gd 12, established similar cross-sectional areas of MLAp, induced comparable vessel/lumen ratios in the spiral arteries, and induced decidual cellularity that was indistinguishable morphologically. The values from the B6 transplanted RAG-$2^{-/-}\gamma_c^{-/-}$ mice were therefore used as the control for all other BM transplant experiments. Engraftment of RAG-$2^{-/-}\gamma_c^{-/-}$ females with BM from IFN-$\gamma^{-/-}$ mice restored normal numbers of uNK cells with morphology typical for gd 12 and induced an MLAp of normal size (Figs. 1 and 2). However, IFN-γ–deficient uNK cells failed to initiate pregnancy-induced remodeling of spiral arteries (Fig. 3). The decidual arterial vessel/lumen ratios in IFN-$\gamma^{-/-}$ BM–engrafted mice were significantly different from the controls but similar to those in nonengrafted RAG-$2^{-/-}\gamma_c^{-/-}$ mice (Fig. 4). Decidua in these mice were either necrotic (Fig. 5) or hypocellular compared with B6 BM–engrafted controls. These results highlight an essential role for uNK-derived IFN-γ in arterial remodeling during pregnancy.

Consistent with previous observations in tgε26 mice (17), RAG-$2^{-/-}\gamma_c^{-/-}$ females receiving BM from SCID mice demonstrated normal density of morphologically typical uNK cells, cross-sectional areas of MLAp, remodeling of decidual arteries, and decidual cellularity similar to that in B6 BM–engrafted controls (Figs. 1–5). This experiment identifies NK cells rather than T cells as the important cell type providing IFN-γ within implantation sites.

Absence of IFN-γ signaling pathways is associated with excessive numbers of small, hypogranular uNK cells, huge MLAp, unmodified arteries, and necrosis in decidua during pregnancy (reference 9 and our unpublished data). To investigate whether IFN-γ receptor signal–disrupted uNK

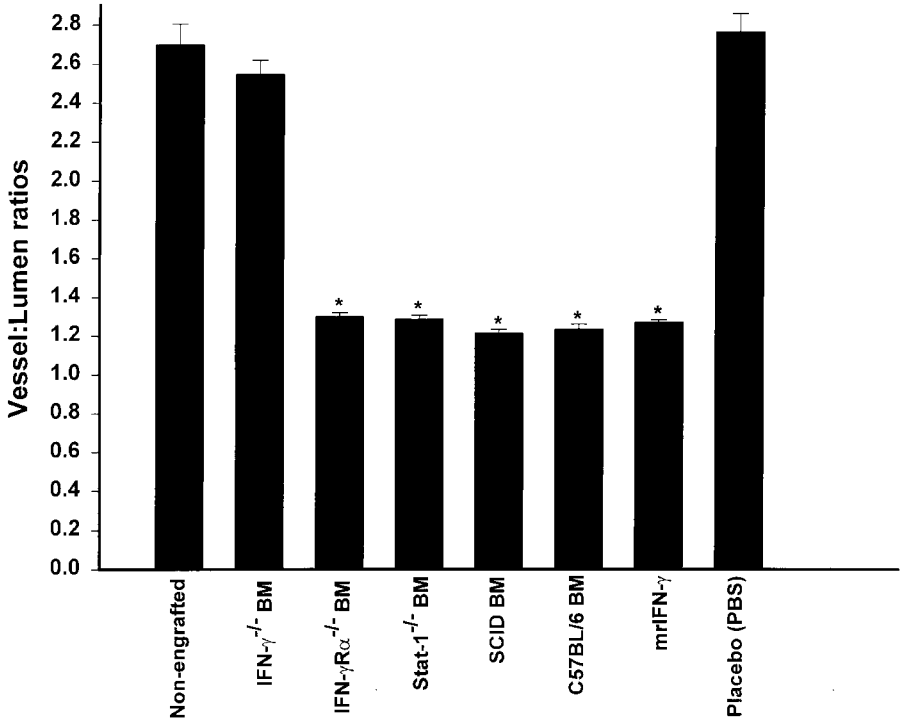

Figure 4. Histogram comparing mean ratios of decidual vessel/lumen diameter in unmanipulated and BM-engrafted RAG-$2^{-/-}\gamma_c^{-/-}$ mice at gd 12. RAG-$2^{-/-}\gamma_c^{-/-}$ mice engrafted with BM from SCID, IFN-γRα$^{-/-}$, or Stat-$1^{-/-}$ mice or treated with mrIFN-γ (*) had lower vessel/lumen ratios compared with those of unmanipulated RAG-$2^{-/-}\gamma_c^{-/-}$ mice. There were significant differences between RAG-$2^{-/-}\gamma_c^{-/-}$ mice engrafted with BM from IFN-$\gamma^{-/-}$ mice, unmanipulated or treated with PBS. The mean decidual vessel/lumen diameter ratio in normal C57BL/6 mice was 1.17 ± 0.11. Values were obtained from 11 central cross-sections per implantation site, 2–3 implantation sites per dam. 4–5 dams were used in each experimental group.

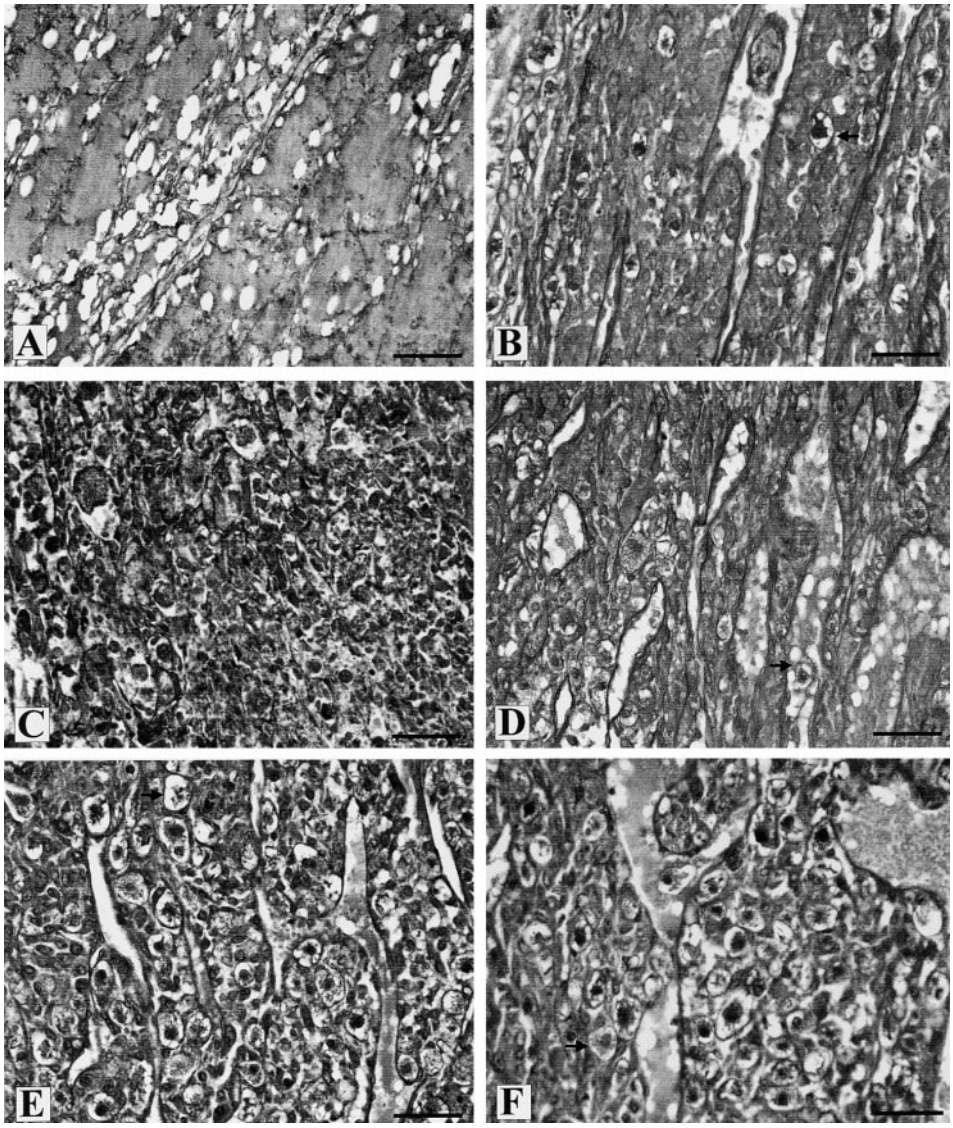

Figure 5. Comparison of decidual morphology in unmanipulated and BM-engrafted RAG-$2^{-/-}\gamma_c^{-/-}$ mice at gd 12. A shows a very hypocellular edematous decidua from an unmanipulated RAG-$2^{-/-}\gamma_c^{-/-}$ mouse. C is decidua from RAG-$2^{-/-}\gamma_c^{-/-}$ mice engrafted with IFN-$\gamma^{-/-}$ BM. Decidua in these recipients was either necrotic (C) or hypocellular (not shown). B–F show normal decidual morphology in RAG-$2^{-/-}\gamma_c^{-/-}$ mice engrafted with B6, SCID, IFN-$\gamma R\alpha^{-/-}$, or Stat-$1^{-/-}$ BM cells, respectively. Excessive numbers of uNK cells (arrows) were present in the decidua of RAG-$2^{-/-}\gamma_c^{-/-}$ recipients of IFN-$\gamma R\alpha^{-/-}$ or Stat-$1^{-/-}$ BM (E and F) compared with control (B). A–F were stained with PAS. Bars, 50 μm.

cells, able to produce IFN-γ but unable to respond to it, could induce normal changes to decidual vessels and tissue, RAG-$2^{-/-}\gamma_c^{-/-}$ females were infused with BM from IFN-$\gamma R\alpha^{-/-}$ or Stat-$1^{-/-}$ mice. Recipients had increased numbers of uNK cells and significantly larger MLAp compared with B6 BM–engrafted controls (Figs. 1 and 2). The IFN-$\gamma R\alpha^{-/-}$ and Stat-$1^{-/-}$ BM–derived uNK cells had low numbers of small cytoplasmic granules and appeared immature (Figs. 1 and 2). These transplants permitted normal, pregnancy-induced remodeling of decidual arteries (Fig. 3) and sustained highly cellular decidua (Fig. 5). Mean vessel/lumen ratios from IFN-$\gamma R\alpha^{-/-}$ or Stat-$1^{-/-}$ BM–engrafted RAG-$2^{-/-}\gamma_c^{-/-}$ females were not significantly different from those in control B6 BM–engrafted mice (Fig. 4).

Effects of Administration of mrIFN-γ on RAG-$2^{-/-}\gamma_c^{-/-}$ Implantation Sites. To determine if remodeling of decidual arteries and normal cellularity of decidua in RAG-$2^{-/-}\gamma_c^{-/-}$ females reconstituted with IFN-γ–competent uNK cells could be fully attributed to IFN-γ, pregnant RAG-$2^{-/-}\gamma_c^{-/-}$ mice were treated with mrIFN-γ that was confirmed to be bioactive (data not shown). In these mice, the decidual arteries underwent pregnancy-induced remodeling, and the decidua had normal cellularity after treatment with all dosages examined (100–1,000 U/d for 6 d; Fig. 6). The arterial vessel/lumen ratios in IFN-γ–treated mice were similar to those in B6 BM–engrafted controls but different from those in pregnant RAG-$2^{-/-}\gamma_c^{-/-}$ mice treated with PBS or left untreated (Fig. 4).

Assessment of Implantation Sites in RAG-$2^{-/-}\gamma_c^{-/-}$ Females Infused with TNF-$\alpha^{-/-}$ or TNF-R1$^{-/-}$ BM Cells. TNF-α is a pleiotropic, uNK cell–derived regulatory cytokine (26). To assess whether cytokine-mediated effects of uNK cells are restricted to IFN-γ, RAG-$2^{-/-}\gamma_c^{-/-}$ females were engrafted with BM from TNF-$\alpha^{-/-}$ or TNF-R1$^{-/-}$ mice. uNK cells and MLAp were present at the implantation sites of reconstituted mice. Density (number of uNK cells per square millimeter) and granularity of uNK cells (Fig. 7) and the cross-sectional areas of MLAp were similar to those in

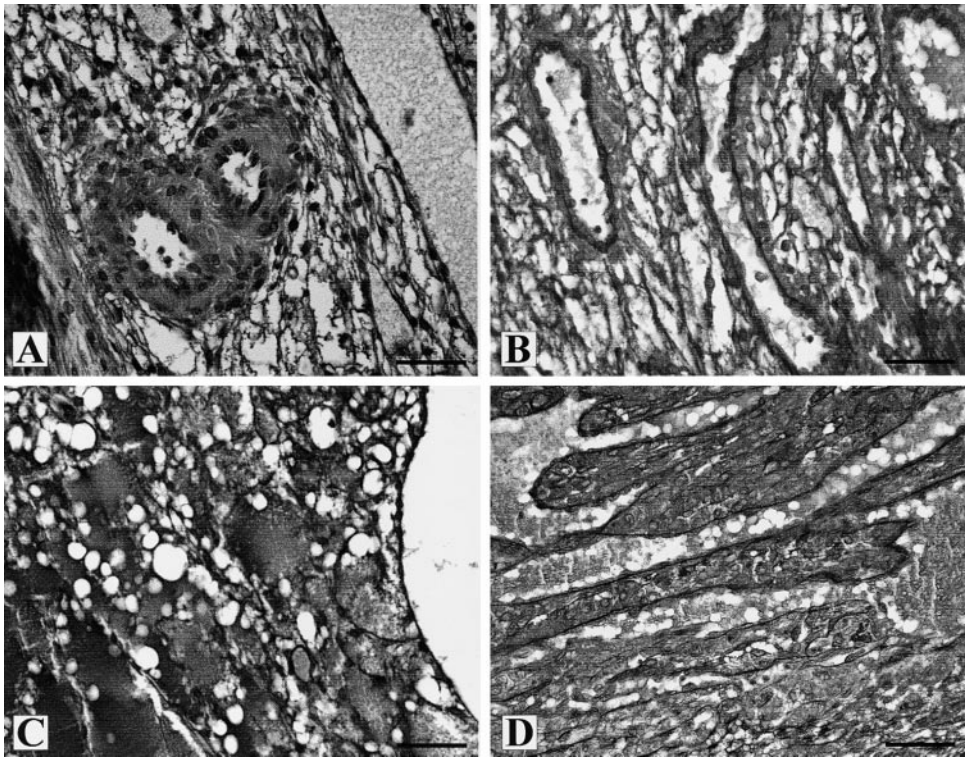

Figure 6. Comparison of decidua and decidual artery morphology in pregnant RAG-$2^{-/-}\gamma_c^{-/-}$ mice treated with placebo (PBS) or mrIFN-γ (1,000 U/d) for 6 d. A and C are cross-sections of unmodified spiral arteries and hypocellular decidua, respectively, from PBS-treated females. B is a cross-section of modified decidual artery from IFN-γ–treated females. D shows normal decidual morphology in IFN-γ–treated mice. A–D were stained with PAS. Bars, 50 μm.

RAG-$2^{-/-}\gamma_c^{-/-}$ females engrafted with B6 BM. Decidua in these recipients were normal histologically, and the decidual arteries had undergone normal pregnancy-induced modification (Fig. 7). The mean arterial vessel/lumen ratio was 1.26 ± 0.21, which is similar to the ratios in B6 BM–engrafted RAG-$2^{-/-}\gamma_c^{-/-}$ controls and different from ratios measured in pregnant, nonengrafted RAG-$2^{-/-}\gamma_c^{-/-}$ females. A summary of the results is shown in Table I.

Discussion

The goal of these experiments was to define the functions of uNK cell–derived IFN-γ during pregnancy. A new animal model was developed based upon manipulation of RAG-$2^{-/-}\gamma_c^{-/-}$ females, the first strain reported to be absolutely devoid of uNK cells (15). It was first necessary to define the amounts of IFN-γ produced at RAG-$2^{-/-}\gamma_c^{-/-}$ implantation sites. Pregnancy induced mesometrial IFN-γ at low but constant concentrations, supporting previous findings in tgε26 mice, an immune-deficient strain having 1% of normal levels of uNK cells (9, 21). This suggests that (a) the low constant production of IFN-γ in tgε26 is not derived from uNK cells, (b) nonlymphoid cells are induced by pregnancy to produce IFN-γ within implantation sites, and (c) uNK cells are the only cell population responsible for the midgestation (gd 10) rise in mesometrial IFN-γ concentrations. Macrophages, neutrophils, and/or decidual cells have been suggested as possible nonlymphoid sources of IFN-γ in the pregnant uterus (8, 27, 28).

Transplantation of IFN-$\gamma^{-/-}$ BM to RAG-$2^{-/-}\gamma_c^{-/-}$ mice generated implantation sites displaying only some of the features seen in homozygously mated IFN-$\gamma^{-/-}$ mice. Although uNK cells appeared typical and their total numbers were appropriately regulated, the decidua had the properties of the donor strain, not the host strain. The major difference between the host (RAG-$2^{-/-}\gamma_c^{-/-}$) and donor strains is that the host mesometrial uterus produces low amounts of IFN-γ, whereas the donor strain does not. This suggests that the terminal differentiation (not initial activation) and population size regulation of uNK cells is mediated by low concentrations of IFN-γ, which, however, are insufficient to influence the vascular target cells. In normal mice, small uNK cells with limited numbers of small granules are seen about gd 8, leading us to suggest that the excessively accumulated small, hypogranular cells reported here are arrested in their maturation by a block in IFN-γ signaling. An alternate explanation is that they are gd-appropriate in maturity but alternatively differentiated. The known roles of IFN-γ in regulation of apoptosis, via IFN regulatory factor 1 and bcl2 (2), are consistent with the observation of excessive uNK cell numbers at gd 10–14 in IFN-$\gamma^{-/-}$ mice but not in RAG-$2^{-/-}\gamma_c^{-/-}$ females transplanted with IFN-$\gamma^{-/-}$ BM. In normal mice, the regulation of uNK cell granularity frequency is probably autocrine, because normal uNK cells produce more IFN-γ than the nonlymphoid cells in the uterus. Engraftment of RAG-$2^{-/-}\gamma_c^{-/-}$ mice with normal B6 or with SCID ($T^-B^-NK^+$) BM restored normal uNK cells, resulting in full correction of the pathology seen in nonmanipulated RAG-$2^{-/-}\gamma_c^{-/-}$ females. This confirmed the central role of uNK cells in these changes but did not define the mechanisms involved.

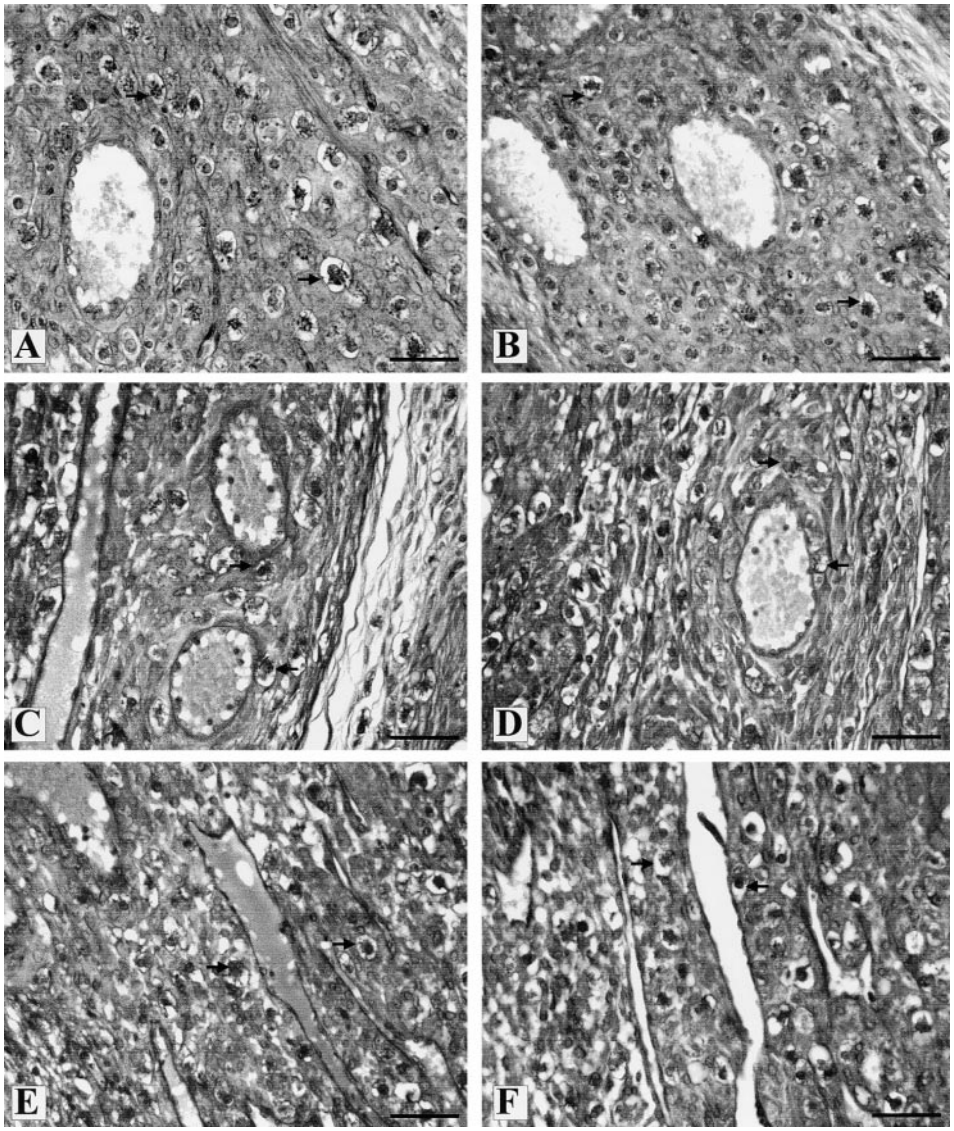

Figure 7. Photomicrographs of implantation site morphology from RAG-$2^{-/-}\gamma_c^{-/-}$ mice engrafted with TNF-$\alpha^{-/-}$ BM (A, C, and E) or TNF-R$1^{-/-}$ BM (B, D, and F). uNK cells were established with normal mature morphology and cell density (A and B) compared with B6 BM–engrafted control. Decidual arteries underwent normal pregnancy-induced remodeling (C and D, compared with Fig. 3 B). Decidua had normal morphology (E and F, compared with Fig. 5 B). Arrows point to uNK cells. A–F were stained with PAS. Bars, 50 μm.

Table I. *Summary of Results*

Experimental group	uNK cell frequency	uNK cell granularity	MLAp size	Decidual morphology	Arterial remodeling
Nonengrafted RAG-$2^{-/-}\gamma_c^{-/-}$	–	–	–	hypocellular	–
B6 (control)	N	N	N	N	N
RAG-$2^{-/-}\gamma_c^{-/-}$ + IFN-$\gamma^{-/-}$ BM	N	N	N	necrotic	–
RAG-$2^{-/-}\gamma_c^{-/-}$ + IFN-γR$\alpha^{-/-}$ BM	increased	decreased	increased	N	N
RAG-$2^{-/-}\gamma_c^{-/-}$ + Stat-$1^{-/-}$ BM	increased	decreased	increased	N	N
RAG-$2^{-/-}\gamma_c^{-/-}$ + TNF-$\alpha^{-/-}$ BM	N	N	N	N	N
RAG-$2^{-/-}\gamma_c^{-/-}$ + TNF-R$1^{-/-}$ BM	N	N	N	N	N
RAG-$2^{-/-}\gamma_c^{-/-}$ + mrIFN-γ	–	–	–	N	N
RAG-$2^{-/-}\gamma_c^{-/-}$ + PBS	–	–	–	hypocellular	–

N, normal.

RAG-2$^{-/-}\gamma_c^{-/-}$ recipients of IFN-γR$\alpha^{-/-}$ or Stat-1$^{-/-}$ BM demonstrated implantation sites that shared only part of the phenotype seen in homozygously mated IFN-γR$\alpha^{-/-}$ (9) or Stat-1$^{-/-}$ mice (our unpublished data). This phenotype was distinct from but complementary to that seen in RAG-2$^{-/-}\gamma_c^{-/-}$ mice engrafted with IFN-$\gamma^{-/-}$ BM. In recipients of IFN-γR$\alpha^{-/-}$ or Stat-1$^{-/-}$ BM, the decidual arteries became modified in a normal manner, as assessed by morphology and morphometric measurements. The established uNK cell population, however, was excessive in number and comprised of small, relatively hypogranular cells. These uNK cells are unimpaired in IFN-γ production; NK cells from IFN-γR$\alpha^{-/-}$ mice produce IFN-γ in amounts similar to those of their congenic partners (29, 30). The correction of both decidua and decidual BV anomalies in RAG-2$^{-/-}\gamma_c^{-/-}$ females engrafted with BM from IFN-γR$\alpha^{-/-}$ or Stat-1$^{-/-}$ mice indicates that uNK cell–derived IFN-γ is the initiator of these changes. Furthermore, IFN-γ–mediated gene regulation in uterine arteries and decidua appears to require higher concentrations of cytokine than the IFN-γ–mediated gene regulation in uNK cells. The immaturity and overgrowth of uNK cells is explained by their inability to respond to IFN-γ due to dysfunction in the signaling pathway involving receptor engagement or Stat-1 activation. This indicates that regulation of uNK cell maturation is by Stat-1–regulated genes.

The results obtained in mrIFN-γ–treated pregnant RAG-2$^{-/-}\gamma_c^{-/-}$ mice confirm that IFN-γ alone, independent of the presence of uNK cells, is sufficient to initiate pregnancy-induced remodeling of decidual arteries and to support integrity of decidua. IFN-γ, in the doses employed, has been reported by others to be highly abortifacient in mice, even with a single treatment (31, 32). One explanation for the survival of fetuses in IFN-γ–treated alymphoid RAG-2$^{-/-}\gamma_c^{-/-}$ females is that exogenous IFN-γ activates lymphocytes in normal mice that mediate abortion. However, daily treatment of fully immune competent B6 mice (n = 4) with 1,000 U of mrIFN-γ for 6 d in the same experiment did not elevate the resorption rates over PBS-treated B6 controls (data not shown). Thus, there appear to be genetic differences in susceptibility to IFN-γ–mediated abortion (33). In previous studies, high dose TNF-α given in combination with IFN-γ induced abortion in B6 mice (34). TNF-α is present in RAG-2$^{-/-}\gamma_c^{-/-}$ mice, suggesting that normal levels of TNF-α combined with high doses of IFN-γ are compatible with healthy pregnancy. Using transfer of TNF-$\alpha^{-/-}$ BM, we were unable to establish a role for TNF-α in initiating the normal pregnancy-induced remodeling of decidual arteries, maintenance of decidual integrity, or uNK cell maturation/senescence.

Pregnancy is associated with extensive uterine remodelling, cell proliferation, and cell invasion. For both uNK cells and decidua, it has been postulated that their major functions are to limit trophoblast invasion (35). This study discounts that idea, as placental size in all experiments matches that in B6 BM–engrafted RAG-2$^{-/-}\gamma_c^{-/-}$ mice and in B6 nonmanipulated pregnancy (data not shown). uNK cell–derived IFN-γ is not necessary for initiation of decidualization (9) or for its development to gd 6 but seems to be essential for decidual maintenance in the second trimester, gd 7–14 (9, 15). It is most surprising that collapse of decidua at midgestation does not lead to pregnancy disruption, whereas failure of decidualization in early gestation results in pregnancy termination (36). It has been documented ultrastructurally that some human uNK cells can form gap junctions with decidual cells and that this promotes survival of both cell types (10). Such data indicate that uNK cells may differ from other NK cell subsets by preferentially using decidua as their supporting stroma. The process of trophoblast invasion into the decidualized tissue is mediated by the balance between extracellular proteolytic enzymes and their inhibitor systems, including α_2-macroglobulin, a known product of mesometrial decidua that is regulated by IFN-γ (2, 37, 38). Dysregulation of these systems in the absence of IFN-γ may promote proteolysis of decidua and its matrix. In RAG-2$^{-/-}\gamma_c^{-/-}$ mice, low amounts of uIFN-γ may be weakly protective from proteolytic enzymes, providing a hypocellular phenotype. In IFN-$\gamma^{-/-}$, IFN-γR$\alpha^{-/-}$, or Stat-1$^{-/-}$ mice, proteolytic action may destroy late decidua gaining necrotic phenotype.

The mechanisms initiating mammalian pregnancy-induced uterine artery remodeling are not known. Adult BV, other than those of the uterus and ovary, are stable (39). Angiopoietin-1 is involved in BV stabilization when ligated to its receptor, Tie-2 (40). Ablation of murine Tie-2 disrupts vessel structure (41). In this study, relatively high concentrations of IFN-γ were required for initiation of uBV remodeling during pregnancy. uNK cells are highly migratory cells, and at gd 10, in mesometrial decidua of normal B6 pregnancy, 5% of uNK cells are in the arteries/arterioles and 25% are associated with the muscular walls of these vessels (our unpublished data and Fig. 3). It is likely that short range, high concentration uNK cell–derived IFN-γ signaling initiates destabilization of the decidual arteries, permitting vessel wall thinning and lumen increase by two complementary mechanisms. The first is indirect IFN-γ–regulated changes to gene expression in target tissues (smooth muscle, endothelial cells, and matrix) (18). The second is direct actions on BV. Human uNK cells express angiopoietin-2 (42), a natural competitive antagonist to the Tie-2 receptor of endothelium (40, 43), and they express NK5 (44), an angiogenic factor. Thus, in addition to initiating uterine arterial instability, uNK cells may play later roles in branching angiogenesis within implantation sites. Actions of host NK cells on allografted vessels are reported as destructive (45, 46). Future studies will be needed to clarify whether unusual aspects of fetal antigen presentation, uterine endothelial cell activation, decidual matrix composition, or the subsets of NK cells involved account for our novel finding that IFN-γ from uNK cells is central in initiation of uterine arterial remodeling.

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