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TGF-β drives the conversion of conventional NK cells into uterine tissue-resident NK cells to support murine pregnancy

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eLife Assessment

The importance of uterine natural killer (NK) cells in reproductive success has been demonstrated in mice and humans; however, it is still unclear how uterine NK cells are developed. In this **important** manuscript, the authors provide **convincing** evidence that TGF-b signaling in NK cells supports normal pregnancy in mice by the conversion of conventional NK cells into uterine tissue-resident NK cells. Previous concerns have been addressed in this revised version.

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Abstract

Tissue microenvironments shape lymphocyte differentiation to align immune function with local physiological demands. Uterine natural killer cells are critical for reproductive success, yet the molecular cues in the uterus that instruct their specialized identities remain incompletely understood. Here, we identify a TGF-β–dependent differentiation pathway by which circulating conventional NK cells convert into uterine tissue-resident NK cells during murine pregnancy. Loss of TGF-β receptor II expression in *Ncr1*-expressing cells disrupted this conversion, markedly reducing tissue-resident NK cells in the gravid uterus. Impaired TGF-β–driven uterine tissue-resident NK cell differentiation during murine pregnancy led to abnormal spiral artery remodeling and increased fetal resorption rates at midgestation, ultimately reducing litter sizes at birth. Collectively, these findings define TGF-β as a pivotal driver of tissue-resident NK cell differentiation in the gravid uterus and establish a mechanistic framework through which the uterine microenvironment programs NK cell identity to meet the physiological demands of gestation.

Introduction

Lymphocyte differentiation in response to local environmental cues is a fundamental mechanism by which the immune system adapts to tissue-specific demands. Outside of circulation, conventional NK (cNK) cells undergo regulated differentiation within tissue microenvironments that impose site-specific transcriptional programs to generate phenotypically and functionally discrete NK cell subsets[1–6]. In the uterus, this process gives rise to uterine NK (uNK) cells–a heterogeneous lymphocyte population composed primarily of tissue-resident NK (trNK) cells, with a minor subset of cNK cells–that are thought to mediate critical gestational adaptations, including placental vascular remodeling, trophoblast differentiation, and fetal development, through the secretion of cytokines and growth factors[2,7–16]. The importance of uNK cells in pregnancy is evidenced by our previous findings demonstrating that their absence results in reduced litter sizes and increased fetal resorption rates in mice[17]. In humans, disruptions in uNK cell abundance or

function have also been associated with serious obstetrical disorders, including recurrent miscarriage and preeclampsia[18–23]. Despite their importance for reproductive success, the mechanisms by which uterine trNK cells differentiate and acquire their specialized functional identities within the gravid uterus remain elusive.

Recent investigations into the developmental origins of uterine trNK cells have suggested that local molecular cues govern their differentiation. Our prior work identified Eomesodermin (Eomes) as a central transcription factor driving the establishment of trNK cells in both the virgin and pregnant murine uterus, suggesting these cells arise from precursors in the cNK cell lineage[17]. In line with this lineage relationship, parabiosis studies in virgin mice demonstrated that cNK cells in the peripheral vasculature can migrate into the uterus and adopt phenotypic characteristics consistent with uterine trNK cells[24]. Parallel findings in humans further support this differentiation pathway as analyses of endometrial biopsies from uterine transplant recipients revealed that uNK cells carry the recipient genotype, indicating a blood-borne origin for human uterine trNK cells[25]. Together, these findings support a model in which uterine trNK cells arise from hematogenous cNK cells that traffic into the pregnant uterus, where local environmental cues orchestrate their terminal differentiation.

While significant progress has been made in defining the developmental origins of uterine trNK cells, the molecular factors that instruct their differentiation within uterine tissues remain incompletely understood. In the virgin uterus, transforming growth factor (TGF)-β may be a central mediator steering the differentiation of uterine trNK cells. In the virgin uterus, trNK cells depend on sustained autocrine TGF-β signaling to maintain their population, suggesting that continuous, cell-intrinsic TGF-β signaling is critical for preserving tissue-specific NK cell identities within the uterine microenvironment[26]. Corroborating single-cell transcriptomic analyses show that, at steady-state, murine uNK cells exhibit distinct transcriptional programs enriched for TGF-β response genes, suggesting that TGF-β imprints the uNK cell compartment to drive trNK cell differentiation[27]. In humans, $CD16^+$ NK cells in the peripheral blood have the potential to convert into $CD16^-$ NK cells that phenotypically resemble decidual NK cells following exposure to TGF-β *in vitro*, providing evidence of a conserved role for TGF-β in promoting the phenotype of trNK cells[28]. Together, these findings position TGF-β signaling as a critical driver of uterine trNK cell identity and functional specialization within the virgin uterus. Whether TGF-β mediates this differentiation during murine pregnancy, however, remains unknown.

In this study, we identified an *in vivo* TGF-β–dependent differentiation process through which circulating cNK cells give rise to uterine trNK cells in the gravid mouse uterus. This differentiation is tightly linked to the physiological adaptations of pregnancy, as disruption of TGF-β signaling in *Ncr1*-expressing cells during murine gestation impaired spiral artery remodeling and increased resorption rates at midgestation, culminating in reduced litter sizes at birth. Collectively, this work defines a mechanistic framework in which TGF-β governs the *in vivo* differentiation and functional specialization of uterine trNK cells, thereby aligning lymphocyte differentiation to the physiological requirements of pregnancy.

Results

Peripheral cNK cells differentiate into trNK cells in the pregnant murine uterus

The pregnant uterus is characterized by three innate lymphoid cell subsets distinguished by the expression of CD49a, CD49b, and Eomes[2,7]. $CD49a^+$ $Eomes^+$ trNK cells and $CD49a^+$ $Eomes^-$ type 1 innate lymphoid cells (ILC1s) reside within uterine tissues, whereas $CD49b^+$ $Eomes^+$ cNK cells circulate through the peripheral vasculature[24]. To determine whether cNK cells migrate into the pregnant uterus, we intravenously administered a fluorescently labeled anti-CD45.2 antibody *in vivo* prior to euthanasia, allowing us to discriminate circulating lymphocytes (CD45.2–PE-$Cy7^+$) from those residing within uterine tissues (CD45.2–PE-$Cy7^-$). $CD49a^+$ $Eomes^+$ trNK cells and $CD49a^+$ $Eomes^-$ ILC1s within implantation sites were not labeled with our circulating antibody, confirming their residency within tissues of the gravid uterus. Interestingly, intravascular labeling of

circulating lymphocytes revealed a population of extravascular CD49b^{+} Eomes^{+} cNK cells within implantation sites at gestational day (gd) 6.5 that increased at gd 14.5, suggesting that peripheral cNK cells extravasate into the pregnant murine uterus (Figure 1 A, B).

To assess whether peripheral cNK cells migrating into the pregnant uterus could differentiate into uterine trNK cells, we adoptively transferred CD45.2^{+} splenic cNK cells into C57BL/6 CD45.1 dams mated with C57BL/6 CD45.1 males at gd 0.5. This mating strategy allowed us to distinguish donor maternal lymphocytes from fetal lymphocytes at the maternal-fetal interface. By gd 10.5, a subset of adoptively transferred splenic cNK cells upregulated CD49a and downregulated CD49b, acquiring a phenotype characteristic of CD49a^{+} Eomes^{+} uterine trNK cells (Figure 1 C, D). Together, these findings reveal a previously unrecognized plasticity of peripheral cNK cells *in vivo* during murine pregnancy, enabling them to convert into uterine trNK cells within the gravid uterus.

TGF-β signaling drives the differentiation of trNK cells in the pregnant murine uterus

Inasmuch as it has been shown that TGF-β can induce an ILC1-like phenotype that can encompass trNK cells[28–31], we sought to examine the possible role of TGF-β in the differentiation of uNK cells. Unlike peripheral cNK cells in the spleen, we found that cNK cells extravasating into the gravid uterus upregulated expression of TGF-β receptor II, suggesting that TGF-β signaling could indeed mediate their differentiation into uterine trNK cells during gestation (Figure 2 A). To test this, *TGF-βRII*$^{fl/fl}$ mice were crossed with *Ncr1*^{iCre} mice to generate mice that lack TGF-βRII on NKp46^{+} NK cells and ILC1s. To ensure we exclude a confounding effect of Ncr1^{iCre} expression, we profiled the uterine innate lymphoid compartment in pregnant Ncr1^{iCre} dams at gestational day 6.5. No differences were observed in the absolute number of trNK cells, cNK cells, or ILC1s relative to wildtype controls (Figure S1 A-D), and implantation site number and resorption rates were likewise unchanged (Figure S1 E-F). These data indicate that Ncr1^{iCre} expression alone does not perturb uterine ILC composition or early pregnancy outcomes. There was no difference noted in the total number and frequency of splenic cNK cells between TGF-βRII$^{Ncr1\Delta}$ and littermate control dams during gestation (Figure 2 B, C). However, in the pregnant uterus, CD49a^{+} Eomes^{-} ILC1s were markedly reduced in implantation sites of TGF-βRII$^{Ncr1\Delta}$ dams, paralleling the reduction of ILC1s previously reported in the virgin uterus of TGF-βRII$^{Ncr1\Delta}$ female mice[26]. Importantly, loss of TGF-β receptor II expression in *Ncr1*-expressing cells also significantly reduced the number of CD49a^{+} Eomes^{+} trNK cells in the gravid uterus of TGF-βRII$^{Ncr1\Delta}$ dams. This reduction of uterine trNK cells was accompanied by a small increase in the absolute number and frequency of CD49b^{+} Eomes^{+} cNK cells within the pregnant uterus of TGF-βRII$^{Ncr1\Delta}$ dams (Figure 2 D, E). Collectively, these findings suggest that a TGF-β–driven differentiation pathway directs the conversion of peripheral cNK cells into uterine trNK cells during murine pregnancy.

Impaired trNK cell differentiation in the absence of TGF-β signaling compromises pregnancy outcomes

Having established that TGF-β signaling drives the differentiation of trNK cells in the pregnant uterus, we next examined whether disrupting this pathway affects pregnancy outcomes. Litter size, pup birth weight, and gestational length at first parturition were compared between littermate control and TGF-βRII$^{Ncr1\Delta}$ dams mated with C57BL/6 CD45.1 males. TGF-βRII$^{Ncr1\Delta}$ dams had reduced litter sizes at birth compared to littermate controls (Figure 3 A). The birth weights of neonatal pups were not affected by the loss of TGF-β receptor II expression in *Ncr1*-expressing cells, with no relationship observed between litter size and pup birth weight (Figure 3 B). Furthermore, gestation proceeded over a similar timeframe in both TGF-βRII$^{Ncr1\Delta}$ dams and littermate control dams (Figure 3 C). The reduction in litter size observed in TGF-βRII$^{Ncr1\Delta}$ dams suggests impaired TGF-β–dependent uterine trNK cell differentiation in the gravid murine uterus disrupts gestational adaptations crucial for fetal survival.

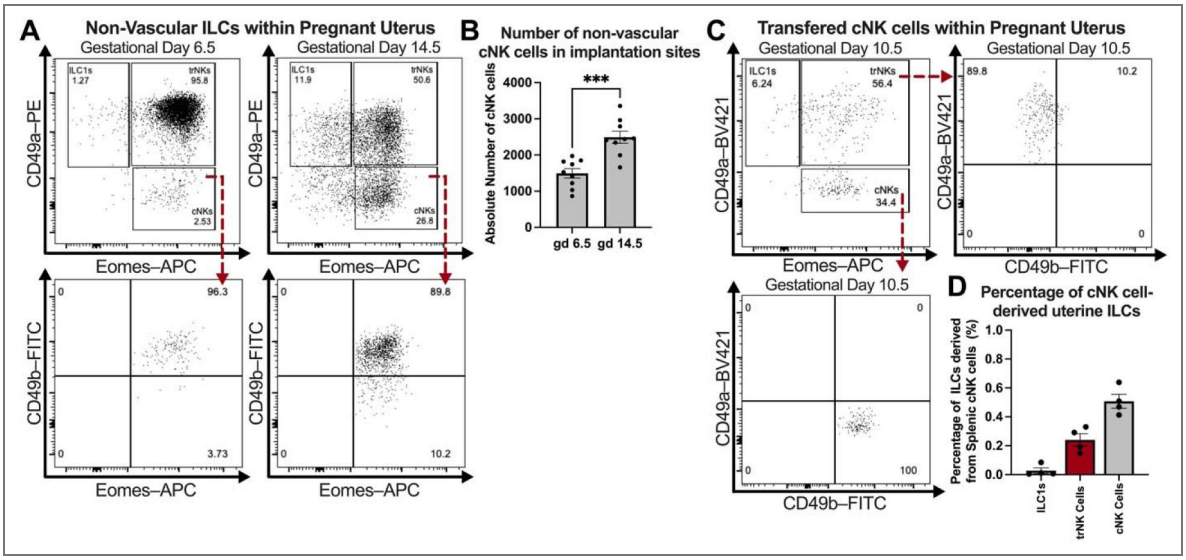


Figure 1. Peripheral cNK cells extravasate into the pregnant uterus and acquire a uterine trNK cells phenotype.

(**A**) Representative flow plots depicting the presence of non-vascular CD49b^{+} Eomes^{+} cNK cells within the gravid uterus of wildtype mice intravascularly labeled with anti-CD45.2 antibody *in vivo* at gds 6.5 and 14.5 (gd 6.5: C57BL/6 dams, *n*=3, implantation sites *n*=9; gd 14.5: C57BL/6 dams, *n*=3, implantation sites *n*=9). Gating strategy: Live, Single Cells; CD3^{-} CD19^{-} CD45.1^{-} CD45.2–PE-Cy7^{-} CD45.2–Pacific Blue^{+} NK1.1^{+} NKp46^{+} cells. (**B**) Absolute cell counts of non-vascular CD49b^{+} Eomes^{+} cNK cells within the gravid uterus of wildtype mice at gds 6.5 and 14.5. (**C**) Concatenated flow plots of implantation sites showing that adoptively transferred cNK cells in pregnant uterus of wildtype dams upregulate CD49a and down regulate CD49b by gd 10.5, acquiring a CD49a^{+} CD49b^{-} Eomes^{+} phenotype characteristic of uterine trNK cells (C57BL/6 dams *n*=4). Here, 2.5x10^{6} CD45.2^{+} CD3^{-} CD19^{-} NK1.1^{+} NKp46^{+} CD49b^{+} splenic cNK cells were adoptively transferred into pregnant C57BL/6-CD45.1 dams at gd 0.5, and the receptor profile of these cells was subsequently assessed at gd 10.5. Gating strategy: Live, Single Cells; CD3^{-} CD19^{-} CD45.1^{-} CD45.2–PE-Cy7^{-} CD45.2–PE^{+} NK1.1^{+} NKp46^{+} cells. (**D**) Proportion of uterine ILC subsets derived from adoptively transferred splenic cNK cells in the pregnant uterus of wildtype dams. Statistics were calculated using unpaired *t tests* with the Mann-Whitney correction. Error bars indicate SEM; *** $p < 0.001$.

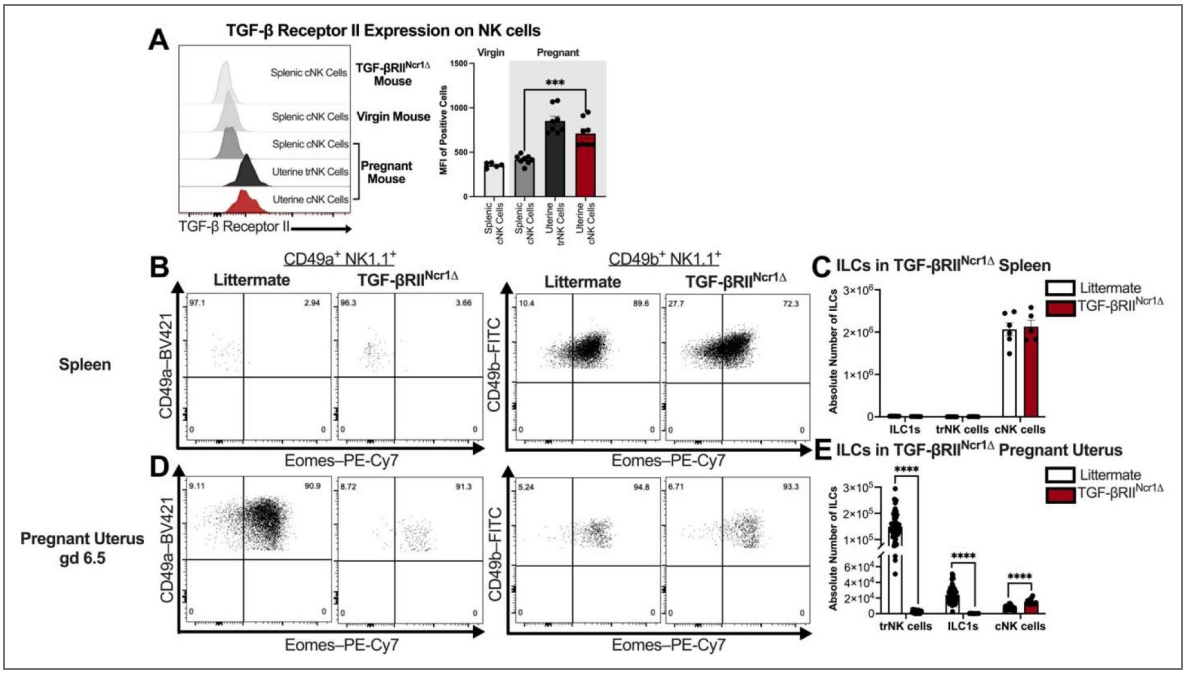


Figure 2. Loss of TGF-β Signaling in *Ncr1*-expressing cells impairs uterine trNK cell differentiation in pregnant mice.

(**A**) Representative histograms depicting TGF-β Receptor II expression on splenic NK cells from virgin TGF-βRII$^{Ncr1\Delta}$ and wildtype mice as well as splenic and uterine NK cell subsets from pregnant wildtype mice at gd 10.5 (virgin TGF-βRII$^{Ncr1\Delta}$ mice, *n*=2; virgin mice: C57BL/6, *n*=5; gd 10.5: C57BL/6 dams, *n*=8, implantation sites *n*=8). MFI, median fluorescent intensity. Gating strategy: Live, Single Cells; CD3$^-$ CD19$^-$ CD45.1$^-$ CD45.2$^+$ NK1.1$^+$ NKp46$^+$ cells. (**B**) Representative flow plots showing the expression of CD49a, CD49b, and Eomes across ILC subsets in the pregnant spleens of littermate control and TGF-βRII$^{Ncr1\Delta}$ dams at gd 6.5 (Littermates, *n*=6; TGF-βRII$^{Ncr1\Delta}$, *n*=5). Gating strategy: Live, Single Cells; CD3$^-$ CD19$^-$CD45.1$^-$ CD45.2$^+$ NK1.1$^+$ NKp46$^+$ cells. (**C**) Absolute cell counts of CD49a$^+$ Eomes$^+$ trNK cells, CD49a$^+$ Eomes$^-$ ILC1s, and CD49b$^+$ Eomes$^+$ cNK cells in the spleens of pregnant littermate control and TGF-βRII$^{Ncr1\Delta}$ dams at gd 6.5. (**D**) Representative flow plots showing the expression of CD49a, CD49b, and Eomes across ILC subsets in the gravid uterus of littermate control and TGF-βRII$^{Ncr1\Delta}$ dams at gd 6.5 (Littermates, *n*=6, implantation sites *n*=54; TGF-βRII$^{Ncr1\Delta}$, *n*=5, implantation sites *n*=15). (**E**) Absolute cell counts of CD49a$^+$ Eomes$^+$ trNK cells, CD49a$^+$ Eomes$^-$ ILC1s, and CD49b$^+$ Eomes$^+$ cNK cells in the gravid uterus of littermate control and TGF-βRII$^{Ncr1\Delta}$ dams at gd 6.5. Statistics were calculated using unpaired *t tests* with the Mann-Whitney correction. Error bars indicate SEM; *** $p < 0.001$; and **** $p < 0.0001$.

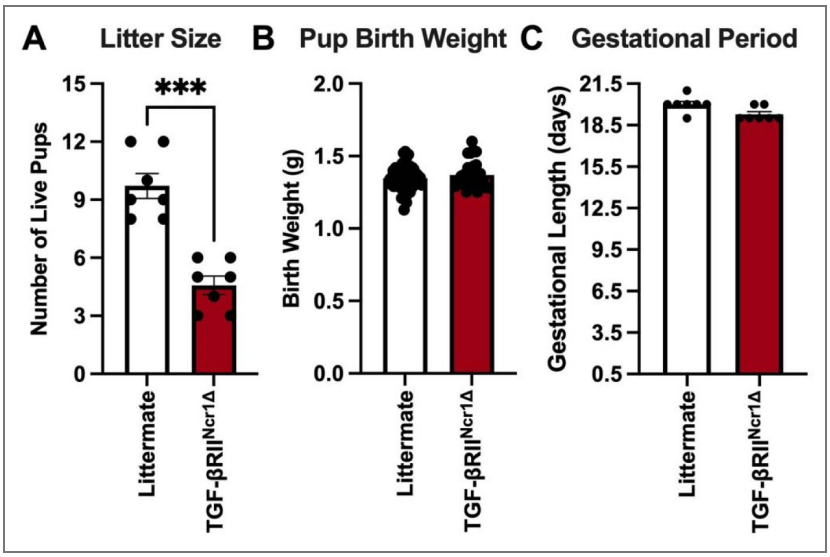


Figure 3. Impaired TGF-β–dependent uterine trNK cells differentiation leads to adverse pregnancy outcomes characterized by reduced litter sizes.

(**A**) Number of live pups at first parturition from littermate control and TGF-βRII$^{Ncr1\Delta}$ dams (Littermates, *n*=7; TGF-βRII$^{Ncr1\Delta}$, *n*=7). (**B**) Pup birth weight in grams (g) from pups birthed by littermate control and TGF-βRII$^{Ncr1\Delta}$ dams (Littermates, *n*=68; TGF-βRII$^{Ncr1\Delta}$, *n*=31). (**C**) Gestational period in days for littermate control and TGF-βRII$^{Ncr1\Delta}$ dams (Littermates, *n*=7; TGF-βRII$^{Ncr1\Delta}$, *n*=7). Statistics were calculated using unpaired *t tests* with the Mann-Whitney correction. Error bars indicate SEM; *** $p < 0.001$.

uNK cells are thought to be critical regulators of placental vasculature remodeling[9,10,12,14], particularly of the decidual spiral arteries, prompting us to investigate whether impaired uterine trNK cell differentiation due to loss of TGF-β signaling affects this process at midgestation. At gd 10.5, TGF-βRII$^{Ncr1\Delta}$ dams had fewer implantation sites and increased resorption rates compared to littermate controls (Figure 4 A, B). Furthermore, stereological quantification of decidual spiral arteries in midsagittal sections of gd 10.5 implantation sites revealed abnormalities in TGF-βRII$^{Ncr1\Delta}$ dams (Figure 4 C). Specifically, decidual spiral arteries from TGF-βRII$^{Ncr1\Delta}$ dams exhibited a 37% reduction in luminal area and a 75% increase in wall thickness, resulting in an increased vessel-to-lumen ratio relative to littermate controls (Figure 4 D). Taken together, these findings indicate that impaired uterine trNK cell differentiation in the absence of TGF-β signaling disrupts decidual spiral artery remodeling, leading to increased fetal resorptions at midgestation and an overall reduction in litter sizes at birth.

Partial reconstitution of uterine trNK cells restores midgestational pregnancy outcomes in TGF-βRII$^{Ncr1\Delta}$ dams

To determine whether restoring uterine trNK cells could rescue the midgestational pregnancy defects observed in TGF-βRII$^{Ncr1\Delta}$ dams, we adoptively transferred wildtype, congenically labeled splenic cNK cells into pregnant TGF-βRII$^{Ncr1\Delta}$ dams at gd 0.5. By gd 10.5, donor cNK cells were detected in the pregnant uterus, where a subset upregulated CD49a and downregulated CD49b, consistent with acquisition of a uterine trNK cell phenotype (Figure 5 A). However, adoptively transferred splenic cNK cells only partially reconstituted the uterine trNK cell population in the gravid uterus of TGF-βRII$^{Ncr1\Delta}$ dams, as evidenced by reduced absolute number and frequency of donor-derived trNK cells in reconstituted TGF-βRII$^{Ncr1\Delta}$ dams (Figure 5 A-C). Notably, this partial reconstitution was sufficient to rescue the gestational defects caused by impaired TGF-β–mediated uterine trNK cell differentiation. Reconstituted TGF-βRII$^{Ncr1\Delta}$ dams exhibited implantation site numbers and fetal resorption rates at gd 10.5 comparable to those observed in littermate controls (Figure 5 D, E). Together, these findings suggest that even partial restoration of the uterine trNK cell in pregnant TGF-βRII$^{Ncr1\Delta}$ dams is sufficient to restore pregnancy outcomes at midgestation, supporting a central role for uterine trNK cells as the principal NK cell subset required for successful murine pregnancy.

Discussion

Building on our previous work indicating that trNK cells in the pregnant uterus are derived from the cNK cell lineage, we now demonstrate that TGF-β signaling during murine gestation directs the differentiation of peripheral cNK cells into uterine trNK cells during murine gestation. This process is crucial for maintaining decidual spiral artery integrity and supporting successful pregnancy outcomes. This study represents the first direct *in vivo* evidence that TGF-β signaling mediates the generation of uterine trNK cells from peripheral cNK cells, uncovering an unrecognized role for TGF-β in shaping the distinct composition of innate lymphocytes at the maternal-fetal interface.

Our adoptive transfer studies demonstrate that uterine trNK cells can arise from a plastic reprogramming of peripheral cNK cells upon entry into the gravid uterus, highlighting the uterus as a dynamic and instructive niche that directs cNK cells to acquire a specialized tissue-specific phenotype. Consistent with our findings, recent human uterine transplantation studies demonstrate that certain subsets of uNK cells can be replenished from recipient-derived cells in the grafted uterus, supporting a model of continuous uNK cell differentiation[25]. Importantly, our studies suggest this phenotypic conversion is not a passive consequence of tissue-residency but is actively instructed by TGF-β signaling in the pregnant uterus. By identifying TGF-β as a central regulator directing uterine trNK cell differentiation during murine gestation, our work provides a mechanistic explanation of the process by which trNK cells acquire specialized tissue-specific phenotypes within the uterus that help sustain pregnancy, reconciling the long-standing paradox of why uterine trNK cells differ so markedly from their circulating counterparts.

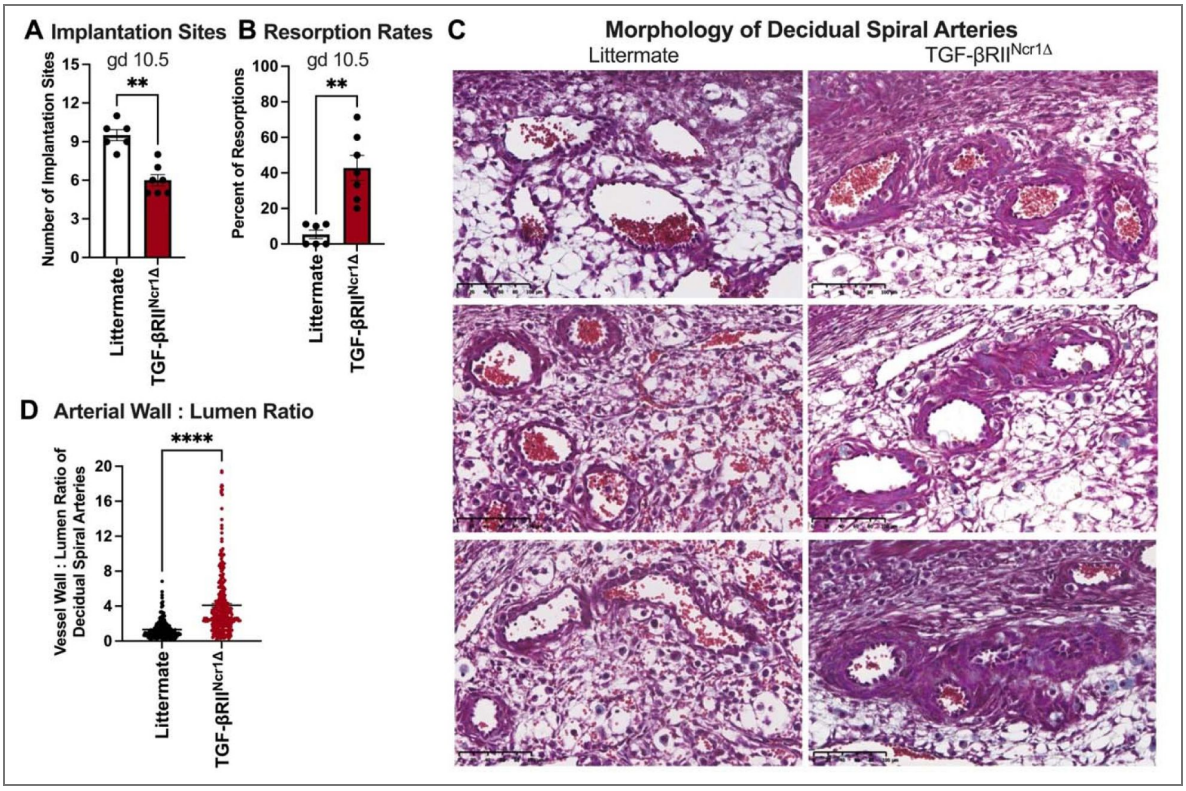


Figure 4. TGF-β–dependent uterine trNK cell differentiation required for proper spiral artery remodeling and fetal survival.

(**A**) At gd 10.5, TGF-βRII$^{Ncr1\Delta}$ dams had fewer implantation sites than littermate control dams (Littermates, *n*=6; TGF-βRII$^{Ncr1\Delta}$, *n*=7). (**B**) Fetal resorption rates in littermate control and TGF-βRII$^{Ncr1\Delta}$ dams at gd 10.5, showing increased resorptions in conditional knockout dams (Littermates, *n*=6; TGF-βRII$^{Ncr1\Delta}$, *n*=7). Resorption rates (RR) were calculated as: RR(%) = (number of resorbed implantation sites/number of total implantation sites) X 100. (**C**) Representative images of gd 10.5 decidual spiral arteries from three littermate control and three TGF-βRII$^{Ncr1\Delta}$ dams stained with Masson's Trichrome (Littermates, *n*=6; TGF-βRII$^{Ncr1\Delta}$, *n*=7; Scale bar, 100μm). (**D**) Spiral artery wall-to-lumen ratio at gd 10.5 implantation sites from littermate control and TGF-βRII$^{Ncr1\Delta}$ dams. Increased wall-to-lumen ratio in TGF-βRII$^{Ncr1\Delta}$ dams indicative of impaired spiral artery remodeling. (Littermates, *n*=6, decidual spiral arteries *n*=257; TGF-βRII$^{Ncr1\Delta}$, *n*=7 decidual spiral arteries *n*=305). Statistics were calculated unpaired *t tests* with the Mann-Whitney correction. Error bars indicate SEM; ** $p < 0.01$; and **** $p < 0.0001$.

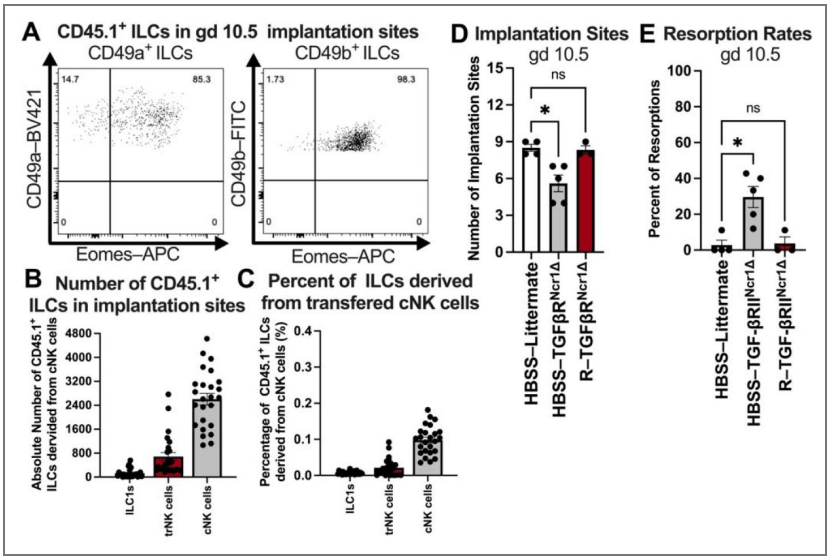


Figure 5. Adoptive transfer of splenic cNK cells partially reconstitutes uterine trNK cells and rescues midgestational pregnancy defects in TGFBRII$^{Ncr1\Delta}$ dams.

(**A**) Representative flow plots showing the expression of CD49a, CD49b, and Eomes across CD45.1$^+$ ILC subsets in gd 10.5 implantation sites of TGF-βRII$^{Ncr1\Delta}$ dams reconstituted with splenic CD45.1$^+$ cNK cells. Briefly, 3.0x10^6 splenic CD45.1$^+$ cNK cells were adoptively transferred into TGF-βRII$^{Ncr1\Delta}$ dams at gd 0.5. By gd 10.5, a portion of adoptively transferred cNK cells in pregnant uterus of TGF-βRII$^{Ncr1\Delta}$ dams upregulated CD49a and downregulated CD49b, acquiring a CD49a$^+$ CD49b$^-$ Eomes$^+$ phenotype characteristic of uterine trNK cells (Reconstituted (R)–TGF-βRII$^{Ncr1\Delta}$, *n*=3, implantation sites, *n*=25). Gating strategy: Live, Single Cells; CD3$^-$ CD19$^-$ CD45.1$^+$ CD45.2$^-$ NK1.1$^+$ NKp46$^+$ cells. (**B**) Absolute numbers of CD45.1$^+$ ILC subsets in gd 10.5 implantation sites from reconstituted TGF-βRII$^{Ncr1\Delta}$ dams (R–TGF-βRII$^{Ncr1\Delta}$, *n*=3, implantation sites, *n*=25). (**C**) Proportion of uterine CD45.1$^+$ ILC subsets derived from adoptively transferred splenic cNK cells in gd 10.5 implantation sites from TGF-βRII$^{Ncr1\Delta}$ dams (R–TGF-βRII$^{Ncr1\Delta}$, *n*=3, implantation sites *n*=25). (**D**) Number of implantation sites and (**E**) fetal resorption rates in reconstituted TGF-βRII$^{Ncr1\Delta}$ dams at gd 10.5 were comparable to those measured in littermate control dams injected intravascularly with HBBS (HBSS–Littermates, *n*=4; R–TGF-βRII$^{Ncr1\Delta}$, *n*=3). Resorption rates (RR) were calculated as: RR(%) = (number of resorbed implantation sites/number of total implantation sites) X 100. Statistics were calculated unpaired *t tests* with the Mann-Whitney correction.

The absence of cNK cell accumulation in the gravid uterus in the setting of impaired TGF-β signaling suggests a defect in tissue retention rather than recruitment. In the absence of TGF-β–mediated cues, circulating cNK cells that enter the uterine vasculature may fail to acquire the molecular programs required for residency and instead continue to transit through the tissue. This is consistent with a model in which TGF-β signaling promotes not only phenotypic conversion but also the acquisition of retention signals necessary for persistence within the uterine microenvironment, reinforcing that acquisition of tissue-residency in the gravid uterus is an actively instructed process.[29,32]

The functional consequences of this TGF-β–dependent pathway of uterine trNK cell differentiation on fecundity are profound. Impaired trNK cell differentiation in the absence of TGF-β signaling resulted in adverse pregnancy outcomes in mice, characterized by reduced litter sizes at first parturition. While our prior studies suggest uNK cells are critical for gestation, how these cells ensure pregnancy success remains poorly understood. In this study, impaired TGF-β–dependent trNK cell differentiation was associated with abnormal spiral artery remodeling and increased fetal resorption at midgestation. Notably, partial reconstitution of the uterine trNK cell compartment in TGF-βRII$^{Ncr1\Delta}$ dams was sufficient to normalize implantation site numbers and fetal resorption rates at midgestation. Together, these findings position uterine trNK cells as key contributors to placental vascular adaptations in the gravid uterus, emphasizing the importance of TGF-β–driven uterine trNK cell differentiation for murine reproductive success. The involvement of uNK cells in decidual spiral artery remodeling has been previously inferred from histological comparisons of implantation sites from immunodeficient mouse models; however, our work expands on these observations by pinpointing the subset of uNK cells involved in decidual angiogenesis[8–11]. Additional studies are necessary to elucidate the molecular mechanisms through which uterine trNK cells remodel decidual spiral arteries in the pregnant mouse uterus.

Interestingly, the inability to fully reconstitute the uterine trNK cell compartment following adoptive transfer suggests that only a subset of circulating cNK cells may be capable of differentiating into trNK cells during pregnancy, or alternatively that trNK cells already present in the virgin uterus may undergo *in situ* proliferation in the gravid uterus. Previous studies from our lab as well as others show that trNK cells within the pregnant murine uterus express marked levels of Ki67, supporting a model in which local proliferation of uterine trNK cells is a major contributor to the uterine trNK cell pool during pregnancy[7,33]. Prior studies have also described hematopoietic precursors within endometrial and decidual tissues that generate uterine trNK cells, suggesting that the compartment may be also sustained by local precursor differentiation[34–36]. Together, these findings suggest that uterine trNK cell ontogeny may be more complex than a single-source model and raise the possibility that distinct developmental pathways may operate at different stages of reproductive life. Therefore, defining the relative contribution and developmental timing of hematogenous versus locally maintained sources *in vivo* could provide relevant insights into the developmental trajectories and transcriptional programs that underlie decidual NK cell heterogeneity.

Notably, a reduction in the total number of implantation sites was also observed in TGF-βRII$^{Ncr1\Delta}$ dams, suggesting impaired TGF-β–dependent uterine trNK cell differentiation compromises implantation success. This finding raises the possibility that uterine trNK cells contribute to pregnancy not only through the remodeling of the placental vasculature but also by promoting uterine receptivity to implantation. In humans, studies of recurrent implantation failure and recurrent miscarriages have similarly implicated uNK cells in establishing endometrial receptivity and subsequently facilitating embryo implantation[22,23,37,38]. Therefore, future investigation is warranted to determine whether TGF-β–dependent alterations in uterine trNK cell–derived signals coordinate the cellular crosstalk underlying embryo implantation.

In addition to its effects on uterine trNK cells, loss of TGF-β receptor II in *Ncr1*-expressing cells substantially reduced ILC1s in the gravid murine uterus. However, the functional relevance of uterine ILC1s in pregnancy appears limited. Our previous findings show that the residual ILC1 population present in the gravid uterus of Eomes$^{Ncr1\Delta}$ dams failed to ameliorate the adverse

pregnancy outcomes observed in this model, suggesting that uterine ILC1s are not required for successful gestation in mice[17]. Thus, the loss of ILC1s in the gravid uterus of TGF-βRII$^{Ncr1\Delta}$ dams is unlikely to account for the pregnancy defects detected in this mouse model.

More broadly, the role of TGF-β as a master regulator that tempers NK cell effector function extends beyond the pregnant uterus, shaping NK cell phenotype and functions across diverse tissue microenvironments. In the tumor microenvironment, TGF-β signaling suppresses NK cell toxicity by driving their conversion toward a ILC1-like phenotype that facilitates tumor immune evasion[30]. Similarly, in the obese murine liver, cNK cells undergo a TGF-β–dependent shift toward a less cytotoxic, ILC1-like state that mitigates tissue injury and protects against nonalcoholic fatty liver disease[31]. Within the gravid murine uterus, this same signaling axis is harnessed to promote maternal-fetal tolerance by reprograming peripheral cNK cells into uterine trNK cells with specialized, proangiogenic functions that sustain fetal development rather than immune activation. Whether a similar TGF-β driven program of uterine trNK cell differentiation is conserved in human pregnancy remains an important question for future investigation. Collectively, these findings position TGF-β as a context-dependent modulator of NK cell identity, fine-tuning their phenotype and function to the physiological needs of the surrounding microenvironment–whether to limit inflammation, permit tumor growth, or ensure reproductive success.

In conclusion, our work supports a model of continuous uterine trNK cell differentiation during murine gestation, in which peripheral cNK cells are recruited to the gravid uterus and converted into trNK cells via TGF-β signaling. This dynamic differentiation pathway ensures that uNK cells are appropriately tuned to the unique physiologic needs of gestation, linking NK cell plasticity directly to reproductive success. Our findings establish TGF-β–driven uterine trNK cell differentiation as a central axis for immune regulation in pregnancy and provide a framework that will be important for future studies exploring how perturbations in this pathway could underlie pregnancy complications. These insights reshape our understanding of NK cell developmental plasticity and highlight uterine immune adaptation as a fundamental component of reproductive fitness.

Materials and Methods

Mice

All mouse studies were performed in accordance with ethical guidelines and animal protocol approved by the Washington University School of Medicine Animal Studies Committee under protocol number *24-0332*. Wild-type *C57BL/6* (stock number 665) and *B6.SJL-Ptprc^{a}Pepc^{b}/BoyJ* (stock number 664) mice were purchased from Charles River Laboratories (Wilmington, MA). *Ncr1^{iCre}* were generously gifted by Eric Vivier at Aix Marseille University, Marseille, France. TGF-βRII$^{Ncr1\Delta}$ mice and littermate controls were generated by crossing *B6;129-Tgfbr2^{tm1Karl}/J* (strain: 012603; The Jackson Laboratory, Bar Harbor, ME) with *Ncr1^{iCre}*mice. Female mice aged 6-8 weeks were used in all mouse studies. All mice were housed in the Laboratory for the Animal Care barrier facility at the Washington University School of Medicine and maintained on a 12-hour light/dark cycle.

Timed-Pregnancies

To distinguish maternal lymphocytes from fetal lymphocytes at the maternal-fetal interface, we mated virgin C57BL/6, littermate control, Ncr1^{iCre} and TGF-βRII$^{Ncr1\Delta}$ female mice with C57BL/6 CD45.1 male mice overnight. The timing of conception was determined by detection of a copulation plug the following morning, which was designated as gd 0.5. Pregnancy outcomes were evaluated by comparing gestational length, litter sizes, and pup birth weight at first parturition between littermate control and TGF-βRII$^{Ncr1\Delta}$ dams. Pregnant dams were dissected at gds 6.5, 10.5, or 14.5 to assess the immune constituents of implantation sites, and at gd 10.5 to assess fetal resorption rates and placental vasculature morphology. Fetal resorption rates were calculated as the percentage of resorbed implantation sites per pregnant uterus.

cNK Cell Adoptive Transfer Studies in Pregnant Dams

Wildtype CD45.2 splenic cNK cells were purified by negative selection (Stem Cell Technologies, Vancouver, Canada) to obtain $CD3^-$ $CD19^-$ $NK1.1^+$ $NKp46^+$ $CD49b^+$ cNK cells with purity of 87-93%. $2.5x10^6$ purified $CD45.2^+$ cNK cells were injected intravascularly via tail vein injection into C57BL/6 CD45.1 pregnant dams at gd 0.5. The phenotype of adoptively transferred cNK cells was then assessed by flow cytometry at gd 10.5.

cNK Cell Adoptive Transfer Studies in Pregnant TGF-βRII$^{Ncr1\Delta}$ Dams

Wildtype $CD45.1^+$ splenic cNK cells were purified by negative selection (Stem Cell Technologies, Vancouver, Canada) to obtain $CD3^-$ $CD19^-$ $NK1.1^+$ $NKp46^+$ $CD49b^+$ cNK cells with purity of 85-92%. $3.0x10^6$ purified $CD45.1^+$ cNK cells were suspended in 300 µl of sterile Hank's Balanced Salt Solution (HBSS) and injected intravascularly via tail vein injection into pregnant TGF-βRII$^{Ncr1\Delta}$ dams at gd 0.5. Littermate control dams were injected with 300 µl of sterile HBSS intravascularly via tail vein injection. The phenotype of adoptively transferred cNK cells in reconstituted TGF-βRII$^{Ncr1\Delta}$ dams was then assessed by flow cytometry at gd 10.5. The number of implantation sites and fetal resorption rates were assessed at gd 10.5. as described above in reconstituted TGF-βRII$^{Ncr1\Delta}$ dams and HBSS-treated littermate control dams.

Intravascular Staining of Circulating Lymphocytes

To distinguish tissue-resident lymphocytes at the maternal-fetal interface from those in circulation, we administered 3 µg of fluorescently labeled anti-CD45.2–PE-Cy7 (104; Invitrogen, Waltham, MA) to pregnant dams intravascularly via tail vein injection 3 minutes prior to euthanasia.

Single Cell Isolations from Different Tissues

Implantation Site Digestion

Pregnant dams were dissected at gds 6.5, 10.5, or 14.5 to assess the immune constituents of individual implantation sites. Each healthy implantation was digested with Liberase TL (167 µg/ml; Sigma-Aldrich, St. Louis, MO) and DNase1 (150 µg/ml; Sigma-Aldrich, St. Louis, MO) for 1 hour at 37°C. Enzymatically digested implantation sites were minced, washed with 10% FBS RPMI media, and resuspended in 3mL of complete R10 media.

Splenic Preparation

Spleens from each pregnant dam were harvested, minced, and filtered through a 70 µm mesh. Splenocyte suspensions were subsequently treated with RBC Lysis Buffer, washed with 10% FBS RPMI media, and resuspended in 5mL of complete R10 media.

Flow Cytometry

Fluorescently-labeled antibodies to the indicated antigens were purchased from the following vendors: Invitrogen (Waltham, MA), which included CD3e (clone 145-2C11), CD19 (1D3), CD45.2 (104), CD45.1 (A20), CD49b (DX5), EOMES (Dan11mag), NKp46 (29A1.4), and Fixable Viability Dye (eFluorTM506); BioLegend (San Diego, CA) which included NK1.1 (PK136); BD Biosciences (Franklin Lakes, NJ) which included CD49a (Ha31/8) and Streptavidin (PE-Conjugate); R&D Systems (Minneapolis, MN) which included TGF-βRII (Biotinylated).

Prior to staining, 5,000 Precision Count Beads (BioLegend, San Diego, CA) were added to each sample to quantify cell numbers. Cells were stained with fixable viability dye (Invitrogen, Waltham, MA) and then stained for cell surface markers in 2.4G2 hybridoma supernatant (anti-FcγRIII) to block Fc receptors. Following surface staining, cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBiosciences; San Diego, CA) according to the manufacturer's instructions and subsequently stained for intracellular molecules. All samples were acquired on a FACS Canto (BD Biosciences, Franklin Lakes, NJ) and analyzed using FlowJo Software 10.8.2 (BD Biosciences, Franklin Lakes, NJ). Maternal uterine cNK cells were defined as

viable singlets, CD3$^-$ CD19$^-$ CD45.1$^-$ CD45.2$^+$ NK1.1$^+$ NKp46$^+$ CD49a$^-$ CD49b$^+$ Eomes$^+$. Maternal uterine trNK cells were defined as viable singlets, CD3$^-$ CD19$^-$CD45.1$^-$ CD45.2$^+$ NK1.1$^+$ NKp46$^+$ CD49a$^+$ CD49b$^-$ Eomes$^+$. Maternal uterine ILC1s were defined as viable singlets CD3$^-$ CD19$^-$ CD45.1$^-$ CD45.2$^+$ NK1.1$^+$ NKp46$^+$ CD49a$^+$ CD49b$^-$ Eomes$^-$.

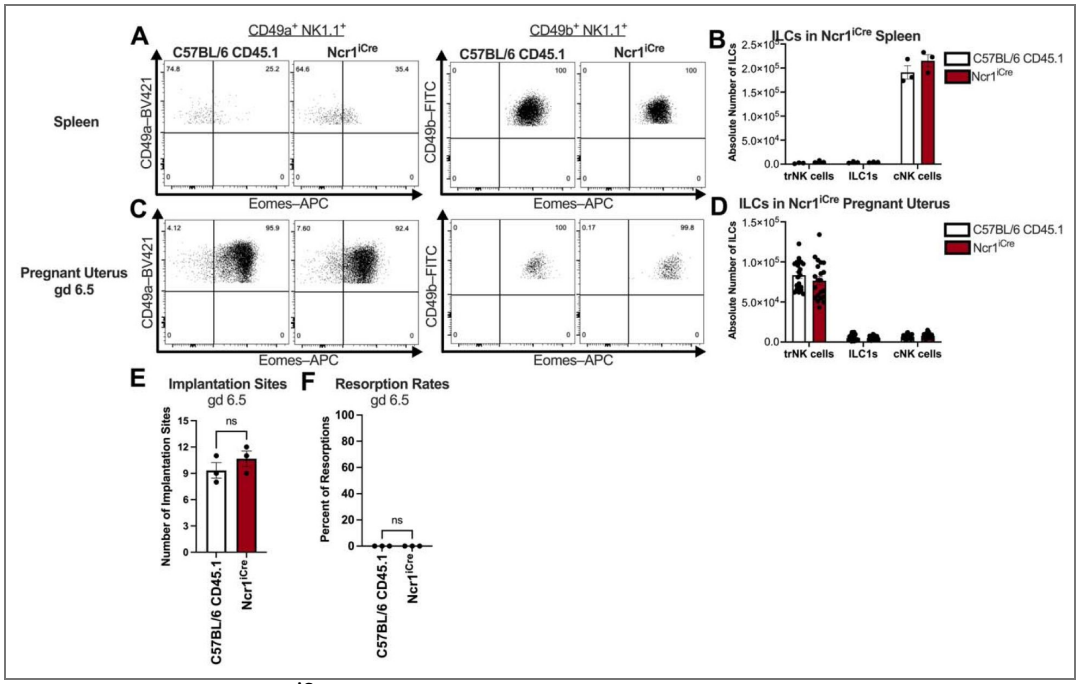
Morphological Analysis of Decidual Spiral Arteries

Placental tissues at gd 10.5 were prepared for histology by fixing uterine horns containing intact implantation sites in 4% paraformaldehyde, followed by paraffin embedding. Thin tissue sections (5 µm) were cut and processed for Masson's Trichrome staining according to manufacturer's instructions. All slides were examined by light microscopy, and images were captured using a NanoZoomer HT2.0 Digital Slide Scanner (Hamamatsu Photonics, Hamamatsu, Japan). The center section of each serially sectioned implantation site was identified, and at least 4 implantation sites per litter were analyzed for each dam. Vessel wall and lumen measurements of decidual spiral arteries were taken from cross-sectional images using NIS-Elements Viewer NDP.view2 4.11.0 Imaging Software (Nikon Microscope). Vessel wall-to-lumen ratios for individual decidual spiral arteries were calculated as the ratio of the outer wall area to the luminal area. Investigators were blinded to sample genotypes during analysis.

Statistical Analysis

Statistical analysis was performed with Prism 10.4.1 (GraphPad software) using unpaired *t tests* with the Mann-Whitney correction. Error bars in figures represent the SEM. Normality was assessed with Prism 10.4.1 (GraphPad software) using Q-Q plots. Sample sizes for each experiment were determined using a significance level (α) of 0.5 and power of 85%. Statistical significance was indicated as follows: ns, not significant; *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$.

Supplementary figures



Supplementary Figure 1. Ncr1^{iCre} expression does not alter uterine ILC composition or early pregnancy outcomes. (**A**) Representative flow plots showing the expression of CD49a, CD49b, and Eomes across ILC subsets in the spleens of wildtype C57BL/6 CD45.1 and Ncr1^{iCre} dams at gd 6.5 (C57BL/6 CD45.1, *n*=2; Ncr1^{iCre}, *n*=3). Gating strategy: Live, Single Cells; CD3$^-$ CD19$^-$ CD45.1$^-$ CD45.2$^+$ NK1.1$^+$ NKp46$^+$ cells. (**B**) Absolute cell counts of CD49a$^+$ Eomes$^+$ trNK cells, CD49a$^+$ Eomes$^-$ ILC1s, and CD49b$^+$ Eomes$^+$ cNK cells in the spleens of pregnant wildtype C57BL/6 CD45.1 and Ncr1^{iCre} dams at gd 6.5. (**C**) Representative flow plots showing the expression of

CD49a, CD49b, and Eomes across ILC subsets in the gravid uterus of wildtype C57BL/6 CD45.1 and Ncr1^{iCre} dams at gd 6.5 (C57BL/6 CD45.1, *n*=3, implantation sites *n*=21; Ncr1^{iCre}, *n*=3, implantation sites *n*=21). (**D**) Absolute cell counts of CD49a^{+} Eomes^{+} trNK cells, CD49a^{+} Eomes^{-} ILC1s, and CD49b^{+} Eomes^{+} cNK cells in the gravid uterus of wildtype C57BL/6 CD45.1 and Ncr1^{iCre} dams at gd 6.5. (**E**) Number of implantation sites and (**F**) fetal resorption rates in Ncr1^{iCre} dams at gd 6.5 were comparable to those measured in wildtype C57BL/6 CD45.1 dams (C57BL/6 CD45., *n*=3; Ncr1^{iCre} dams, *n*=3). Resorption rates (RR) were calculated as: RR(%) = (number of resorbed implantation sites/number of total implantation sites) X 100. Statistics were calculated unpaired *t tests* with the Mann-Whitney correction.

Data availability

Flow cytometry data will be deposited in FlowRepository upon publication.

Acknowledgements

We extend our gratitude to the members of the Yokoyama Laboratory for their insightful discussions and constructive feedback. Additionally, we thank the Alafi Neuroimaging Core for access to the NanoZoomer HT2.0 Digital Slide Scanner, supported by the shared instrumentation grant NCRR1S10RR027552. This work was supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development of the National Institutes of Health under award number 1F30HD118750-01A1.

Additional information

Authors' Contributions

J.D.B., performed experiments; acquisition and analysis of data; drafting of the manuscript; J.D.B., D.M.N., and W.M.Y, study concept and design; interpretation of data; critical revision of the manuscript; L.Y., technical support; W.M.Y., provided supervision; obtained funding. All authors approved of the final version of this manuscript.

Abbreviations used in this paper include

cNK: conventional NK
Eomes: Eomesodermin
Gd: gestational day
ILC: innate lymphoid cell
ILC1: type 1 innate lymphoid cell
TGF-β: transforming growth factor-β
trNK: tissue-resident NK
uNK: uterine NK

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Peer reviews

Reviewer #1 (Public review):

This is an excellent paper from Dr. Yokoyama and colleagues. The experiments are technically demanding, given the very low cell numbers and the challenges of working with implantation sites at gestational days 6.5, 10.5, and 14.5. Overall, the impact of TGF-β receptor II deficiency in the NK lineage on uterine trNK cell numbers and litter size is convincing, and the authors' conclusions are well supported by the data. Less convincing, however, is the claim that the decrease in trNK cells is compensated by an increase in cNK cells; rather, the absence of TGF-β receptor II appears to result in an overall reduction of NK/ILC1 cells.

Comments on revised version:

I thank the authors for addressing all my comments from my initial review.

https://doi.org/10.7554/eLife.109878.2.sa2

Reviewer #2 (Public review):

In their manuscript "TGF-β drives the conversion of conventional NK cells into uterine tissue-resident NK cells to support murine pregnancy", Yokoyama and colleagues investigate the role of Tgfbr2 expression by NK cells in the formation of tissue-resident uterine NK cells and subsequent importance in murine pregnancy. By transferring congenic splenic conventional NK cells into pregnant mice, they show conversion of circulating NK cells into uterine ivCD45 negative tissue-resident NK cells. When interfering with the formation of uterine trNK cells, spiral artery remodelling was impaired, fetal resorption rates were increased, and litter sizes were reduced.

Generally, this is a research topic of high interest, yet the manuscript is lacking detailed mechanistical insights and some questions remain open. At the current state, the data represent an interesting characterisation of the Tgfbr2-fl/fl Ncr1-Cre mice in pregnancy, but considering 1) the recent publication by the group (Ref#17) on the role of Eomes+ cNK cells during pregnancy, 2) the previously described role of Tgfbr2 and autocrine TGFb expression for uterine NK cell differentiation in virgin mice (also cited by the authors), and 3) the well-known relevance of uterine NK cells during pregnancy, additional experiments addressing the specific role of Tgfb during pregnancy would help to improve novelty and significance of the manuscript.

Comments on revised version:

In their revised version of the manuscript and their point-by-point response, the authors have very carefully addressed and discussed all of our concerns and suggestions.

https://doi.org/10.7554/eLife.109878.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Public review):***
>
> *(1) Figure 1A and B: Although a trend is evident, it does not appear that the absolute number of cNK cells at day 14 is significantly changed from day 6.5?*

We thank the reviewer for this careful observation. We had not originally performed a statistical comparison between the number of cNK cells present at gds 6.5 and 14.5. We have now conducted the appropriate statistical analysis for this dataset and found that the absolute number of cNK cells at day 14.5 is in fact significantly different from day 6.5 (p = 0.0005; unpaired *t test*, Mann-Whitney correction). The figure and corresponding legend have been updated to reflect this analysis. Please see Figure 1B:

"Statistics were calculated using unpaired *t tests* with the Mann-Whitney correction. Error bars indicate SEM; *** $p < 0.001$."

> *(2) Figure 2E: The authors state, "This reduction of uterine trNK cells was accompanied by a concomitant increase in the absolute number and frequency of CD49b+Eomes+ cNK cells within the pregnant uterus of TGF-βRIINcr1Δ dams (Figure 2 D, E). The number of cNK cells appears relatively low (visually ~1,000-1,300), and although the difference is statistically significant, its physiological relevance is unclear. More importantly, this modest increase does not correlate with the marked decrease in trNK and ILC1 populations, as cNK cells do not appear to accumulate. In my opinion, the conclusion "Collectively, these findings indicate that a TGF-β-driven differentiation pathway directs the conversion of peripheral cNK cells into uterine trNK cells during murine pregnancy" should be slightly toned down.*

We thank both reviewers for this suggestion. Regarding the absence of cNK cell accumulation in the absence of TGF-β signaling, we suggest that this may be related to the normal passage of cNK cells circulating in the placenta, i.e., these cells may not have acquired signals to remain in the uterus and are simply continuing to pass through and not accumulating. Nonetheless, we have rephrased our wording in to address this concern as follows:

"This reduction of uterine trNK cells was accompanied by a small increase in the absolute number and frequency of CD49b^{+} Eomes^{+} cNK cells within the pregnant uterus of TGF-βRII$^{Ncr1\Delta}$ dams (Figure 2 D, E). Collectively, these findings suggest that a TGF-β–driven differentiation pathway directs the conversion of peripheral cNK cells into uterine trNK cells during murine pregnancy."

"The absence of cNK cell accumulation in the gravid uterus in the setting of impaired TGF-β signaling suggests a defect in tissue retention rather than recruitment. In the absence of TGF-β–mediated cues, circulating cNK cells that enter the uterine vasculature may fail to acquire the molecular programs required for residency and instead continue to transit through the tissue. This is consistent with a model in which TGF-β signaling promotes not only phenotypic conversion but also the acquisition of retention signals necessary for persistence within the uterine microenvironment, reinforcing that acquisition of tissue-residency in the gravid uterus is an actively instructed process [29,32]."

> *(3) Figures 2-4: It is unclear whether the littermate controls are floxed mice or floxhet-Ncr1iCre mice? This distinction is important, as Ncr1iCre expression itself could potentially lead to a phenotype.*

To address these concerns, we characterized the uterine innate lymphoid cell compartment in the pregnant uterus of Ncr1^{icre} dams at gestational day 6.5. We did not observe a difference in the absolute number and frequency of trNK cells, cNK cells, and ILC1s in the gravid uterus of Ncr1^{icre} dams compared to wildtype CD45.1 C57BL/6 mice. Additionally, the number of implantation sites and resorption rates in Ncr1^{icre} dams was comparable to wildtype CD45.1 C57BL/6 mice. Together these data indicate that Ncr1^{icre} expression itself does not influence the phenotype we report in TGF-βRII$^{Ncr1\Delta}$ dams. These additional findings have been included in Supplementary Figure 1 and in the text as follows:

"To ensure we exclude a confounding effect of $Ncr1^{iCre}$ expression, we profiled the uterine innate lymphoid compartment in pregnant $Ncr1^{iCre}$ dams at gestational day 6.5. No differences were observed in the absolute number of trNK cells, cNK cells, or ILC1s relative to wildtype controls (Figure S1 A-D), and implantation site number and resorption rates were likewise unchanged (Figure S1 E-F). These data indicate that $Ncr1^{iCre}$ expression alone does not perturb uterine ILC composition or early pregnancy outcomes."

> ***Reviewer #1 (Recommendations for the authors):***
>
> *(1) Figure 1C &D: The adoptive transfer experiment is convincing. As a minor point, why is the gate setting for Eomes different between panels 1C and 1D?*

To clarify the phenotype of the adoptively transferred cNK cells, we included two additional gates depicting the expression of CD49a and CD49b in unlabeled (non-vascular) trNK cells and cNK cells in the pregnant uterus Please see the revised Figure 1C and revised figure legend:

"(C) Concatenated flow plots of implantation sites showing that adoptively transferred cNK cells in pregnant uterus of wildtype dams upregulate CD49a and down regulate CD49b by gd 10.5, acquiring a $CD49a^+$ $CD49b^-$ $Eomes^+$ phenotype characteristic of uterine trNK cells (C57BL/6 dams *n*=4). Here, $2.5x10^6$ $CD45.2^+$ $CD3^-$ $CD19^-$ $NK1.1^+$ $NKp46^+$ $CD49b^+$ splenic cNK cells were adoptively transferred into pregnant C57BL/6-CD45.1 dams at gd 0.5, and the receptor profile of these cells was subsequently assessed at gd 10.5. Gating strategy: Live, Single Cells; $CD3^-$ $CD19^-$ $CD45.1^-$ CD45.2–PE-$Cy7^-$ CD45.2–PE^+ $NK1.1^+$ $NKp46^+$ cells."

> *(2) Figure 3: Has the pup ratio male/female changed?*

We did not observe a statistically significant difference in the female-to-male pup ratio between groups.

> ***Reviewer #2 (Public review):***
>
> *(1) The authors suggest cNK extravasation and local differentiation into iv- trNK. Can it be estimated how much this process contributes to the trNK pool vs. a potential local proliferation of already existing trNK? How do absolute numbers of CD49a+ Eomes+ trNK change during pregnancies? (In Figure 1A, the cell numbers of CD49a+ Eomes+ trNK seem to go down dramatically between gd 6.5 and 14.5). The plot in 1B could also include absolute numbers of ILC1s and trNKs. Would recruited cNK cells compensate for a potential loss of CD49a+ Eomes+ trNK?*

Our prior work as well as others have tracked the changes in uterine trNK cells, cNK cells, and ILC1s over the course of murine pregnancy. Consistent with these studies, the absolute number of uterine $CD49a^+$ $Eomes^+$ trNK cells peaks during early pregnancy (roughly between gds 5.5 7.5) and subsequently declines until term. The decrease in uterine trNK cells between gd 6.5 and gd 14.5 observed in Figure 1A is therefore consistent with the known physiological contraction of the decidual NK compartment as pregnancy progresses. Thus, it is unlikely that cNK cells recruited within the uterine tissue compensate for the loss of $CD49a^+$ $Eomes^+$ trNK cells observed. To address the reviewer's request, we have now included the absolute number of uterine trNK cells and ILC1s in Figure 1–please see updated Figure 1C and D and corresponding figure legend (provided below). With respect to the relative contribution of cNK cells extravasation vs local proliferation of trNK cells, our data do not allow us to quantitatively distinguish between these mechanisms. Moreover, previous studies have demonstrated that uterine trNK cells express Ki67, suggesting that they exhibit proliferative activity during this period. Thus, we hypothesize that both local proliferation of existing trNK cells and recruitment of circulating cNK cells contribute to the population of uterine trNK cells during early pregnancy.

"(C) Concatenated flow plots of implantation sites showing that adoptively transferred cNK cells in pregnant uterus of wildtype dams upregulate CD49a and down regulate CD49b by gd 10.5, acquiring a CD49a^{+} CD49b^{-} Eomes^{+} phenotype characteristic of uterine trNK cells (C57BL/6 dams *n*=4). Here, 2.5x10^{6} CD45.2^{+} CD3^{-} CD19^{-} NK1.1^{+} NKp46^{+} CD49b^{+} splenic cNK cells were adoptively transferred into pregnant C57BL/6-CD45.1 dams at gd 0.5, and the receptor profile of these cells was subsequently assessed at gd 10.5. Gating strategy: Live, Single Cells; CD3^{-} CD19^{-} CD45.1^{-} CD45.2–PE-Cy7^{-} CD45.2–PE^{+} NK1.1^{+} NKp46^{+} cells. (D) Proportion of uterine ILC subsets derived from adoptively transferred splenic cNK cells in the pregnant uterus of wildtype dams. Statistics were calculated using unpaired *t tests* with the Mann-Whitney correction. Error bars indicate SEM; ***$p < 0.001$."

Barahona, J.D., Yang, L. and Yokoyama, W.M., 2025. Eomesodermin defines uterine NK cells crucial for pregnancy success in mice. The Journal of Immunology, 214(10), pp.2549-2556.

Filipovic, I., Chiossone, L., Vacca, P., Hamilton, R.S., Ingegnere, T., Doisne, J.M., Hawkes, D.A., Mingari, M.C., Sharkey, A.M., Moretta, L. and Colucci, F., 2018. Molecular definition of group 1 innate lymphoid cells in the mouse uterus. Nature Communications, 9(1), p.4492.

> *(2) Figure 1C: 2.5 Mio cNK cells have been transferred, but only very few cells can be detected within the uterus (concatenated FACS plot shown). What may represent the limit to generate uterine trNK out of cNK? Is the niche supporting cNK-trNK differentiation limited? Is it only a specific subset of (splenic) cNK capable of differentiating into trNK? Is gd 0.5 the optimal timepoint for the transfer? Is there continuous recruitment of cNK into the uterus and differentiation into trNK, or is it enhanced at specific timepoints of pregnancy? Could there be local proliferation of cNK-derived trNK? This could be studied by proliferation dye dilution of WT cNK cells in this transfer-setup.*

We recognize that transferring cNK cells at gestational day 0.5–prior to placental formation–may partially account for the low uterine reconstitution observed. At this time point, the local signals necessary for efficient recruitment and retention of cNK cells in the uterus may not yet be fully established, potentially resulting in preferential homing to peripheral tissues such as the spleen and liver. Consistent with this possibility, we do observe a robust population of adoptively transferred cNK cells in the spleen and liver of our pregnant dams. We decided to transfer cNK cells at gestational day 0.5 to ensure that the cells were present at throughout most of early pregnancy, particularly during implantation and the initial stages of decidualization. We also did not transfer cells before mating to minimize the number of mice that did not get pregnant. Additionally, performing the transfer at this early time point minimized repeated manipulation of pregnant dams, as procedural stress itself has been shown to affect physiological processes of gestation and could thereby confound the pregnancy outcomes we were assessing. Furthermore, Filipovic et al. 2018 previously showed that both trNK cells and cNK cells in the pregnant uterus expressed Ki67 at gestational 9.5, suggesting that there could be local proliferation of cNK-derived trNK cells in the gravid uterus that could limit the migration of circulating cNK cells into this microenvironment. We have discussed in more depth in our discussion section as follows:

"Interestingly, the inability to fully reconstitute the uterine trNK cell compartment following adoptive transfer suggests that only a subset of circulating cNK cells may be capable of differentiating into trNK cells during pregnancy, or alternatively that trNK cells already present in the virgin uterus may undergo *in situ* proliferation in the gravid uterus. Previous studies from our lab as well as others show that trNK cells within the pregnant murine uterus express marked levels of Ki67, supporting a model in which local proliferation of uterine trNK cells is a major contributor to the uterine trNK cell pool during pregnancy [7,32]. Prior studies have also described hematopoietic precursors within endometrial and decidual tissues that generate uterine trNK cells, suggesting that the compartment may be also sustained by local precursor differentiation [33-35]. Together, these findings suggest that

uterine trNK cell ontogeny may be more complex than a single-source model and raise the possibility that distinct developmental pathways may operate at different stages of reproductive life. Therefore, defining the relative contribution and developmental timing of hematogenous versus locally maintained sources *in vivo* could provide relevant insights into the developmental trajectories and transcriptional programs that underlie decidual NK cell heterogeneity."

Zhai, Q.Y., Wang, J.J., Tian, Y., Liu, X. and Song, Z., 2020. Review of psychological stress on oocyte and early embryonic development in female mice. Reproductive Biology and Endocrinology, 18(1), p.101.

Wiebold, J.L., Stanfield, P.H., Becker, W.C. and Hillers, J.K., 1986. The effect of restraint stress in early pregnancy in mice. Reproduction, 78(1), pp.185-192.

Sánchez-Rubio, M., Abarzúa-Catalán, L., Del Valle, A., Méndez-Ruette, M., Salazar, N., Sigala, J., Sandoval, S., Godoy, M.I., Luarte, A., Monteiro, L.J. and Romero, R., 2024. Maternal stress during pregnancy alters circulating small extracellular vesicles and enhances their targeting to the placenta and fetus. Biological Research, 57(1), p.70.

Filipovic, I., Chiossone, L., Vacca, P., Hamilton, R.S., Ingegnere, T., Doisne, J.M., Hawkes, D.A., Mingari, M.C., Sharkey, A.M., Moretta, L. and Colucci, F., 2018. Molecular definition of group 1 innate lymphoid cells in the mouse uterus. Nature Communications, 9(1), p.4492.

> *(3) The authors should consider inducible Tgfbr2 deletion (e.g. with Tamoxifen-inducible Cre) to enable development of the uterine NK compartment in virgin mice and only ablate trNK differentiation during pregnancy. This could help to estimate the turnover of cNK into trNK, or to understand if constant cNK recruitment is required to form the uterine trNK compartment during pregnancy.*

Thank you for this suggestion. We did initially consider incorporating a mouse model with a tamoxifen-inducible deletion of the TGF-βRII to examine the differentiation of peripheral cNK cells into uterine trNK cells more precisely. However, the administration of tamoxifen during murine pregnancy has well-established deleterious effects on implantation, fetal viability, and placentation, which would confound our interpretations of any adverse pregnancy outcome observed in our studies. Because our goal was to assess NK cell-specific contributions to murine gestation without introducing additional pregnancy-related perturbations, we elected to use an $Ncr1^{iCre}$ – based mouse model in our studies.

Ved, N., Curran, A., Ashcroft, F.M. and Sparrow, D.B., 2019. Tamoxifen administration in pregnant mice can be deleterious to both mother and embryo. Laboratory animals, 53(6), pp.630-633.

Sun, M.R., Steward, A.C., Sweet, E.A., Martin, A.A. and Lipinski, R.J., 2021. Developmental malformations resulting from high-dose maternal tamoxifen exposure in the mouse. PLoS One, 16(8), p.e0256299.

Ilchuk, L.A., Stavskaya, N.I., Varlamova, E.A., Khamidullina, A.I., Tatarskiy, V.V., Mogila, V.A., Kolbutova, K.B., Bogdan, S.A., Sheremetov, A.M., Baulin, A.N. and Filatova, I.A., 2022. Limitations of tamoxifen application for in vivo genome editing using Cre/ERT2 system. International Journal of Molecular Sciences, 23(22), p.14077.

> *(4) Did the authors consider transfer of Tgfbr2-floxed Ncr1-Cre cNK in the same setup as in Fig. 1C? This experiment could confirm the requirement of Tgfbr-dependent signaling for cNK to trNK conversion during pregnancy versus effects of Tgfb signals on trNK numbers in the uterus at steady state (before pregnancy).*

We thank the reviewer for this mechanistically insightful suggestion. We did consider performing reciprocal transfer experiments using TGF-βRII$^{fl/fl}$ Ncr1^{icre} cNK cells in the same adoptive transfer system as in Figure 1C. Our current adoptive transfer experiments already directly address this question. Transfer of congenically labeled wild-type splenic cNK cells into TGF-βRII$^{Ncr1\Delta}$ dams at gestational day 0.5 resulted in partial reconstitution of the uterine trNK compartment and, importantly, this was sufficient to rescue the adverse pregnancy outcomes observed at midgestation. These findings indicate that TGF-β–competent cNK cells can differentiate and function appropriately within the pregnant uterine environment, supporting a requirement for TGF-β–dependent signaling in cNK-to-trNK conversion during pregnancy. Because restoration of TGF-β–sufficient cNK cells rescues these pregnancy outcomes, we believe this experiment functionally demonstrates the importance of TGF-β signaling in this process and therefore did not pursue reciprocal transfer of TGF-βRII–deficient cNK cells.

“Partial reconstitution of uterine trNK cells restores midgestational pregnancy outcomes in TGF-βRII$^{Ncr1\Delta}$ dams

To determine whether restoring uterine trNK cells could rescue the midgestational pregnancy defects observed in TGF-βRII$^{Ncr1\Delta}$ dams, we adoptively transferred wildtype, congenically labeled splenic cNK cells into pregnant TGF-βRII$^{Ncr1\Delta}$ dams at gd 0.5. By gd 10.5, donor cNK cells were detected in the pregnant uterus, where a subset upregulated CD49a and downregulated CD49b, consistent with acquisition of a uterine trNK cell phenotype (Figure 5 A). However, adoptively transferred splenic cNK cells only partially reconstituted the uterine trNK cell population in the gravid uterus of TGF-βRII$^{Ncr1\Delta}$ dams, as evidenced by reduced absolute number and frequency of donor-derived trNK cells in reconstituted TGF-βRII$^{Ncr1\Delta}$ dams (Figure 5 A-C). Notably, this partial reconstitution was sufficient to rescue the gestational defects caused by impaired TGF-β–mediated uterine trNK cell differentiation. Reconstituted TGF- βRII$^{Ncr1\Delta}$ dams exhibited implantation site numbers and fetal resorption rates at gd 10.5 comparable to those observed in littermate controls (Figure 5 D, E). Together, these findings suggest that even partial restoration of the uterine trNK cell in pregnant TGF-βRII$^{Ncr1\Delta}$ dams is sufficient to restore pregnancy outcomes at midgestation, supporting a central role for uterine trNK cells as the principal NK cell subset required for successful murine pregnancy.”

> *(5) Figures 2D/E: The authors should state that ILC1s are reduced in the virgin uterus of female Tgfbr2-floxed or Tgfb1-floxed Ncr1-Cre mice and cite the relevant work (the Ref #29 discussed in this context did not show that?). It would be helpful to include an analysis of all three uterine ILC subsets in steady state. This could help to answer the question if the cNK cell changes are pregnancy-specific or a general phenomenon in Tgfbr2-floxed Ncr1-Cre mice.*

We thank the reviewer for this important comment and for noting the miscitation. We regret the error and have corrected the reference in the revised manuscript to cite the appropriate study demonstrating reduced ILC1s in the virgin uterus of Tgfb1$^{fl/fl}$ Ncr1^{iCre} mice {Sparano, C. et al. 2024. Autocrine TGF-β1 drives tissue-specific differentiation and function of resident NK cells. Journal of Experimental Medicine, 222(3), p.e20240930}. Please see Line 148. Importantly, the steady-state ILC compartment in virgin Tgfb1$^{fl/fl}$ Ncr1^{iCre} mice has already been carefully characterized in the previously published work, including analysis of all three uterine ILC subsets. Because the steady-state uterine ILC landscape in this mouse model has already been established by Sparano, C. et al. 2024, our study focuses specifically on the pregnancy-associated changes in the uterine ILC landscape occurring in the absence of TGF-β signaling in *Ncr1*-expressing cells and their subsequent effects on gestational outcomes. In the absence of TGF-β signaling there appears to be a higher frequency of cNK cells in both the virgin uterus and pregnant uterus, suggesting that this is more of a general phenomenon.

"However, in the pregnant uterus, CD49a^{+} Eomes^{-} ILC1s were markedly reduced in implantation sites of TGF-βRII$^{Ncr1\Delta}$ dams, paralleling the reduction of ILC1s previously reported in the virgin uterus of TGF-βRII$^{Ncr1\Delta}$ female mice [26]."

> *(6) Figure 2E: Please phrase more carefully about the "concomitant increase" of cNKs, since this increase is much less pronounced compared to the very strong reduction (absence) of trNKs in Tgfbr2-floxed Ncr1-Cre mice. Do the authors suggest that cNKs are halted at this stage and cannot differentiate into trNK, based on these data?*

We thank both reviewers for this suggestion, and we have rephrased our wording to address this concern as follows:

"This reduction of uterine trNK cells was accompanied by a small increase in the absolute number and frequency of CD49b^{+} Eomes^{+} cNK cells within the pregnant uterus of TGF-βRII$^{Ncr1\Delta}$ dams (Figure 2 D, E). Collectively, these findings suggest that a TGF-β–driven differentiation pathway directs the conversion of peripheral cNK cells into uterine trNK cells during murine pregnancy."

Please also see our response to Reviewer #1, Comment #2.

> *(7) Can the reduced litter size and the abnormal spiral artery formation be rescued by transfer of WT cNK into Tgfbr2-floxed Ncr1-Cre mice?*

We thank the reviewers for this interesting question. In subsequent experiments, we transferred congenically labeled, splenic cNK cells from wildtype female mice into TGF-βRII$^{Ncr1\Delta}$ dams at gestational day 0.5. We only observed partial reconstitution of uterine trNK cell population; however, the number of viable implantation sites and resorption rates in reconstituted TGF-βRII$^{Ncr1\Delta}$ dams were comparable to the number of viable implantation sites and resorption rates in HBSS-treated littermate controls at gestational day 10.5. Given that partial reconstitution of the uterine trNK cell compartment in reconstituted TGF-βRII$^{Ncr1\Delta}$ dams was sufficient to rescue the defects in implantation site number and fetal resorption rates observed at midgestation, we hypothesize that this level of restoration may permit patrial but functionally sufficient spiral artery remodeling to reestablish maternal-fetal blood flow adequate to support fetal viability, although spiral artery remodeling was not directly assessed in this transfer study.

"Partial reconstitution of uterine trNK cells restores midgestational pregnancy outcomes in TGF-βRII$^{Ncr1\Delta}$ dams

To determine whether restoring uterine trNK cells could rescue the midgestational pregnancy defects observed in TGF-βRII$^{cr1\Delta}$ dams, we adoptively transferred wildtype, congenically labeled splenic cNK cells into pregnant TGF-βRII$^{Ncr1\Delta}$ dams at gd 0.5. By gd 10.5, donor cNK cells were detected in the pregnant uterus, where a subset upregulated CD49a and downregulated CD49b, consistent with acquisition of a uterine trNK cell phenotype (Figure 5 A). However, adoptively transferred splenic cNK cells only partially reconstituted the uterine trNK cell population in the gravid uterus of TGF-βRII$^{Ncr1\Delta}$ dams, as evidenced by reduced absolute number and frequency of donor-derived trNK cells in reconstituted TGF-βRII$^{Ncr1\Delta}$ dams (Figure 5 A-C). Notably, this partial reconstitution was sufficient to rescue the gestational defects caused by impaired TGF-β–mediated uterine trNK cell differentiation. Reconstituted TGF-βRII$^{Ncr1\Delta}$ dams exhibited implantation site numbers and fetal resorption rates at gd 10.5 comparable to those observed in littermate controls (Figure 5 D, E). Together, these findings suggest that even partial restoration of the uterine trNK cell in pregnant TGF-βRII$^{Ncr1\Delta}$ dams is sufficient to restore pregnancy outcomes at midgestation, supporting a central role for uterine trNK cells as the principal NK cell subset required for successful murine pregnancy."

> ***Reviewer #2 (Recommendations for the authors):***
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> *(1) Figure 1C: The shown gate seems to "cut" into the CD49b staining; staining for all transferred cells should be shown; have cNK cells been stained in parallel with the same panel to provide a positive and compensation control?*

To clarify the phenotype of the adoptively transferred cNK cells, we included two additional gates depicting the expression of CD49a and CD49b in unlabeled (non-vascular) trNK cells and cNK cells in the pregnant uterus Please see the revised Figure 1C.

"(C) Concatenated flow plots of implantation sites showing that adoptively transferred cNK cells in pregnant uterus of wildtype dams upregulate CD49a and down regulate CD49b by gd 10.5, acquiring a CD49a^{+} CD49b^{-} Eomes^{+} phenotype characteristic of uterine trNK cells (C57BL/6 dams *n*=4). Here, 2.5x10^{6} CD45.2^{+} CD3^{-} CD19^{-} NK1.1^{+} NKp46^{+} CD49b^{+} splenic cNK cells were adoptively transferred into pregnant C57BL/6-CD45.1 dams at gd 0.5, and the receptor profile of these cells was subsequently assessed at gd 10.5. Gating strategy: Live, Single Cells; CD3^{-} CD19^{-} CD45.1^{-} CD45.2–PE-Cy7^{-} CD45.2–PE^{+} NK1.1^{+} NKp46^{+} cells."

> *(2) Figure 2A: The authors could include an isotype control or a staining in a genetic knockout as a control staining.*

Thank you for this suggestion. As suggested, we included staining in a genetic TGF-βRII$^{Ncr1\Delta}$ knockout as additional control staining. Please see the revised Figure 2A.

"Representative histograms depicting TGF-β Receptor II expression on splenic NK cells from virgin TGF-βRII$^{Ncr1\Delta}$ and wildtype mice as well as splenic and uterine NK cell subsets from pregnant wildtype mice at gd 10.5 (virgin TGF-βRII$^{Ncr1\Delta}$ mice, *n*=2; virgin mice: C57BL/6, *n*=5; gd 10.5: C57BL/6 dams, *n*=8, implantation sites *n*=8). MFI, median fluorescent intensity. Gating strategy: Live, Single Cells; CD3^{-} CD19^{-} CD45.1^{-} CD45.2^{+} NK1.1^{+} NKp46^{+} cells."

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