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T-bet Fate Mapping Reveals Gestational Stage-Specific Transcriptional Adaptation of Decidual NK Cells

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ABSTRACT

Problem: Natural killer (NK) cells are critical regulators of immune balance at the maternal–fetal interface. T-bet (Tbx21) is a key transcription factor shaping NK cell effector functions, yet its role in decidual NK (dNK) cell adaptation across gestation remains unclear.

Method of Study: We used a T-bet fate-mapping mouse model ($Rosa26^{RFP} \times Tbx21^{Cre}$) to track developmental and functional reprogramming of NK cells in the uterus, decidua, and placenta throughout pregnancy. Analyses included flow cytometry, bulk RNA sequencing of fate-mapped cells, and single-cell transcriptomic profiling of CD45+Lineage- immune populations at mid and late gestation.

Results: We found that NK cells with a history of T-bet expression (RFP+) progressively downregulate T-bet in a tissue and gestation-specific manner, particularly within decidual and placental compartments. Despite this loss, RFP+ cells retained core NK cell markers and altered their lineage identity towards ILC2 or ILC3 fate. Bulk transcriptomic analysis revealed that T-bet downregulation is associated with dampened IFN- γ , and cytotoxic pathways and increased expression of tissue-residency associated transcriptional regulators. Single-cell RNAseq revealed a gestational transition in dNK subset composition, with a decline in cytotoxic tissue-resident NK cells and expansion of regulatory and conventional NK subsets by late gestation.

Conclusions: These findings identify a novel transcriptional program that shapes NK cell plasticity in response to T-bet downregulation across gestation. Rather than undergoing lineage diversion, dNK cells adapt to the decidual environment via transcriptional compensation and subset redistribution during pregnancy. This work sheds light on the temporal coordination of innate immune function relevant to pregnancy success.

1 | Introduction

Pregnancy represents a unique immunological challenge, requiring a finely tuned balance between immune tolerance and defense. The decidua, the maternal endometrium transformed during pregnancy, serves as an immunological interface between two disparate individuals (mother and fetus). Despite advances in

our understanding of this system, the cellular-level mechanisms required for maternal–fetal crosstalk remain a significant knowledge gap. Decidual natural killer (dNK) cells, the most abundant lymphocytes in the decidua during early and mid-pregnancy, play a pivotal role in establishing and maintaining a successful pregnancy [1]. Functionally and phenotypically distinct from peripheral natural killer cells (pNK), dNK cells are thought

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to undergo stage-specific adaptations through modulation of inflammatory programs to support pregnancy [2–4].

One critical distinction between pNK and dNK cells lies in their transcriptional regulation and effector potential [4, 5]. In both human and mice, pNK cells, which predominantly reside in the spleen and circulation, are characterized by high cytotoxic potential and robust expression of effector molecules including perforin, granzyme B, and IFN- γ [6, 7]. In contrast, dNK cells display markedly reduced cytotoxicity but an enhanced capacity to produce a diverse array of immunomodulatory and proangiogenic factors including VEGF, IL-8, and PlGF [3, 8–10, 11]. It is now also apparent that dNK cells are not a single cell type, as single-cell RNA sequencing (scRNAseq) of first-trimester and term dNK cells has revealed three distinct subsets, dNK1, dNK2, and dNK3, classified by receptor repertoires and transcriptional profiles [5, 12], potentially reflecting their specialized roles in immune regulation and tissue adaptation at the maternal–fetal interface.

Key transcriptional regulators of NK cell development and functional specialization are T-box family members T-bet (gene: *Tbx21*) and Eomesodermin (gene: *Eomes*, henceforth both will be referred to as Eomes), which act in a coordinated yet distinct manner to guide lineage commitment, maturation, and effector programming [13]. In pNK cells, combined deletion of *Tbx21* and *Eomes* results in a complete loss of mature NK cells, whereas individual deletion of either gene causes a partial developmental block [14]. T-bet is required during the terminal stages of NK cell maturation, where it drives the expression of cytotoxic effector molecules and surface receptors necessary for immune defense [15, 16]. In murine models, T-bet-deficient (*Tbx21*^{−/−}) mice exhibit increased NK cell numbers in the bone marrow but significantly reduced frequencies in peripheral tissues such as spleen, liver, and blood, but not in uterus [17]. These pNK cells often retain an immature CD27⁺CD11b⁺ phenotype [15, 17]. Single-cell RNA sequencing further demonstrated that over 65% of NK cells lacking T-bet exhibited pronounced upregulation of genes characteristic of immature NK cells [18]. Mechanistically, T-bet has been proposed to drive NK cell maturation by synergizing with Zeb2 to activate a maturation-specific transcriptional program, while concurrently upregulating S1P5 to promote the egress of mature NK cells from the bone marrow into circulation [15, 19, 20, 21]. Additionally, it reinforces NK cell maturation by repressing immature gene expression programs through the mTORC2-Akt^{S473}-FoxO1-T-bet axis [18]. By contrast, Eomes is essential for early NK cell lineage specification and the maintenance of core identity markers [13, 22, 23]. The interdependence of these transcription factors is underscored by murine studies showing that NK precursors lacking Eomes fail to develop into NK cells in the absence of T-bet, as demonstrated by the complete loss of NK cells in *Tbx21*^{−/−} *Eomes*^{fl/fl} *Vav-Cre*⁺ mice [14, 15]. These findings indicate that both Eomes and T-bet are essential for NK cell development and for maintaining lineage commitment. In humans, a similar transcriptional gradient is observed, where terminally differentiated CD57⁺CD56^{dim} NK cells express the highest levels of T-bet and the lowest levels of Eomes [7, 24]. However, direct evidence for the functional necessity of T-bet in human NK cell development remains limited. A report of a patient with a rare autosomal recessive microcephaly syndrome involving chromosomal translocation and silencing of Eomes

transcripts demonstrated that numbers of cells with an NK phenotype could develop normally in the absence of Eomes, though functional data were lacking [25, 26].

Tissue-specific variation in T-bet expression further reflects the heterogeneity of NK cell ontogeny. TrNK cells, found in non-lymphoid organs such as the liver, salivary glands, Peyer's patches, skin, and uterus, exhibit divergent T-bet and Eomes expression profiles [1, 27]. Liver-resident NK cells and skin-resident NK cells are strictly dependent on T-bet for their development and maintenance, conversely, trNK cells in the salivary glands and uterus express high levels of Eomes and are developmentally independent of T-bet [13, 26, 27]. Sojka et al. demonstrated that uterine trNK cells possess distinguishing features that set them apart not only from pNK cells but also from trNK populations in other tissues such as the liver and skin, which develop in a T-bet-independent manner [27, 28].

T-bet expression itself is regulated by multiple signaling pathways and transcription factors. Cytokine-mediated signals, particularly interleukin-15 (IL-15) [29], play a central role by activating STAT5, which directly promotes *Tbx21* transcription. Transcription factors, including ETS1 and members of the TOX family [30, 31], further contribute to the initiation of T-bet expression during early stages of NK cell lineage commitment. Once induced, T-bet acts as a central orchestrator of a broad gene expression program that governs terminal NK cell maturation and acquisition of effector functions (e.g., IFN- γ). During pregnancy, dNK cells utilize IFN- γ and other cytokines to promote trophoblast invasion and regulate vascular remodeling, thereby establishing a supportive environment at maternal–fetal interface [8, 32, 33]. Studies in pNK cells have shown that while early IFN- γ production is independent of T-bet, sustained secretion under prolonged stimulation requires T-bet expression [17]. In addition to its role in cytokine production, T-bet supports the cytolytic activity of NK cells by directly binding to the promoters of key effector genes such as *Prfl*, *Gzmb*, and *Runx1* [15, 17]. Moreover, T-bet has been identified as a key regulator of trNK cell persistence. In T-bet-deficient mice, the number of trNK cells, characterized by CD49a⁺CD49b[−] phenotype and distinct from circulating CD49a[−]CD49b⁺ pNK cells, was significantly reduced [27]. Beyond the NK lineage, T-bet plays a broader role in regulating immune cell plasticity and lineage stability. In diverse tissue contexts, including the intestinal lamina propria, liver, and inflamed mucosa, T-bet drives the trans-differentiation of ILC3 and ILC2 subsets into ILC1-like cells by repressing *ROR γ t* and inducing IFN- γ expression [34, 35, 36].

Despite these well-established functions of T-bet in peripheral and tissue-resident NK cell subsets, how T-bet is regulated and functions within dNK cells during pregnancy remain poorly understood. Given the unique phenotype and specialized functions of dNK cell subsets, this study intends to reveal the details of dNK cell transcriptional regulation as they adapt to evolving pregnancy. We integrate high-dimensional polychromatic flow cytometry with sorted bulk and single cell transcriptomic profiling to analyze the role of T-bet in dNK cells at the maternal–fetal interface as they adapt to evolving pregnancy. To specifically examine the cellular history and transcriptional adaptation of T-bet⁺ dNK cells before and at commencement of pregnancy we

employ the T-bet fate-mapping to identify these cells throughout the rest of gestation. We aimed to address two central questions:

1. How is T-bet regulated in dNK cells across pregnancy?
2. What is the transcriptional and lineage landscape of dNK cells in the context of T-bet downregulation with advancing gestation?

2 | Methods

2.1 | Mice

Female and male C57BL/6J (B6) mice were purchased from Jackson laboratory (Bar Harbor, ME, cat# 000664). Transgenic mouse model, loxP-flanked STOP-RFP reporter mouse strain (B6;129S6Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J; henceforth B6.Rosa, Jackson Lab, cat# 007914) were crossed with Tbet-Cre reporter strain (B6.CBA-Tg(Tbx21-cre)1Dlc/J, henceforth B6.Tbet^{Cre}, Jackson Lab, cat# 024507) to produce B6.Rosa^{RFP}/Tbet^{Cre} mouse model, respectively. These animals have a floxed STOP codon in front of the bright RFP transgene (tdTomato), which is removed (allowing RFP synthesis) in cells that have expressed the T-bet transcription factors at any prior time. B6.Tbet^{Cre} animals were backcrossed to C57BL/6 for at least 2 generations at Jackson Laboratories and were backcrossed an additional 5 generations prior to breeding with B6.Rosa animals, with resulting Rosa/Tbet^{Cre} animals being backcrossed into the B6 background for at least 8 generations.

B6.Rosa^{RFP}/Tbet^{Cre} females (6–13 weeks) were syngeneically mated with C57BL/6J (B6) males. The day when a vaginal plug was detected in a timed mating was counted as gestational day (GD) 0.5. Virgin mice and the mice at various specified GD (early: 6, 7, 8, mid:12, 13, 14, late: 16, 17,18 days) were sacrificed, and GD of each embryo/decidua/placenta/uterus saved for analysis. Detailed dissociator settings for the different types of tissue are provided in the [Supplementary Methods](#).

2.2 | Tissue Processing and Immune Cell Isolation

Mouse uterine horns were excised, and individual implantation sites were separated. Placentae and fetal membranes were gently peeled using fine forceps, and the maternal layer directly attached to the placenta, corresponding to the decidua basalis, was microdissected with fine scissors. Decidua, embryos (GD6-8 only), placentae, and uteri were collected and minced with scissors in RMPI 1640 containing collagenase type V (Worthington Biochem, cat# LS005282)/DNase I (Worthington Biochem, cat# LS006344). These specimens were then loaded in gentleMACS C tube (Miltenyi Biotec Inc. San Diego, CA, cat# 120-005-331), and a specially adapted tissue dissociation program run in gentleMACS Dissociator for 30 min (Miltenyi Biotec Inc. San Diego, CA, cat# 130-096-427) [37]. Spleen, thymus and Peyer's patches (used as control tissues) were mechanically dissociated in RMPI 1640 containing 10% heated FBS in gentleMACS C tube, by running corresponding programs for different tissue types in gentleMACS Dissociator.

After dissociation, homogenates were filtered through 70 µm cell strainer, and red cells of splenic or thymic (as needed) specimens were lysed with ACK lysis buffer (Life Technologies, cat# A10492-01). Single cell suspension obtained was used for downstream applications [37, 38].

2.3 | Flow Cytometry Labeling

Single cell suspensions were first labeled with LIVE/DEAD fixable blue stain (Invitrogen, cat# L34962) or Zombie NIR Fixable Viability Kit (Biolegend, cat# 423105) and subsequently a cocktail of fluorochrome-conjugated monoclonal antibodies (list in Table S2) according to the manufacturer's instructions. Briefly, antibodies were diluted in BD Horizon Brilliant™ Stain Buffer (BD Biosciences, San Jose, CA, cat# 566349) and used to label cells for 30 mins, washed, and fixed with 4% formaldehyde (TED PELLA, Inc., cat# 1805) for 5 mins before washout using stain buffer (BD, cat# 554656). Transcription factor assessment for intracellular staining was done using BD Pharmingen Transcription Factor Buffer Set (BD, cat# 562574). UltraComp eBeads were used for compensation (eBioscience, cat # 01-222-42). The number of mice used for flow cytometry analyses was summarized in Table S1.

2.4 | Data Analysis

Flow cytometry data were acquired using two instruments: the BD LSRFortessa (BD Biosciences) and the Cytek Aurora spectral cytometer. The LSRFortessa is equipped with five lasers (UV 355 nm, Violet 405 nm, Blue 488 nm, Yellow/Green 561 nm, and Red 640 nm) and a 20-detector configuration. Photomultiplier tube (PMT) voltage settings were standardized using SPHERO Rainbow Calibration Particles (Spherotech, Cat# RFP-30-5A). In parallel, spectral data were acquired using the Cytek Aurora, a five-laser, 67-channel spectral flow cytometer configured as follows: 355 nm (16 channels), 405 nm (16 channels), 488 nm (14 channels), 561 nm (10 channels), and 640 nm (8 channels). The Aurora supports sample acquisition from both 5 mL round-bottom tubes. Spectral data were unmixed using SpectroFlo software (version 3.2.1) with a reference matrix generated from single-stained controls.

Manual gating analysis was performed using FlowJo v10.10 (BD Biosciences, Franklin Lakes, NJ, USA). Statistical analysis was conducted manually using GraphPad Prism version 7 (GraphPad Software Inc., La Jolla, CA, USA). One-way ANOVA followed by Tukey's multiple testing adjusted post-hoc analysis was used to determine statistical significance ($p < 0.05$). All data are represented as mean $\pm$ SEM. All the statistical details were summarized in Tables S3–S6.

2.5 | Bulk RNA Sequencing

2.5.1 | Library Preparations and Sequencing

RNA was isolated from sorted CD19- TCRB- CD11c- CD3e- CD45+ RFP+ cells from uteri/decidua of B6.Rosa^{RFP}/Tbet^{Cre}; 2 virgin mice, 2 mice at GD 6.5, 1 mouse at GD 14.5, and 1 mouse at GD 18.5 using the Qiagen RNEasy Micro Kit. RNA libraries were created

using the Takara SMART-Seq v4 Ultra-Low Input RNA kit and sequenced on Illumina platforms by the University of Wisconsin Gene Expression Center in collaboration with the UWBC DNA Sequencing Facility, Madison, Wisconsin.

2.5.2 | Computational Analysis

Fastq files were trimmed and aligned to the mm10 assembly using STAR aligner (Table S7). Count matrices were then analyzed for differential gene expression using DESeq2, comparing early and late gestation (GD 6.5 and GD 18.5). Heatmaps were created with ComplexHeatmap. Adjusted *p*-values were generated via Bonferroni-correction (Table S8).

2.6 | Single Cell Sequencing

2.6.1 | Library Preparations and Sequencing

Single-cell suspensions of sorted CD19⁺ TCRB⁺ CD11c⁺ CD3e⁺ Ly6G⁺ CD45⁺ cells from WT C57BL/6J mouse decidua at GD 13.5 and 18.5 (*n* = 1 sample per time point) were submitted to UW Biotechnology Center. Following quality control (QC), high-quality transcriptomes were retained for analysis with the following retention metrics: for GD 13.5, 2671 cells were retained (from 3039 raw); for GD 18.5, 2254 cells were retained (from 2450 raw). Libraries were prepared and cells sequenced using the 10x Chromium Single Cell 3' platform at the University of Wisconsin Gene Expression Center in collaboration with the UWBC DNA Sequencing Facility, Madison, Wisconsin.

2.6.2 | Single Cell Transcriptomics Data Preprocessing

Sequencing data were processed with CellRanger count v3.1.0. scRNA sequences were aligned to the mm10 assembly. Filtered gene matrices were analyzed using Seurat v5.1.0 except where otherwise specified. ScDbtFinder (Bioconductor) was used to annotate cells as singlets or doublets in individual gene matrices, and then both samples were merged into a single Seurat object. For initial quality control, cells were retained that had between 200 and 6000 features, lower than 2% mitochondrial transcripts, total RNA count below 4000 and were annotated as singlets by scDbtFinder. A total of 4925 cells passed QC (Figures S4A, B and S5). Counts were depth-normalized and log-transformed, the top 2000 variable features determined, and counts scaled. Harmony was used for dimensionality reduction and batch correction. A k-nearest-neighbor graph was constructed (FindNeighbors, FindClusters; Leiden clustering with resolution 0.5) and visualized using uniform manifold approximation and projection (RunUMAP).

2.7 | Downstream Analysis

Clusters were annotated based on gene expression and using a combination of canonical cell type marker genes as well as highly expressed genes identified by rank-sum tests (FindMarkers and FindAllMarkers). Genes used to determine cluster identities are found in the relevant figures. Differential gene expres-

sion analyses were carried out using Model-based Analysis of Single-cell Transcriptomics (MAST) implemented through Seurat FindMarkers and plotted with EnhancedVolcano. Heatmaps were created with ComplexHeatmap, with expression data pseudobulked via Aggregate Expression.

3 | Results

3.1 | Gestational Dynamics of T-bet Fate-Mapped dNK Cells

Given the pivotal role of T-bet in NK cell maturation, and to longitudinally track the dNK cells with a history of T-bet expression we generated a fate-mapping strain B6.Rosa^{RFP}/Tbet^{Cre} as described in Materials and Methods. We first confirmed that wild-type and B6.Rosa^{RFP} control mice exhibited no RFP signal, whereas B6.Rosa^{RFP}/Tbet^{Cre} mice showed distinct RFP⁺ cells, confirming that RFP expression faithfully marks cells that have ever expressed T-bet (Figure 1A, B). RFP⁺ cells were readily detected in the nonpregnant (virgin) uterus, comprising roughly one-quarter of the uterine leukocytes (Figure 1B, C), consistent with the presence of tissue-resident T-bet⁺ populations. Consistent with their identity as predominantly conventional natural killer (cNK) cells, maternal spleen Lin-CD45⁺ cells showed very high RFP proportion while RFP⁺ cells were scarcer in Peyer's patches and essentially absent in thymus (Figure 1D), supporting the specificity of the RFP signal for T-bet expressing innate lineages in peripheral and mucosal lymphoid tissues. Notably, virtually all NK1.1⁺ lineage negative (NK1.1⁺CD45⁺CD3⁺CD19⁺Ly6g⁺CD11c⁺, Lin⁻) cells in the virgin animals were RFP⁺. We observed a population of T-bet⁺ cells that lacked NK1.1 expression. While murine uterine NK cells are classically known as PAS⁺ and DBA⁺ [28, 39], we did not assess PAS or DBA reactivity in this study. Consequently, in this study, we cannot classify the NK1.1-T-bet⁺ cells as dNK cells. (Figure S1A, B).

Next, to determine the dynamics of RFP⁺ cells (T-bet fate mapped) across pregnancy, we examined the uterus, decidua, and placenta of B6.Rosa^{RFP}/Tbet^{Cre} mice. At early gestation (GD 6–8), RFP⁺ cells expanded significantly in the decidua compared to the virgin uterus (Figure 1C, E). Beyond early pregnancy however, data revealed a significant stepwise reduction in RFP⁺ cells in maternal (decidual) but not fetal (placental) compartments (*p* < 0.05, Figure 1C, E). These data agree with the previously described enrichment of dNK cells in early pregnancy, with increased proportions of other immune cells towards the end of pregnancy [37]. In the embryo, the Lin-CD45⁺RFP⁺ population (Figure 1E) likely reflects the ontogeny of ILC1s and NK progenitors arising in the fetal liver.

3.2 | T-bet Expression Is Plastic at the Maternal-Fetal Interface

To examine T-bet protein expression dynamics and assess for lineage plasticity within the fate-mapped population, we analyzed T-bet and alternative lineage-defining factors in RFP⁺ cells across gestation. As expected, RFP⁺ cells were largely T-bet protein-expressing (Figure 2A), confirming that the Cre-driven reporter

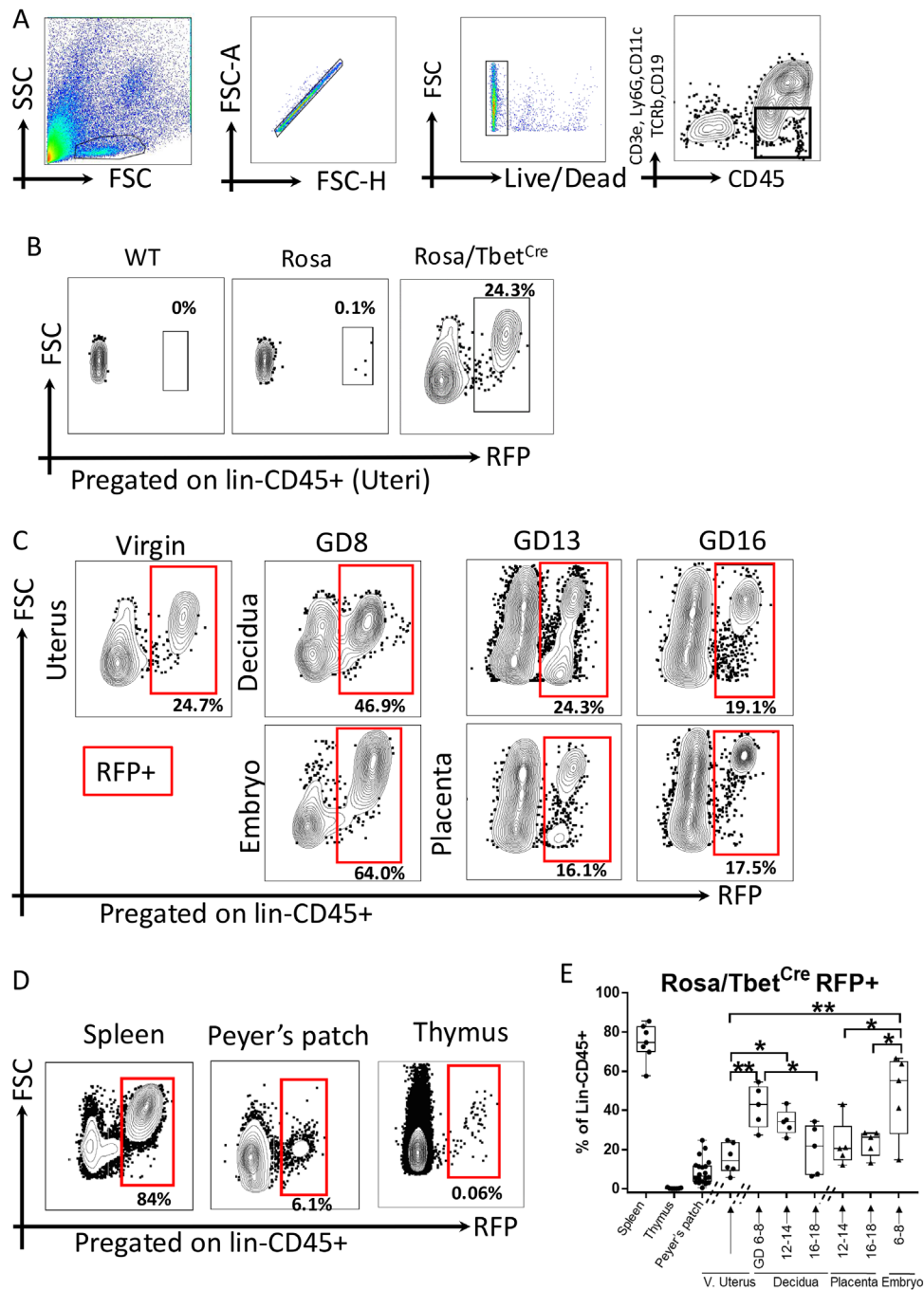


FIGURE 1 | Flow cytometry gating strategy and dynamics of RFP+ cells in Rosa/TbetCre mouse model. (A, B) The proportions of RFP+ cells, defined as lin^{neg} (CD3^{neg}TCRb^{neg}CD19^{neg}CD11c^{neg}LY-6G^{neg}CD45⁺) RFP+ (A), were shown in different mouse model, wild type (B6), Rosa, Rosa/TbetCre(B). (C) RFP+ cells were identified in the mouse virgin uterus, decidua (top panel), embryo, and placenta (lower panel) across murine gestation in Rosa/TbetCre mouse models. (D) RFP+ cells were identified in control tissues; spleen, Peyer's patch and thymus in Rosa/TbetCre mouse model. (E) The proportions of RFP+ cells in mouse virgin uterus, decidua, embryo, and placenta across mouse gestation, as well as in control tissues, in Rosa/TbetCre mouse model. $n = 3-6$ (6 independent experiments), the exact number of animals used were summarized in Table S1. The data of virgin uterus were compared with decidua group or placenta (include embryo) group, respectively. Statistical analysis was performed using ANOVA followed by post-hoc Tukey analysis. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$.

effectively marks cells with T-bet and notably, there were no RFP+ROR γ t+ cells.

Next, we examined protein levels of T-bet and Eomes (Figure 2B), ROR γ t (Figure S2A), and GATA3 (Figure S2B) across pregnancy. To our surprise we observed a progressive loss of T-bet protein in

decidual RFP+ cells as pregnancy advanced (GD12-14 and GD16-18, $p < 0.0001$; Figure 2B, C). In contrast, Eomes was persistently expressed across gestation (Figure 2B, D).

Since the acquisition of T-bet is associated with alteration in surface phenotype amongst gut ILC3s, we investigated whether

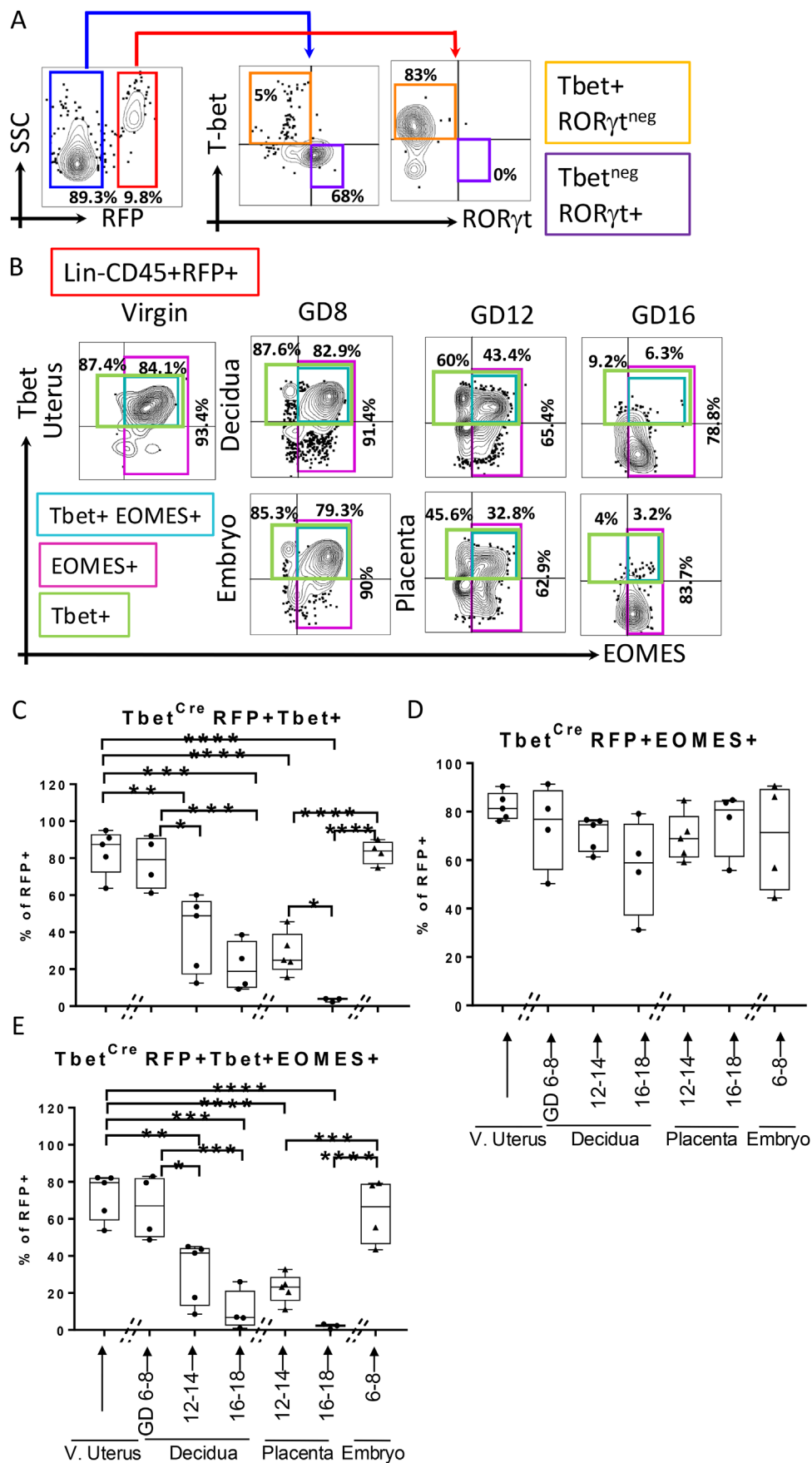


FIGURE 2 | Legend on next page.

FIGURE 2 | Stepwise loss of T-bet expression among RFP+ cells during gestation in Rosa/TbetCre mice. (A) Flow cytometry gating strategy to confirm high T-bet protein expression in RFP+ cells (Left panel). Co-expression analysis of T-bet and ROR γ t of RFP+ and RFPneg populations (middle and right panels) revealed distinct populations of Tbet+ROR γ tneg and TbetnegROR γ t+ cells. (B) Representative flow cytometry plots showing dynamic expression of Tbet and Eomes in LinnegCD45+RFP+ cells in virgin uterus and across gestation in decidua, placenta, and embryo compartments. (C–E) Quantification of T-bet and Eomes expression in RFP+ cells across tissues and gestational time points: (C) Frequency of TbetCreRFP+Tbet+ cells shows a significant decline in decidua and placenta at mid to late gestation compared to virgin uterus and early gestation. (D) Frequency of TbetCreRFP+EOMES+ cells remains relatively stable across gestation. (E) Frequency of double-positive TbetCreRFP+Tbet+EOMES+ cells significantly declines in decidua and placenta at GD12–16. Data are presented as mean $\pm$ SD. Statistical significance was determined using one-way ANOVA with post hoc testing. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

the loss of T-bet in the decidual RFP+ cells results in alteration of NK1.1 or DX5 (CD49b) expression. NK1.1 and DX5 levels were stable in RFP+ cells that have lost T-bet expression (Figure S1), suggesting that loss of T-bet does not result in surface phenotype instability. We then asked if loss of T-bet expression amongst RFP+ cells in B6.RosaRFP/Tbet^{Cre} animals was accompanied by acquisition of ROR γ t, analogous to plasticity seen in the gut [34, 40]. However, this was not the case, with few, if any RFP+Tbet neg cells expressing ROR γ t (Figures S3A and 2D). Similarly, we show no evidence of ILC1–ILC2 transition, as GATA3 was not detected either amongst RFP+Tbet neg cells (Figure S2B, E).

Finally, to test the hypothesis that loss of T-bet is a feature of the maternal–fetal interface, but not of systemic immune cells, we examined RFP+ cells in Peyer's patches from the same animals. Loss of Tbet protein was not observed in RFP+ cells of Peyer's patches at matched gestational ages (Figure S3), indicating that it is specific to decidua, and not a global change or processing artifact.

3.3 | Fate-Mapped Cells Undergo Transcriptional Downregulation of Tbx21 Without Lineage Diversion and Reveal Compensatory Transcriptional Programs

As T-bet is downregulated, while trans-differentiation to GATA-3 (ILC2) or ROR γ t (ILC3) expressing lineages is not occurring, how the transcriptional landscape of dNK cells adapts to loss of this master transcription factor was next assessed. T-bet fate mapper strains were used to specifically capture transcriptomic profiles of cells with current or historical T-bet expressions, thereby enriching Tbet-associated programs at the population level. Consequently, we tracked RFP+ cells at whole-transcriptome level across gestation. At least 33664 of RFP+CD45+Lin- decidua/uterine origin (virgin, GD6.5, GD14.5, and GD18.5) were sorted by flow cytometry, RNA extracted, reverse-transcribed and sequencing performed (RNAseq strategy in Figure 3A and Suppl Figure 4) across gestational timepoints. While flow cytometry demonstrated continued expression of Eomes, ROR γ t, and GATA3 in subsets containing those proteins (Figure 2B, D, S1A, B), transcriptomic analysis revealed partial transcriptional downregulation of Eomes and Rorc, in addition to the expected decrease in Tbx21 (gene encoding Tbet) expression (Figure 3A, B).

Loss of Tbx21 was associated with broad transcriptional reprogramming but not lineage diversion. Transcripts involved in IFN- γ signaling, Ifng, Ifngr1, Ifngr2, Stat1, Irf1, and Jak1, exhibited a biphasic expression pattern: they were downregulated from

early to mid-gestation (GD6.5–GD14.5), followed by partial or robust re-expression at late gestation (GD18.5), especially surprising given well known role of Tbet as key transcription factor driving IFN- γ synthesis. In contrast, genes associated with homing and tissue residency demonstrated a progressive increase in expression over time (Figure 3C). First, integrin-encoding genes (Itga4, Itgal, Itgb1) and chemokine receptors (Ccr7, Cxcr4) were upregulated during mid-to-late gestation, suggesting enhanced adhesive and localization capacity within the decidua. Cxcr6 expression showed a modest upward trend, while Ccr5 steadily declined and Cxcr3 remained minimally expressed throughout (Figure 3C). Cytotoxicity-related genes were also dynamically regulated and displayed distinct temporal patterns. Prfl and Gzmb were expressed at high levels early in gestation, followed by a marked decline by GD14.5–18.5. In contrast, Lta followed a trajectory similar to Gzma and Tnf, characterized by early repression followed by partial restoration (re-expression) at term (GD18.5). Collectively, these transcriptional changes suggest that early-gestation dNK cells are more cytotoxic (high granzyme expression), whereas late-gestation NK cells adopt a more regulatory or tissue-remodeling phenotype, with increased expression of receptors for tissue retention or egress (e.g., CCR7, CXCR4; Figure 3C, D).

To explore compensatory transcriptional programs that may emerge as Tbet activity diminishes, we next examined genes significantly upregulated during mid-to-late gestation. Differential expression analysis revealed that several transcripts, Adgre5 (CD97), Sgk1, Fhl1, and Rasd2, were consistently and significantly elevated at these stages (Figure 3E). These genes, associated with immune modulation, stress signaling, and tissue remodeling, point to the activation of alternative pathways that may sustain NK cell adaptation within the decidua. This shift was further observed in the transcription factor profile (Figure 3F and S4D), where progressive downregulation of Tbx21 coincided with increased expression of FoxO1, Zeb2, Prdm1, and Arntl. Taken together, these data support a model in which transcriptional compensation by alternative regulatory factors enables dNK cells to maintain tissue-supportive and noncytotoxic functions during late gestation in the absence of Tbet.

3.4 | Single-Cell Transcriptomic Analysis Reveals Shifts in NK Cell Subsets Across Gestation

To determine if cellular heterogeneity, rather than intrinsic alteration in gene expression may explain the gestational adaptation in dNK cells associated with Tbet downregulation in the decidua, we used WT C57BL/6J mice to enable unbiased FACS-sorting of CD45+Lin- decidua immune cells without reporter influence or

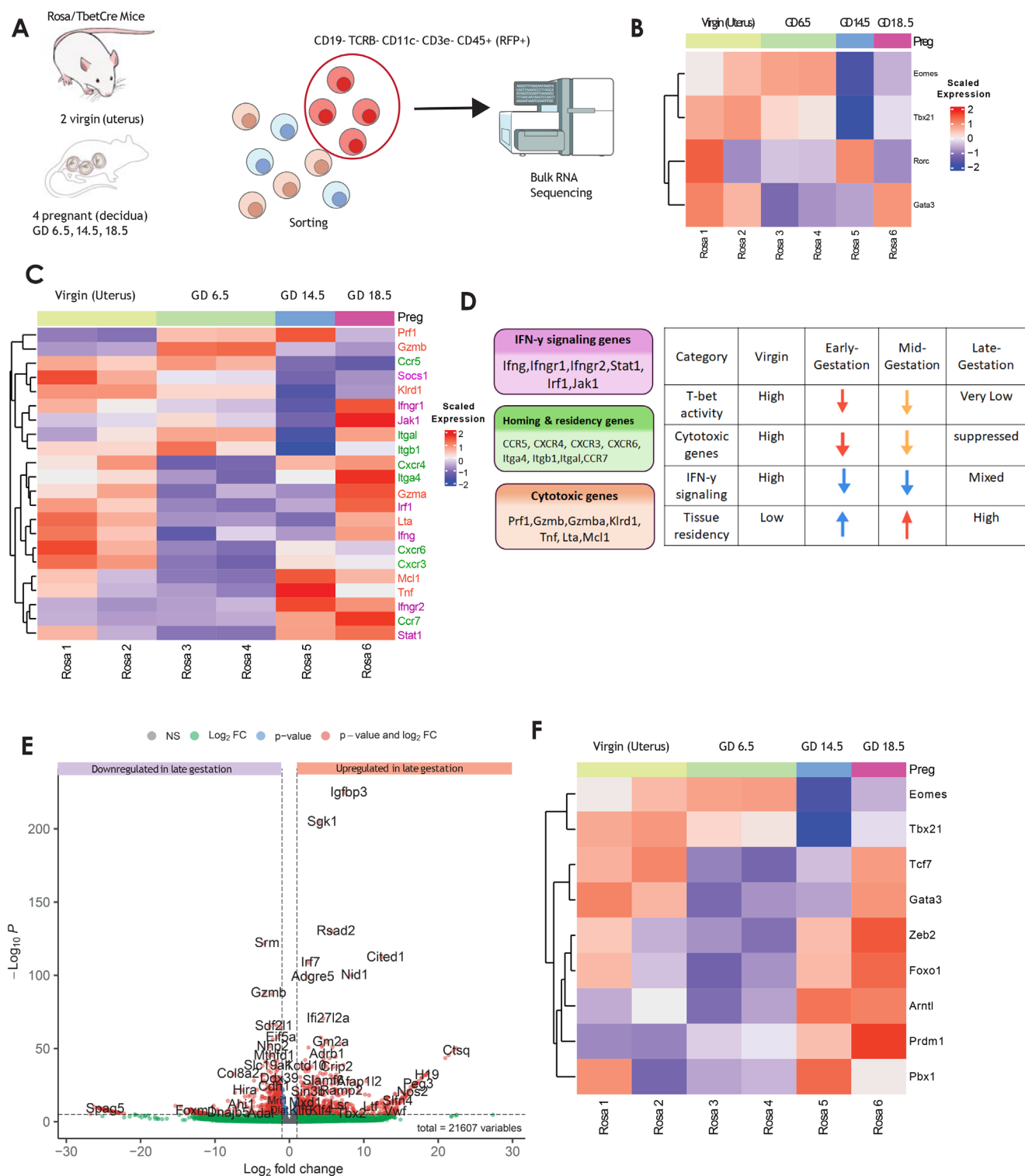


FIGURE 3 | Bulk RNA-seq transcriptional profiling of RFP+ cells across gestation. (A) Experimental workflow: RFP+ Lin^{neg}CD45⁺ cells of decidual or uterine origin were FACS-sorted from virgin mice (Rosa 1, Rosa 2) and pregnant dams at GD 6.5 (Rosa 3, Rosa 4), GD 14.5 (Rosa 5), and GD 18.5 (Rosa 6), followed by RNA extraction and sequencing. (B) Heatmap showing scaled expression *Eomes*, *Tbx21*, *Rorc*, *Gata3* in individual biological replicates across gestational stages. (C) Heatmap of differentially expressed genes associated with NK cell function, including IFN- γ signaling, tissue residency, and cytotoxicity, across individual samples from virgin and pregnant groups. Expression values are z-score scaled per gene. (D) Table summarizes trends of transcriptionally regulated genes. Genes were grouped into IFN- γ signaling, homing and residency, and cytotoxicity modules. in T-bet activity, cytotoxic gene expression. (E) Volcano plot of differentially expressed genes between GD18.5 and GD6.5. Significantly upregulated genes in late gestation (red) and downregulated genes (blue) are labeled; non-significant changes are shown in green. Horizontal dashed line indicates significance threshold (p -value), and vertical dashed lines indicate log₂ fold-change cutoffs. (F) Heatmap of scaled expression of additional transcription factors implicated in dNK cell adaptation across biological replicates across different gestational stages.

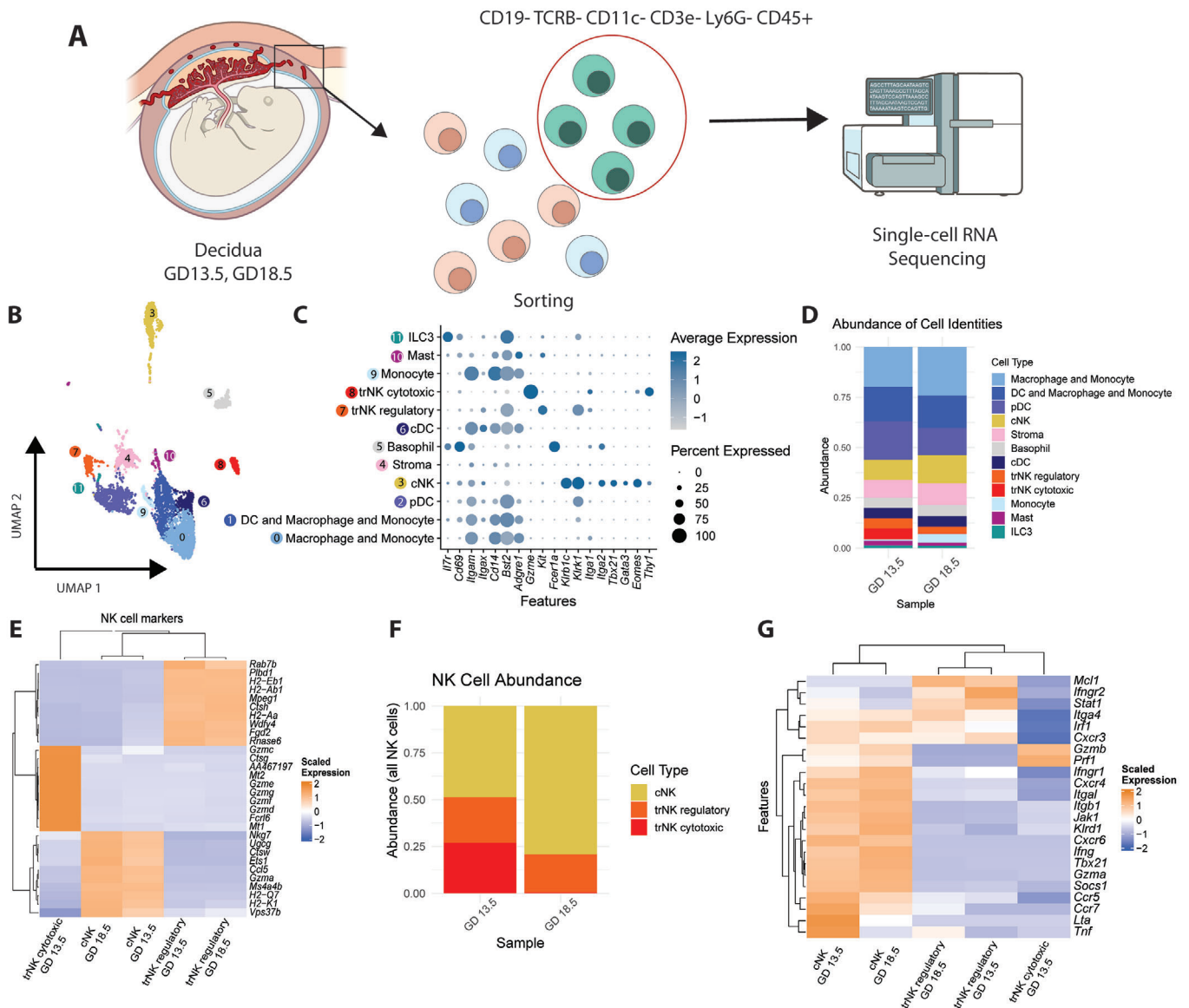


FIGURE 4 | Single-cell transcriptomic analysis of decidual LinnegCD45+ populations across gestation. (A) Single-cell RNA sequencing workflow: decidual tissue sampling of CD45+Linneg cells from pregnant uterus at GD 13.5 ($n = 1$) and GD 18.5 ($n = 1$), followed by FACS sorting and subsequent transcriptomic analysis. (B) Uniform Manifold Approximation and Projection (UMAP) plot showing immune cell clustering. (C) Dot plot displaying marker gene expression for each identified immune cell cluster, where color indicates average expression and dot size indicates percentage of cells expressing the gene. (D) Stacked bar plot showing the relative abundance of immune cell types at GD 13.5 and GD 18.5. (E) Heatmap of marker gene expression for three dNK cell subsets across gestational stages. (F) Stacked bar plot showing the proportion of the three dNK cell subtypes at GD 13.5 and GD 18.5. (G) Heatmap of differentially expressed genes in dNK cells between GD 13.5 and GD 18.5.

enrichment bias. ScRNAseq was performed at mid (GD13.5) and late (GD18.5) gestation (Figure 4A). We profiled the RNA expression of immune cell populations using unsupervised clustering of the transcriptomes, which resolved 13 transcriptionally distinct clusters (Figure 4B, C). Cell types were annotated based on canonical marker expression (Figure 4D), including three major NK cell populations: conventional-like NK cells (cNK; cluster 3), cytotoxic tissue-resident NK cells (trNK-cytotoxic; cluster 8), and regulatory tissue-resident NK cells (trNK-regulatory; cluster 7). Additional clusters included dendritic cells (pDC, cDC), monocytes, mast cells, decidual stromal, basophils, and ILC3s. Among NK subsets, cNK cells were characterized by high expression of Eomes, Klrk1c, and Klrk1, reflecting a circulating phenotype. In

contrast, trNK-cytotoxic cells exhibited elevated Tbx21, Gzme, and Thy1, indicative of cytotoxic and pro-inflammatory functions, while trNK-regulatory cells were distinguished by Bst2, Kit, and Klrk1, suggesting a regulatory role. Concurrent with the downregulation of T-bet (Tbx21) from mid to late gestation, there was a marked shift in dNK cell subset composition, characterized by the expansion of cNK-like and tissue-resident regulatory NK (trNK-regulatory) populations, accompanied by a decline in trNK-cytotoxic cells (Figure 4E, G). This compositional transition was not accompanied by substantial transcriptional reprogramming within the cNK or trNK-regulatory subsets, both of which retained their defining gene expression profiles across gestation. (Figure 4F, H). Notably, trNK-cytotoxic cells were only

detected at GD 13.5, limiting our ability to directly analyze their transcriptional dynamics at later gestational stage.

Gene expression analysis revealed that trNK-regulatory cells were enriched for transcripts associated with immune regulation and homeostasis, including *Socs1*, *Ccr5*, and *Lta*, supporting their skewing toward their immunomodulatory phenotype. Meanwhile, cNKs maintained expression of key cytotoxic and adhesion related genes such as *Itgal*, *Itgb1*, and *Stat1* at GD 18.5.

Together, these findings suggest that from mid to late gestation, dNK cells undergo coordinated changes in subset abundance and transcriptional reprogramming. The observed downregulation of T-bet is associated with the contraction of cytotoxic NK programs and the emergence of regulatory NK phenotypes which may contribute to the supportive role of NK in maintaining normal pregnancy.

4 | Discussion

Decidual NK cells are central regulators of maternal–fetal immune tolerance and placental development, yet the details of their transcriptional regulation across gestation are elusive. Earlier studies demonstrated that Dolichos biflorus agglutinin (DBA) lectin/ periodic acid–Schiff (PAS) staining represents one of the simplest widely used methods for identifying NK cells in the pregnant mouse uterus [41, 42]. However, the contemporary research increasingly employs flow cytometric and transcriptomic approaches to define uterine NK cells with greater precision [37, 38]. Yadi et al., 2008 showed that peripheral and nonpregnant endometrial NK cells typically exhibit NK1.1+DX5+ phenotype and are unreactive to DBA, whereas uterine NK (uNK) cells can be subdivided into DBA-NK1.1+DX5+ and DBA+NK1.1-DX5-subsets [43].

In contrast, subsequent studies revealed that exclusive reliance on DBA staining is insufficient, as DBA primarily labels a classic uNK subset but does not comprehensively capture the entire uNK cell pool, particularly during early gestation [2, 44]. These studies showed that a substantial proportion of NK1.1+ cells are already present at early gestational stages, whereas DBA+ cells become prominent later in pregnancy.

Furthermore, transcriptional profiling of flow-sorted DBA+ and DBA- uNK cells at mid-gestation showed distinct gene expression patterns that DBA+ cells correspond to NK1.1-DX5- subsets, while DBA- cells align with NK1.1+DX5+ phenotypes [39]. Complementary work by Lima et al. further revealed that these NK subsets differ in *Ly49* receptor expression and angiogenic function [45]. These findings indicate that NK1.1 and DBA lectin mark distinct uNK cell populations where the DBA+ and NK1.1+ NK cell subsets represent phenotypically and functionally distinct population that overlap only partially throughout the pregnancy.

In our study, we primarily used NK1.1 expression to characterize dNK cells. Although we did not perform co-staining for DBA lectin or PAS, the NK1.1-T-bet+ cells observed in Figure S1 most likely correspond to the NK1.1-DBA+ dNK subset that predominates at mid-to-late gestation.

T-bet and Eomes jointly coordinate NK cell development and effector functions in both peripheral and tissue-resident compartments. Recently, Barahona et al. demonstrates that Eomes is required for uterine CD49a+ trNK cells development [46], however the dynamics and relationship between T-bet and Eomes modulation in broader dNK cells across gestation is not understood. By combining a T-bet fate-mapping model with high-dimensional flow cytometry and transcriptomic analysis, we provide a comprehensive view of how T-bet and related transcriptional programs unfold in dNK cells throughout pregnancy. Using T-bet fate mapping we were surprised to show that T-bet expression in dNK cells is progressively downregulated across normal pregnancy, uncovering unrecognized level of transcriptional plasticity within this subset.

Given that immune-derived and trophoblast-derived lineages share overlapping transcriptional programs, accurate interpretation of our results required a clear distinction between these compartments. Recent lineage-tracing studies have demonstrated that Eomes and GATA-3 are not merely early developmental markers but persist as functional regulators of the trophoblast lineage. Specifically, Eomes-expressing trophoblast progenitors persist into mid-to-late gestation, contributing to both the junctional and labyrinthine zones [47, 48], while GATA-3 remains essential for the differentiation of labyrinth trophoblast progenitors (LaTPs) into syncytiotrophoblasts [49, 50].

However, these stromal lineages are CD45-negative, whereas the transcription factor T-bet is strictly restricted to the hematopoietic lineage. To preclude signal contamination, we rigorously gated on CD45+ leukocytes, thereby ensuring the expression profile was exclusive to the immune infiltrate and effectively excluding resident trophoblast sources.

T-bet plasticity supports the concept that dNK cells have unique biology, distinguishing them from peripheral mature NK cells which typically exhibit a stable T-bet expression as a hallmark of terminal differentiation [13, 15, 17, 51]. Indeed, mature dNK cells retain the capacity to modulate T-bet expression to adapt to pregnancy associated cues, which was not a global pregnancy phenomenon, as NK cells from Peyer's patches-maintained expression. This localized regulation is consistent with ours [12] and others [1] previous findings, which demonstrated distinct transcriptional and functional adaptations of dNK cells compared to their peripheral or lymphoid tissue counterparts.

T-bet downregulation in dNK cells was not accompanied by trans-differentiation toward alternative ILC lineages as neither ROR γ t nor GATA3 was induced in RFP+ cells. This contrasts gut-resident ILCs, where co-expression of lineage-defining transcription factors and intermediate states reflect plasticity and transitions between subsets [52, 53] adapting to local conditions.

We next determined how T-bet downregulation correlates with transcriptional reprogramming in dNK cells; we performed gestational stage-specific transcriptomic profiling of Tbet fate-mapped sorted RFP+ cells. There is a gestation-associated transition from cytotoxic to regulatory states, marked by coordinated repression of effector programs and induction of genes associated with tissue residency and immune modulation, all while preserving core NK cell identity. This transition is accompanied by compensatory

transcriptional responses that maintain functional integrity in pregnancy. Specifically, we showed that T-bet downregulation during mid-gestation coincides with suppression of key IFN- γ signaling genes, including *Ifng*, *Stat1*, *Irf1*, and *Jak1*. Intriguingly, despite sustained T-bet repression, these transcripts partially rebound at term, demonstrating a previously unrecognized biphasic regulation of the IFN- γ axis. While this pattern initially aligns with earlier findings that T-bet is dispensable for IFN- γ initiation but required for its sustained expression in pNK cells [15], our results expand this model by implicating alternative, T-bet-independent mechanisms in IFN- γ reactivation in dNK cells near parturition.

Human studies have shown that repeated pregnancies can generate “pregnancy-trained” dNK cells with enhanced NKG2C and LILRB1 expression and increased IFN- γ and VEGF-A production [54]. However, no comparable studies have been reported in murine models. In our study, all experiments were performed exclusively in nulliparous mice, and no comparison was made with parous pregnancies. It is therefore intriguing to speculate that a similar “training” mechanism during early pregnancy might prime dNK cells for the later biphasic IFN- γ activation pattern.

We confirm the findings of Zhang et al., who demonstrated that T-bet regulates cytotoxic gene expression during pNK cell maturation by sustaining genes such as *Prfl* and *Gzma* [13]. We observed high levels of *Prfl* and *Gzmb* early in gestation, reflecting cytotoxic programming driven by T-bet and/or Eomes. Expression declined by mid-gestation, coinciding with the progressive reduction in T-bet levels. This divergence suggests that dNK cells undergo a context-dependent attenuation of the Eomes-T-bet axis, limiting cytotoxic potential in favor of immunoregulatory and tissue-supportive functions as pregnancy progresses. Concurrently, we observed a coordinated upregulation of integrins (*Itga4*, *Itga1*, *Itgb1*) and chemokine receptors (*Ccr7*, *Cxcr4*), supporting tissue residency. Interestingly, NK cell egress from the bone marrow and lymphoid tissues is at least partly allowed by T-bet [20, 55], supporting the concept that dNK residence in the decidua is maintained by T-bet downregulation.

Given T-bet’s established role in driving pNK cells maturation and effector functions of, its selective downregulation in the decidua unveiled a previously unexamined question: what alternative transcriptional programs sustain dNK cell identity and function in the absence of this canonical regulator? We analyzed gene expression changes accompanying the decline in T-bet and identified a cohort of upregulated transcripts, including, IGFBP3, *Adgre5*, *Sgk1*, *Fhl1*, and *Rasd2*, that are not classically associated with NK effector function but are involved in cell adhesion, metabolic adaptation, and stress responses. IGFBP3 (insulin-like growth factor-binding protein 3) was the most strongly upregulated transcript in late gestation. Previous studies have shown that IGFBP3 is hormonally regulated by progesterone and BMP2, further implicating it in promoting endometrial function and embryonic development by modulating IGF activity [56, 57]. Together with other IGFBPs, they concentrate IGFs near their receptors and enhance IGFs activity [58]. In human, IGFBP3 mRNA was expressed in both the decidua and certain trophoblasts. It is thought that IGFBPs produced by maternal

decidua interact with IGFs produced by fetal intermediate trophoblasts to mediate bidirectional cell-to-cell communication at maternal–fetal interface [59]. *Adgre5/CD97* is predominantly expressed on various leucocytes, where it mediates leukocyte adhesion to the endothelium and modulates immune cells interactions [60–62]. In the context of pregnancy, human ScRNA seq data (The Human Protein Atlas, [proteinatlas.org](https://www.proteinatlas.org)) reveal that across all tissues, the resident or infiltrated bone marrow-derived immune cells exhibit the highest *Adgre5*, whereas extravillous trophoblasts display low expression levels [60]. Comparing normal controls, *Adgre5*-CD55 interaction between trophoblasts and immune cells is significantly altered in gestational diabetes [63, 64]. However, the specific role of *Adgre5* in dNK cells across the gestation remains unclear. In our results, the observed upregulation of *Adgre5* may facilitate dNK cells adhesion and retention within decidual microenvironment. *Sgk1* encodes serum- and Glucocorticoid- kinases that regulate ion transport, stress responses, and signaling pathways including AKT/FOXO and NF- κ B axes [65–67]. Beyond immune regulation, *Sgk1* reciprocally influences the Th17/Treg balance and contributes to endometrial receptivity and, subsequently in pregnancy maintenance [68, 69]. Human studies have shown that low levels of *Sgk1* in the endometrium are associated with pregnancy failure [70]. The decidua and villi of patients with unexplained recurrent spontaneous abortion (URSA) had lower expression levels of *Sgk1* than that with normal pregnancies [71]. Furthermore, animal studies have also shown that blocking the gene expression of *Sgk1* in mice can lead to miscarriage [72]. Recently, Xiaoqian Di, et al. demonstrated that activation of *Sgk1* signaling improves decidualization responses in these patients [73]. *Fhl1* (Four and a Half LIM Domain Protein 1) is a transcriptional co-regulator that activating downstream pathways involved in endometrial receptivity and blastocyst adhesion. Prior studies showed that upregulation of *Fhl1* at early gestation is essential for establishing and maintaining uterine receptivity, whereas interfering with its expression in the mouse uterus significantly inhibited embryo implantation [74, 75]. In humans, similar to *Sgk1*, studies have shown that *Fhl1* expression is markedly decreased in patients with URSA [75]. *Rasd2* (also known as *cytomegalovirus-inducible gene 5*) encodes an interferon-inducible antiviral protein that belongs to the S-adenosyl-L-methionine (SAM) superfamily of enzymes. *Rasd2* is one of interferon-stimulated genes (ISG) that regulate the IFN response. A recent study demonstrated that *Rasd2* acts as a pathogenic ISG at maternal fetal interface in patients with systemic lupus erythematosus (SLE) as it induces lipid accumulation in placenta and inhibits angiogenesis [76]. Moreover, Ding et al. identified L-chicoric acid (LCA), an HIV-1 integrase inhibitor as a potential therapeutic against *Rasd2*-mediated placental lipid accumulation and impairment of vascular development, thus alleviating adverse pregnancy outcomes [76, 77]. However, to our knowledge, no direct mechanistic evidence currently links T-bet with these genes through a shared signaling pathway or transcriptional regulation suggesting that a compensatory pathway may be activated to maintain NK cell effector function and tissue homeostasis as pregnancy progresses. Additionally, adaptation appears to engage both canonical (as ZEB2 and PRDM1/Blimp-1) [15, 19, 78, 79] and newly implicated transcriptional regulators: FoxO1 and Arntl. These transcription factors were not previously linked to the maintenance of dNK cell identity and coordinated upregulation suggests the emergence of a novel compensatory regulatory network within the decidual microenvironment.

As sorting and bulk RNA-seq cannot resolve whether transcriptional changes reflect uniform reprogramming across all dNK cells or shifts in subset composition throughout gestation, we next performed single cell RNA sequencing of the lineage-negative compartment at two gestational timepoints. Data revealed a pronounced shift in subset composition between mid and late pregnancy. Specifically, a trNK-cytotoxic subset, enriched for *Tbx21*, *Gzme*, and *Thy1*, was detected exclusively at GD 13.5, whereas by GD18.5, both cNK and trNK-regulatory populations had expanded significantly. The maintenance of distinct gene expression profiles in cNK and trNK-regulatory subsets suggests that these cells do not undergo major transcriptional reprogramming but rather reflect a selective expansion of pre-existing cell types suited for the later stages of pregnancy. This may be driven by niche-specific signals within the decidual environment which preferentially promote the proliferation of regulatory NK subsets already primed for this identity [2]. Supporting this, trNK-regulatory cells displayed enrichment of transcripts such as *Socs1*, *Ccr5*, and *Lta*. *Socs1* encodes a suppressor of cytokine signaling that negatively regulates the JAK/STAT pathway, particularly limiting IFN- γ driven activation [80]. In both human and murine NK cells, its upregulation contributes to limiting proinflammatory signaling and maintaining immune tolerance. Recently, a human study demonstrated that *Socs1* was among the most strongly upregulated genes in NK cells from pregnant women, both in peripheral blood and decidua. The study also showed that SOCS1⁺ NK cells exhibited enrichment for *AREG*, a gene linked to regulatory and tissue-resident NK phenotypes [81]. *Ccr5* is a chemokine receptor that mediates responses to CCL3 (MIP-1 α), CCL4, and RANTES. Although migration, distribution and activation of murine uterine NK cells appear independent of CCR5 and MIP-1 α signaling [82], CCR5 signaling may be important for accumulation of regulatory T subsets within the pregnant uterus [83]. In humans, the frequency of CCR5⁺ NK cells in the peripheral blood of third-trimester pregnancies is significantly higher than nonpregnant controls. In addition, CCR5 expression is predominantly enriched on CD56^{bright} NK cells but also detected to a lesser degree on CD56^{dim} subsets in pregnant blood [81]. *Lta* (lymphotoxin a) is a member of the TNF superfamily that is essential for pNK cells differentiation and trafficking [84, 85]. Mouse studies have shown that *Lta* and its receptor are not required for the differentiation and proper localization of NK cells within the pregnant uterus [86]. However, in human studies, *Lta* polymorphism is associated with idiopathic recurrent pregnancy loss [87]. Together, these transcriptional changes accompanied declining T-bet expression enabling trNK-regulatory cells in promoting local immune tolerance and tissue maintenance during late gestation [33, 88].

Pregnancy-driven transcriptional regulation of dNK cells is a novel direction in understanding spatially restricted orchestration of immune function. Herein, we integrated quantitative protein analysis with single-cell transcriptomics (scRNA-seq) to comprehensively delineate the composition of lineage-negative cells and interrogate their associated transcriptional programs. We further complemented our analysis with bulk RNA-seq, which incorporated larger sample sizes and deeper transcriptome coverage, to investigate how the loss of T-bet alters gene expression profiles in cells with a defined history of T-bet expression (RFP⁺ status). Since all matings in this study were performed syngeneically, specifically B6.RosaRFP/Tbet^{Cre}

females were mated to C57BL/6J males, both sharing the same C57BL/6 genetic background, our future direction will aim to investigate the potential contribution of paternal alloantigen to gestation-dependent changes in dNK cells. Additionally, the key question of what drives T-bet loss in the decidua along with the upstream signals regulating these events (e.g., TGF- β signaling, and pregnancy hormones) remain an open direction for future studies. Disruption of these pathways may impair cell fate stability and subset composition, which in turn, may impact risk of pregnancy complications such as preeclampsia or fetal growth restriction. However, it remains unknown whether similar fate plasticity and compensatory mechanisms are relevant to human dNK cell function.

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Ethics Statement

We confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The animal study was approved by University of Wisconsin Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Conflicts of Interest

The authors declare no competing interests.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Table S1: Number of animals for each statistical figure. **Table S2:** Antibodies used to label innate lymphoid cells **Table S3:** Related to Figure 1. Statistical comparisons for RFP+ cells in Rosa/TbetCre mouse model. **Table S4:** Related to Figure 2. Statistical comparisons for transcription factor within RFP+ cells in Rosa/TbetCre mouse model. **Table S5:** Related to Figure S1. Statistical comparisons for transcription factor within RFP+ cells in Rosa/TbetCre mouse model. **Table S6:** aji70220-sup-0006-TableS6.xlsx **Table S7:** Statistics for genes included in Figure 3C **Table S8:** Statistics for genes included in Figure 3C **Supporting File 9:** aji70220-sup-0009-FigureS1.pdf **Supporting Figure 1:** Flow cytometry gating strategy and dynamics of surface markers within RFP+ cells in Rosa/TbetCre mouse model. (A) T-bet and NK1.1 expression within linnegCD45+RFP+ cells were identified in the mouse virgin uterus, decidua (top panel), embryo, and placenta (lower panel) across mouse gestation, respectively. (B) T-bet and DX5 expression within linnegCD45+RFP+ cells were identified in the mouse virgin uterus, decidua (top panel), embryo, and placenta (lower panel) across mouse gestation, respectively. **Supporting File 11:** aji70220-sup-0011-Figures2A-B.pdf **Supporting File 12:** aji70220-sup-0012-FigureS2C-D.pdf **Supporting Figure 2:** Flow cytometry gating strategy and dynamics of transcription factors within RFP+ cells in Rosa/TbetCre mouse model. (A–B) Representative bivariate plots derived from the same parent gate (Lin[−]CD45+RFP+) identifying transcription factor expression in the mouse virgin uterus, decidua (top panel), embryo, and placenta (lower panel) across gestation. (A) Identification of Tbet+RORytneg and TbetnegRORyt+ subsets. (B) Assessment of GATA-3 protein expression (plotted against RORyt), demonstrating that the T-bet fate-mapped (RFP+) population remains GATA-3 protein-negative despite T-bet downregulation. The proportions of Tbet+RORytneg(C), TbetnegRORyt+(D), and GATA-3+RORytneg cells(E) out of linneg CD45+ RFP+ cells in murine virgin uterus, decidua, embryo, and placenta across gestational age, (F) Flow cytometry gating strategy for NK cells defined as LinnegCD45+ NK1.1+. (G) RFP+ NK cell frequencies in virgin uterus and decidua GD 8, 13, and 16. A decline in RFP+ NK cells is observed as gestation progresses. These gating strategy figures were not obtained from the same experiment. $n = 3–5$ (5 independent experiments), the exact

number of animals used were summarized in supplementary table

1. The data of virgin uterus were compared with decidua group or placenta (include embryo) group, respectively. Statistical analysis was performed using ANOVA followed by post-hoc Tukey analysis. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$. **Supporting File 14:** aji70220-sup-0014-FigureS3A-B.pdf **Supporting File 15:** aji70220-sup-0015-FigureS3-C-E.tif **Supporting Figure 3:** Flow cytometry analysis of RFP+ cells in Peyer’s patches in the Rosa/TbetCre mouse model across gestation. (A) Representative gating strategy to identify T-bet and RORyt expression within RFP+ and RFPneg populations from Peyer’s patch. (B) Flow cytometry dot plots of LinnegCD45+RFP+ cells from virgin uterus and GD 8, 12, and 16. Top row displays EOMES and T-bet coexpression; middle row shows T-bet and RORyt expression; bottom row depicts GATA-3 and RORyt expression. (C–H) Quantification of the frequency of indicated subsets within TbetCre RFP+ cells from virgin uterus and across gestational stages GD 6–18. **Supporting File 17:** aji70220-sup-0017-FigureS4.pdf **Supporting Figure 4:** Experimental workflow identifying LinnegCD45+ cells in the murine decidua via bulk and single-cell RNA sequencing. a. Workflow for single-cell and bulk transcriptome profiling of murine decidua including harvest, dissociation, cell staining and FACS sorting for both methods. ScRNAseq libraries were then prepared via 10x Chromium Single Cell 3’ kit. RNA from cells for bulkRNAseq was isolated using the Qiagen RNEasy Micro Kit and RNA libraries were prepared using the Takara SMARTSeq v4 Ultra Low Input RNA Kit. Libraries from both scRNAseq and bulkRNAseq were sequenced using the 10x Illumina NovaSeq 6000. b. Analysis pipeline of scRNAseq and bulkRNAseq via quality control, normalization and analysis of subsequent data. c. Diagram illustrating the decidual-placental interface in murine pregnancy. B, B cells; cNK, conventional natural killer cells; DC, dendritic cells; ILC1, innate lymphoid type 1 cells; ILC2, innate lymphoid type 3 cells; ILC3, innate lymphoid type 3 cells; LP, lymphoid precursor cells; LTi, lymphoid tissue inducer cells; Ma, macrophages; Mo, monocytes; MP, myeloid precursor cells; RBCs, red blood cells; T, T cells; trNK, tissue-resident natural killer cells. d. Heatmap showing scaled expression (z-score) of target genes regulated by transcription factors depicted in Figure 1E in uterine/decidual NK cells from virgin mice and at GD 6.5, 14.5, and 18.5. Genes displayed are broadly associated with stress signaling, immune modulation, and tissue remodeling, reflecting downstream effects of the transcriptional changes observed in Figure 1E. Red indicates higher expression and blue indicates lower expression relative to the mean (scale: -2 to $+2$). **Supporting File 19:** aji70220-sup-0019-FigureS5.pdf **Supporting Figure 5:** Quality control metrics are consistent across samples. A) Total transcript counts detected per cell for each sample following filtering. B) Total features detected per cell for each sample following filtering. C) Percent mitochondrial transcripts detected per cell for each sample following filtering. D) Distribution of cells by sample.