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TGFb1 suppresses the activation of distinct dNK subpopulations in preeclampsia

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ABSTRACT

Background: Decidual natural killer (dNK) cells are the predominant lymphocytes accumulated at the maternal-fetal interface. Regulatory mechanism of dNK cells in preeclampsia, a gestational complication characterized by high blood pressure and increased proteinuria occurring after 20 weeks pregnancy, is not completely understood.
Methods: Multi-parameter flow cytometry is applied to investigate the phenotype and function of dNK cells freshly isolated from decidual samples or conditionally cultured by TGFb stimulation.
Findings: In preeclampsia, we documented elevated numbers of $CD56^+$ $CD3^-$ dNK cells in close proximity to $Foxp3^+$ regulatory T (Treg) cells within the decidua. In vitro experiments using dNK cells from early gestation showed that dNK activation (IFNG, IL-8 and CD107a) can be downregulated by Treg cells. The expression of these markers by dNK cells was significantly lower in preeclampsia. We also observed a positive correlation between the expression of dNK activation receptors (NKp30 and NKG2D) and the expression of IFNG in specific dNK subsets. TGFb levels are increased in the decidua of preeclamptic pregnancies. We analyzed co-expression of activation (IFNG/IL-8/CD107a) and angiogenic (VEGF) markers in dNK cells. TGFb treatment reduced while blockade of TGFb increased co-expression of these markers.
Interpretation: Our findings suggest that elevated decidual TGFb1 supresses the activation of specific subsets of dNK which in turn contributes to the uteroplacental pathology associated with the onset of preeclampsia.

1. Introduction

Preeclampsia is a major cause of maternal/child mortality occurring in 3–7% of all pregnancies and presents long term impact on human health [1,2]. Many studies have reported the inflammatory changes in the maternal circulation of preeclamptic women and the disturbance of molecular pathways in preeclamptic placentae [3,4]. Although significant correlations were found between preeclampsia pathology and maternal serum sFLT1/PlGF levels [5], the contribution of the maternal decidua has been largely neglected. Decidualization, which involves a dramatic morphological and functional differentiation of human endometrial stromal and immune cells, plays an important role in promoting placenta formation to support pregnancy. Since defective decidualization was associated with the development of preeclampsia [6], it is essential to investigate the fundamental cellular interactions occurring within the maternal fetal interface of preeclamptic deciduae.

$CD56^+$ decidual NK (dNK) cells are a key contributor to the maternal-fetal immune recognition and placental angiogenesis [7–9].

In early gestation (6–20wk), dNK cells accumulate densely in decidua and their number decreases at term (37 + wk) in normal pregnancy [10,11]. However, most information on dNK cells at midterm and late gestation (20–37wk) is based on studies of pathological pregnancies which show inconsistencies in the number (elevated or decreased) of dNK in the placental bed of preeclamptic pregnancies [12,13]. As the onset of preeclampsia occurs during this period, a qualitative and quantitative study of dNK cells may be informative for determining the mechanisms underlying the development of preeclampsia.

Animal studies suggest that the terminal maturation of NK cells can be suppressed by blocking TGFb1 signalling [14], which is a critical factor for maternal regulatory T (Treg)-mediated immune suppression [15]. The sustained expansion of Treg cells is essential for maintaining immune tolerance to the developing fetus. The proportion of peripheral Treg cells, regardless of their marker CD25, CD127 and/or Foxp3, increases after implantation [16]. In mice models, Treg depletion at early or later gestational stage results in pregnancy failure [17]. Several studies have shown that human Treg cells isolated from preeclamptic patients have abnormal phenotypic composition [18,19], suggesting that decidua-resident Treg cells may have a distinguishable role on immune homeostasis at the maternal fetal interface. However, it remains unclear how the interaction of Treg and dNK cells affects human pregnancy.

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Research in context

Evidence before this study

Preeclampsia (PE) is a gestational disease characterized by de-novo development of concurrent hypertension and proteinuria. It is a syndrome affecting multiple organ systems in both mother and fetus. Preeclampsia has been associated with abnormalities of the immune system systemically and within the placenta. In the systemic circulation, natural killer (NK) cells are the minor population (about 5%) of blood lymphocytes; while in the decidua they accumulate and become the dominant population of lymphocytes (about 70%) during normal pregnancy. While some studies show abnormal numbers of peripheral blood NK (pbNK) cells in preeclampsia, there is limited data on decidual resident NK (dNK) cells, including their phenotype and function. Regulatory T (Treg) cells influence NK cell function, through actions mediated by the multifunctional cytokine, transforming growth factor beta (TGFb). TGFb signalling can also regulate Treg cell function depending upon the tissue microenvironment. Our research sought to clarify the role of TGFb on dNK cell function in the context of preeclampsia.

Added value of this study

We performed flow cytometry to investigate the quantitative and qualitative features of dNK cells, including the expression of surface markers and functional factors, in preeclampsia. Higher numbers of dNK cells were found in preeclampsia in comparison to normal term pregnancy; however, the angiogenic function of these dNK cells is suppressed by the presence of Treg cells. We further demonstrated that dNK function and phenotype is modified by TGFb, due to the accumulation of decidual Treg cells. In the presence of elevated levels of TGFb, the functional heterogeneity (cytotoxicity and angiogenesis) of dNK is reduced, while blockade of TGFb signalling improved dNK-mediated angiogenesis. We also characterized the profile of dNK and Treg cells in the decidua in both healthy and pathological pregnancies (preeclampsia and pre-term birth). While the number of dNK and Treg cells was increased in preeclampsia, their functionality, as defined by expression of markers of angiogenesis and cytotoxicity, was reduced.

Implications of all the available evidence

We report phenotypic and functional abnormalities of dNK cells in preeclampsia. Our results suggest that elevated decidual TGFb1 could selectively suppress the activation of dNK subsets which may result in an unbalanced uteroplacental immune response and contribute to the onset of preeclampsia. These findings provide a foundation for further mechanistic studies of decidua-resident immune cells in pathologic pregnancies and of potential therapeutic strategies to target these cells.

TGFb1 can inhibit the development and differentiation of human NK cells by facilitating the transition of CD16$^+$ peripheral blood (pb)NK cells to CD16$^-$ population [20]. TGFb1 treatment, in combination with hypoxia and a demethylating agent, can transform normal pbNK cells into noncytotoxic and proangiogenic NK similar to dNK cells [21]. In contrast to peripheral NK cells, dNK cells exist in a distinct microenvironment and exhibit a distinct phenotype, and the impact of TGFb on the differentiation of dNK subpopulations is not known. This is important since multiple lines of evidence underscore the association between the expression levels of placental TGFb, serum TGFb receptors (endoglin), and the severity of preeclampsia [22–25]. Therefore, in this study, we characterized the

distribution of the dNK sub-populations in the decidua of preeclamptic pregnancies and determined the role of TGFb signalling in modulating the activation of these dNK subsets.

2. Experimental procedures

2.1. Patients

All samples were collected by the Research Centre for Women's and Infants' Health (RCWIH) BioBank at Mount Sinai Hospital (Toronto, Canada) following informed consent. Early pregnancy deciduae were obtained from healthy women undergoing elective termination at 6-20wk pregnancy. Normal term decidual samples were obtained from healthy women who gave birth from 37 to 42wk of pregnancy. Deciduae from patients diagnosed with preeclampsia (blood pressure > 140/90 mmHg with concurrent proteinuria) or without preeclamptic complications (20–37wk) were also collected. The demographic and clinical characteristics of all patients were provided in Supplementary Table 1. This study was approved by the Institutional Research Ethics Board at Mount Sinai Hospital (Toronto, Canada).

2.2. Sample preparation

Decidual samples were first identified macroscopically and rinsed with HBSS as previously reported [10,43,44]. Tissue was cut into small pieces (~1 mm^3) and shaken for 30 min at 37 °C in a rotating incubator in Ca, Mg-free HBSS with EDTA, DTT and Hepes. The decidual leukocytes were isolated by Ficoll gradient (GE Healthcare). To obtain purified dNK cells, magnetic-activated cell sorting (MACS) was performed using CD56 microbeads (Miltenyi Biotech, CA).

2.3. Flow cytometry staining

Decidual leukocytes were stained with LIVE/DEAD® fixable cell stain kit (L/D-violet; Invitrogen), followed by non-specific blocking with serum-free protein block (Dako, CA). To investigate dNK phenotype, cells were stained with the following antibodies for 30 min at 4 °C: mouse anti-human CD45-APC/Cy7, CD56-PE/Cy7, CD3-Alexa Fluor 700 (BD Pharmingen, CA), NKp30 (NKp30)-PE, CD335 (NKp46)-PE/Cy5, NKp44 (NKp44)-Alexa647 or NKp80-PE, CD244 (2B4)-PE/Cy5 and NKG2D (NKG2D)-APC (Beckman Coulter, CA). To study Treg cells, decidual leukocytes were stained with CD45-APC/Cy7, CD3-Alexa Fluor 700, CD4-APC, CD8-PE and CD25-PE/Cy5; then fixed, permeabilized and stained for 30 min with anti-human Foxp3-FITC (eBioscience, CA).

For intracellular staining, cells were stimulated with a cytokine cocktail (rhIL-12, 10 ng/ml; rhIL-15, 10 ng/ml; rhIL-18, 50 ng/ml) overnight, then GolgiPlug (BD Pharmingen) was added for another 4 h at 37 °C. Cells were harvested and stained for surface markers. Subsequently these cells were fixed, permeabilized (Cytofix/Cytoperm Plus Kit; BD Biosciences, CA) and stained with anti-human IFNG-FITC (BD Pharmingen, CA) and VEGF-APC, IL-8-PE (R&D Systems, MN) to assess intracellular cytokine expression. In some experiments CD107a-PE/PCy5 (BD Pharmingen) was included to evaluate dNK degranulation.

Flow cytometric data were acquired with a Gallios flow cytometer (Beckman Coulter). Offline data analyses were performed by FlowJo V10 (TreeStar) or Kaluza 1.3 (Beckman Coulter) software. The complex maps of decidual immune cells were plotted by t-Distributed Stochastic Neighbour Embedding (t-SNE) [45], which reduced dimensionality of multi-color flow cytometry data into a 2-dimensional data space (tSNE-X vs tSNE-Y). Concatenating graphs are generated from two samples in each group. Manually-gated viable CD45$^+$ lymphocytes were overlaid into the tSNE plots using FlowJo plugin. Distribution of different markers are visualized in the continent-like mapping structure.

2.4. In vitro regulatory T cell stimulation

CD4$^+$CD25$^+$CD127$^{dim/-}$ decidual Treg cells were enriched for in vitro experiments [46,47] (Miltenyi Biotech). In brief, suspensions of decidual leukocytes were processed through LD separation columns (Miltenyi Biotech) to deplete non-CD4$^+$ and CD127^{high} cells; then positive magnetic separation was conducted to enrich decidual CD4$^+$CD25$^+$CD127$^{dim/-}$ Treg cells. Autologous decidual NK and Treg cells (purity > 90%) from 6-20wk pregnancies were co-cultured (10:1) in 24-well plates for 18 h in the presence of cytokines rhIL12/15/18. The expression of IFNG, IL-8, VEGF and CD107a by dNK cells was quantified by flow cytometry.

2.5. Immunohistochemistry

Human decidual samples were fixed in 4% PFA and paraffin embedded. Serial sections were cut at 5 μm, deparaffinized in xylene, rehydrated through an ethanol gradient. Endogenous peroxidase activity was blocked by incubation of the sections in 3% hydrogen peroxide (Fisher Scientific) for 30 min. Antigen retrieval was performed by microwave using Target Retrieval Solution (Dako). After 30 min incubation with Dako protein blocking solution, sections were incubated overnight at 4 °C with monoclonal mouse anti-human CD56 (Dako; 1:200) and Foxp3 (Abcam; 1:100). Sections were washed in PBS and incubated with biotinylated rabbit anti-mouse IgG (Dako; 1:200). Subsequently, sections were incubated with HRP substrate (Universal LSAB®-HRP Kit, Dako), developed using diaminobenzidine (Dako) and counterstained with Gill's Haematoxylin (Sigma). Photomicrographs were obtained using a Leica DMIL LED microscope.

2.6. TGFb function assay

For in vitro experiments to examine TGFb function, decidual NK cells were cultured for 24 h with either rhTGFb1 (5 ng/ml; R&D Systems) or TGFb receptor kinase inhibitor SB431542 (SB; 1 μg/ml; Sigma-Aldrich).

2.7. Angiogenesis assay

MACS enriched CD56$^+$ dNK cells were cultured with rhTGFb or SB. Conditioned supernatants were collected and added to 96-well plates coated with Matrigel containing 1.5×10^4 Human Uterine Microvascular Endothelial Cells (HUtMEC; PromoCell). After 16 h, HUtMEC branching was examined by light contrast microscopy and images were captured. Total number of branching points and tubes were counted manually from the photomicrography.

2.8. Bioplex measurement

Bio-Plex Pro™ TGFb assay was conducted according to manufacturer's instructions (Bio-Rad Laboratories Inc). Deciduae were homogenized in bicine buffer and total protein was measured by Bradford assay kit (Bio-Rad Laboratories Inc). HCl activated samples were loaded in a 96-well plate containing magnetic beads conjugated with antibodies to TGFb1/2/3. After incubation, the results were analyzed by a Luminex 200 cytometer and Bioplex HTF (Bio-Rad Laboratories Inc). Data analysis was performed with Bio-Plex Manager software (v5.0; Bio-Rad Laboratories Inc).

2.9. Statistical analysis

Statistical analyses were performed by SPSS23 (IBM, NY) and R software (3.4.3). Multiple comparisons between study groups were conducted using Mann-Whitney U test or Kruskal–Wallis test followed by Dunn's test. Pearson correlation was performed and plotted by R packages (PerformanceAnalytics, corrplot). Principal components analysis
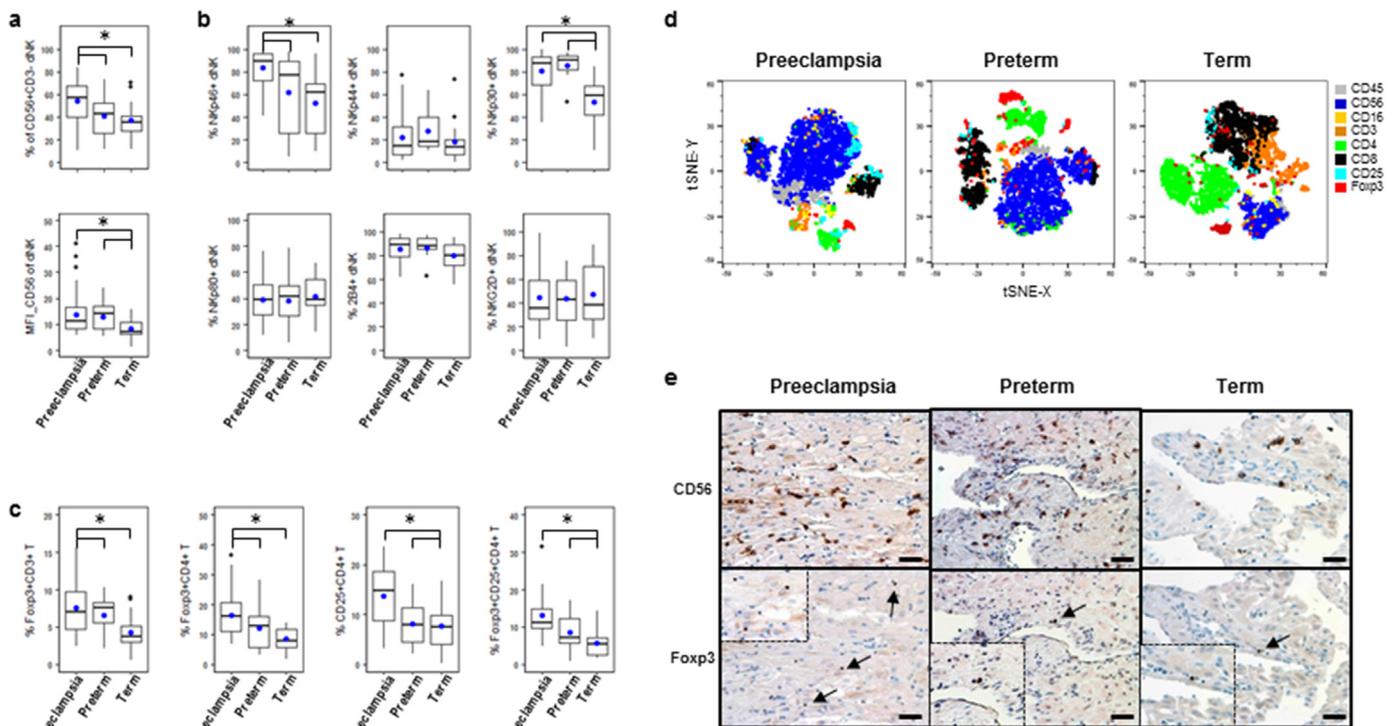
Fig. 1. Phenotypic characteristics of decidual NK and Treg cells in preeclampsia. a) Percentage of CD56$^+$CD3$^-$ dNK cells and CD56 MFI in preeclampsia, preterm and normal term pregnancies. b) The expression level (%) of surface receptor NKp46, NKp44, NKp30, NKp80, 2B4 and NKG2D on dNK cells. c) Phenotypic character of decidual Treg cells by their CD4, CD25 and Foxp3 expression. d) Visual illustration of distinct sub-populations of CD45$^+$ decidual lymphocytes in preeclampsia, preterm and term pregnancies using t-SNE mapping. e) Immunohistochemical staining for CD56 and Foxp3 expression in human deciduae. Spatial proximity of CD56$^+$ dNK and Foxp3$^+$ Treg (arrows) cells was found in deciduae of preeclampsia, preterm and normal term pregnancies. Bar = 50 μm. n = 61 (preeclampsia), 26 (preterm) and 23 (term). *, p < 0.05 when using Kruskal–Wallis test followed by Dunn's test.
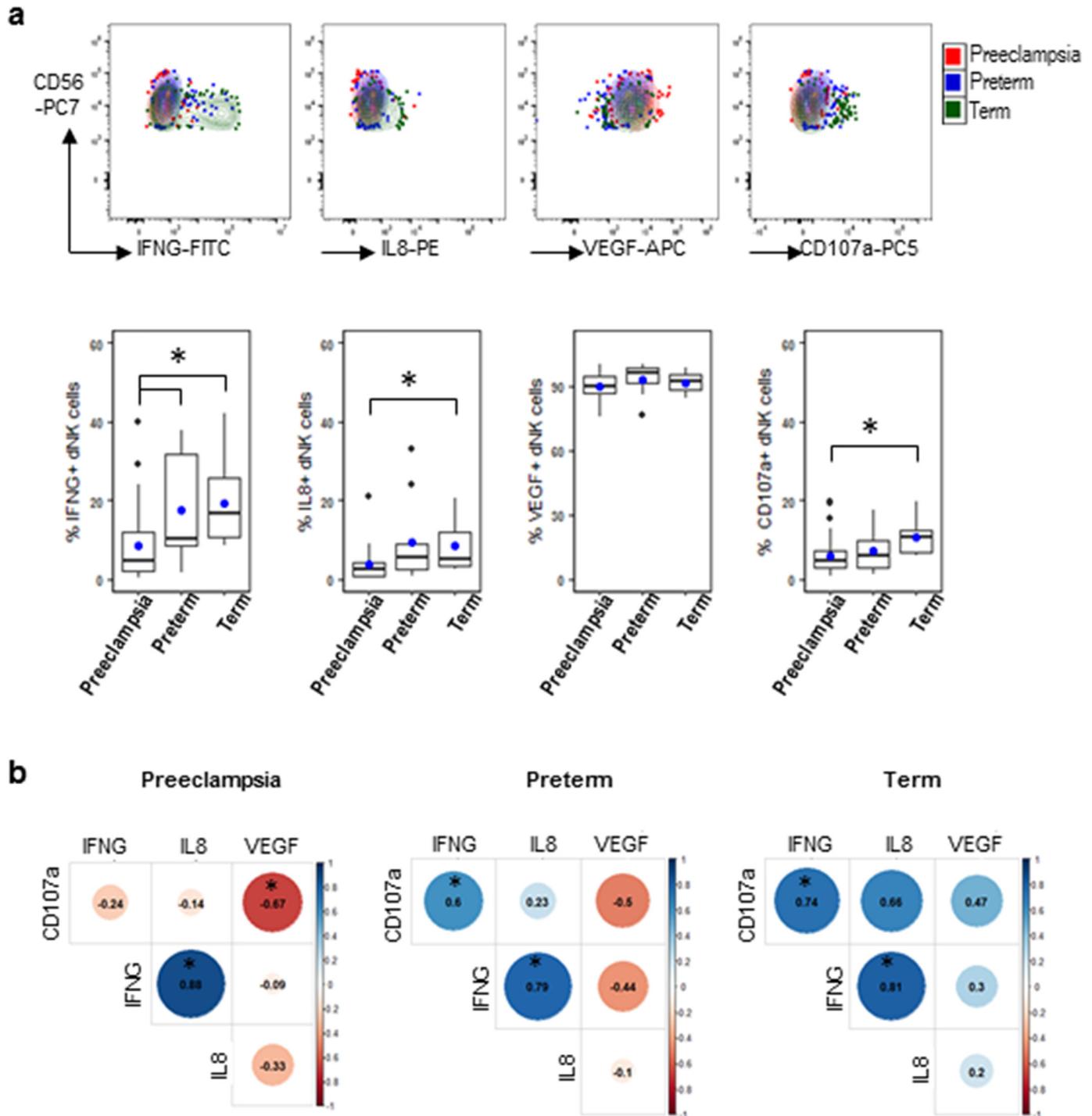
Fig. 2. Functional characteristics of decidual NK in preeclampsia. a) Representative contour plots shown the function of CD56$^+$CD3$^-$ dNK cells by overlaying the expressions of IFNG, IL-8, VEGF and CD107a from preeclamptic, preterm and normal term pregnancies. Data are presented by boxplots and overlaid with mean values as blue diamond symbols. *p < 0.05 when using Kruskal–Wallis test followed by Dunn's test. b) Functional correlation between angiogenic and cytotoxic factors of dNK cells in preeclampsia, preterm and term pregnancies. n = 61 (preeclampsia), 26 (preterm) and 23 (term). The legend color shows the correlation coefficients and the corresponding colors. *p < 0.05 determined by Pearson correlation.

was performed and graphed by related R packages (princomp, ggbiplot, ggplot). Statistical significance was assumed when p < 0.05.

3. Results

3.1. Phenotypic and functional changes of decidual resident NK cells in preeclamptic pregnancy

To capture the characteristics of dNK cells in the decidual niche, we performed multidimensional flow cytometry analysis (Supplementary Fig. 1a). Preeclampsia patients had significantly higher percentage of CD56$^+$CD3$^-$ dNK cells than preterm or normal term patients (Fig. 1a). In addition, the relative antigen density of CD56, measured by median fluorescence intensity (MFI) level, on individual dNK cells in preeclampsia was significantly higher than that from term pregnancy (Fig. 1a). A higher level expression of NKp46 was found in preeclamptic dNK cells than that in preterm or term pregnancy (p < 0.05, Kruskal–Wallis test followed by Dunn's test; Fig. 1b). The expression of NKp30 on dNK cells in preeclampsia was similar to preterm but was significantly higher than that in normal term pregnancy (Fig. 1b). The expression of other
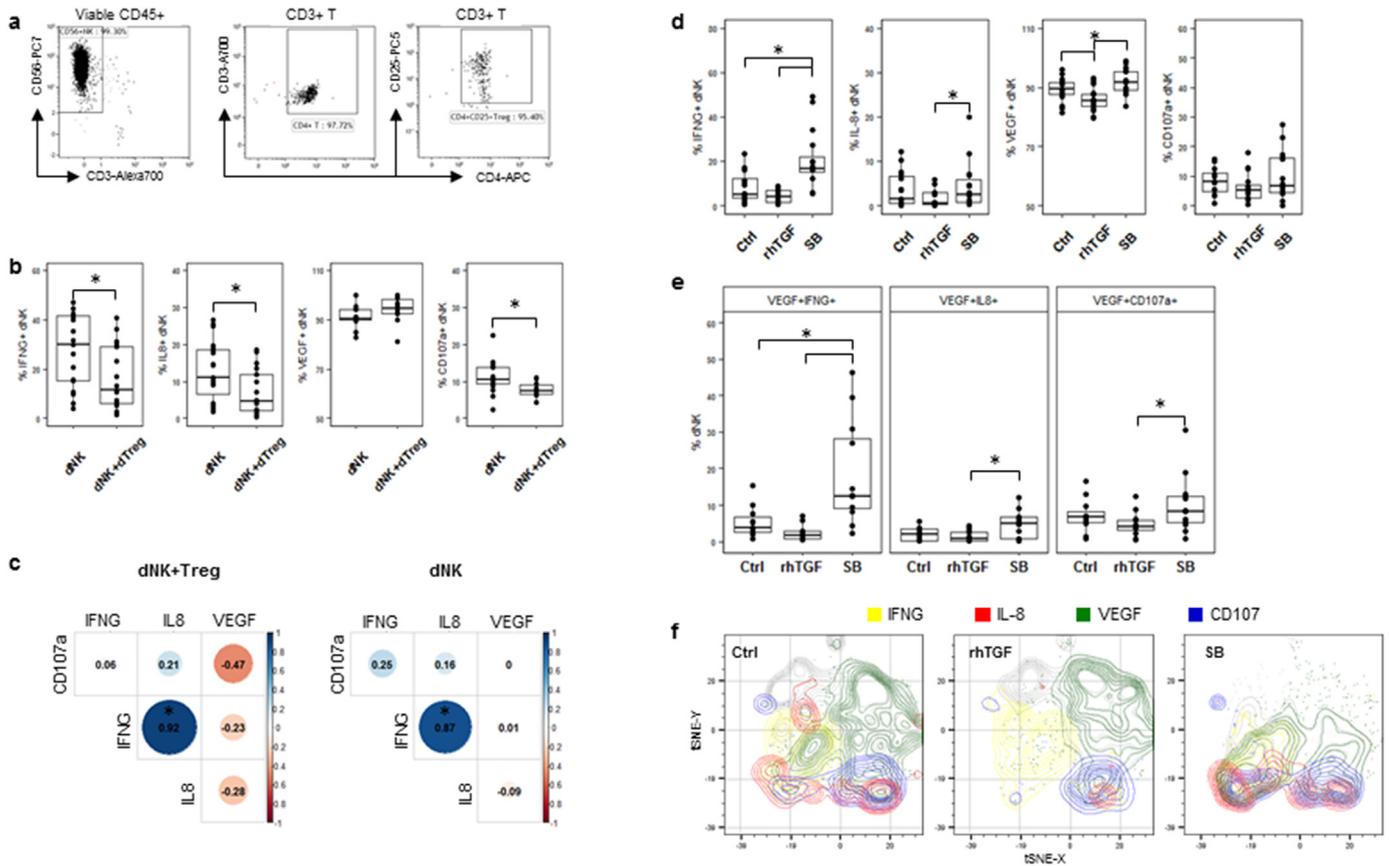
Fig. 3. Functional heterogeneity of dNK cells can be affected by autologous Treg cells and TGFb1. a) Decidual NK and Treg cells were isolated from early pregnancy and enriched by MACS. The purity of isolated population was assessed by flow cytometry. Representative plots of 15 independent experiments were shown. b) Function of dNK cells after co-culture with autologous Treg at ratio of 10:1 in the presence of rhIL12/15/18. *p < 0.05 when using Mann-Whitney U test. c) Correlation between angiogenic and cytotoxic factors of dNK cells with or with Treg cells. The legend color shows the correlation coefficients and the corresponding colors. *p < 0.05 determined by Pearson correlation. d) Freshly isolated dNK cells from early pregnancy were stimulated with rhTGFb1 (rhTGF) or TGF inhibitor (SB) in the presence of rhIL12/15/18. Ctrl, control group. e) The co-expression of VEGF with IFNG, IL-8 or CD107a was assessed by 2-D flow cytometry showing that TGFb blockade improved dNK function. n = 17. *, p < 0.05 when using Kruskal–Wallis test followed by Dunn's test. f) tSNE plots visualized that functional divergence of dNK cells were decreased by rhTGFb1, while improved by TGFb inhibition (SB).

NK receptors NKp44, NKp80, 2B4 and NKG2D were similar between three groups.

Decidua-resident Treg cells were assessed by surface marker CD4$^+$/CD25$^+$ and intra-nuclear transcription factor Foxp3 (Supplementary Fig. 1b). In preeclamptic decidua, Treg subsets, featured as CD3$^+$/CD4^{+-}Foxp3$^+$, CD4$^+$CD25$^+$ or CD4$^+$CD25$^+$Foxp3$^+$ cells, were more frequent than that in preterm or term pregnancy (p < 0.05, Kruskal–Wallis test followed by Dunn's test; Fig. 1c). In addition, tSNE mapping revealed distinct CD45$^+$ lymphocyte populations in preeclamptic and preterm, term pregnancies (Fig. 1d). Preeclamptic decidua had distinct pattern of clusters identified by NK and T cell markers, in comparison to preterm and normal term pregnancy. Immunohistochemical staining further demonstrated that in preeclampsia, preterm or term pregnancy, CD56$^+$ dNK and Foxp3$^+$ Treg cells were located in close proximity in the deciduae (Fig. 1e).

In comparison to term pregnancy, preeclamptic dNK cells had a significant lower expression of IFNG, IL-8 and CD107a (Fig. 2a). In all three groups, the majority of dNK cells were positive for the angiogenic factor VEGF and no remarkable differences were found (Fig. 2a). In addition, upon stimulation with PMA, which bypasses the upstream activation signal, preeclamptic dNK had significantly lower levels of expression of IFNG and CD107a than dNK cells from term pregnancy (Supplementary Fig. 2a), indicating that the intrinsic function of dNK cells was profoundly impaired in preeclampsia. Furthermore, strong positive correlations of IFNG and CD107a expression by dNK were identified in preterm and term pregnancy (p < 0.05, Pearson correlation), but not in preeclampsia (Fig. 2b). A significant negative correlation between

VEGF and CD107a expression was only shown in preeclampsia (Fig. 2b), suggesting that both dNK angiogenic ability and cytotoxic potential are conditionally regulated during pregnancy.

3.2. Selective suppression of dNK function by autologous Treg cells or exogenous TGFb1

To test whether the functional change of dNK could be induced by autologous Treg cells, we conducted a co-culture experiment using purified CD56$^+$ dNK and CD4$^+$ CD25$^+$CD127$^{dim/-}$ Treg cells (Fig. 3a&b). Treg cells significantly reduced the expression of IFNG, IL-8 and CD107a but not VEGF by dNK cells (Fig. 3b). These results indicate that the accumulation of decidual Treg cells could selectively inhibit the function of individual dNK cells and alter their angiogenic capability. Treg cell did not alter the relationship among angiogenic factors of IFNG and IL-8 in dNK cells (Fig. 3c). However, a negative correlation between VEGF and CD107a was noted when dNK were co-cultured with Treg cells although no significant difference was detected.

As Treg cells are known to produce TGFb1 and their number was increased in preeclamptic decidua (Fig. 1c), we considered the possibility that decidual TGFb1 may regulate dNK cell characteristics even when they had no direct interaction with local Treg cells (Fig. 1e). The proportions of IFNG$^+$ or IL-8$^+$ dNK cells were modestly decreased by rhTGFb1 but were significantly upregulated when they were treated with the TGFb inhibitor SB (Fig. 3d). Notably, VEGF expression by dNK cells was suppressed by rhTGFb1 but increased by TGFb blockade (Fig. 3d). The functional diversity of dNK cells, identified by co-expression of VEGF
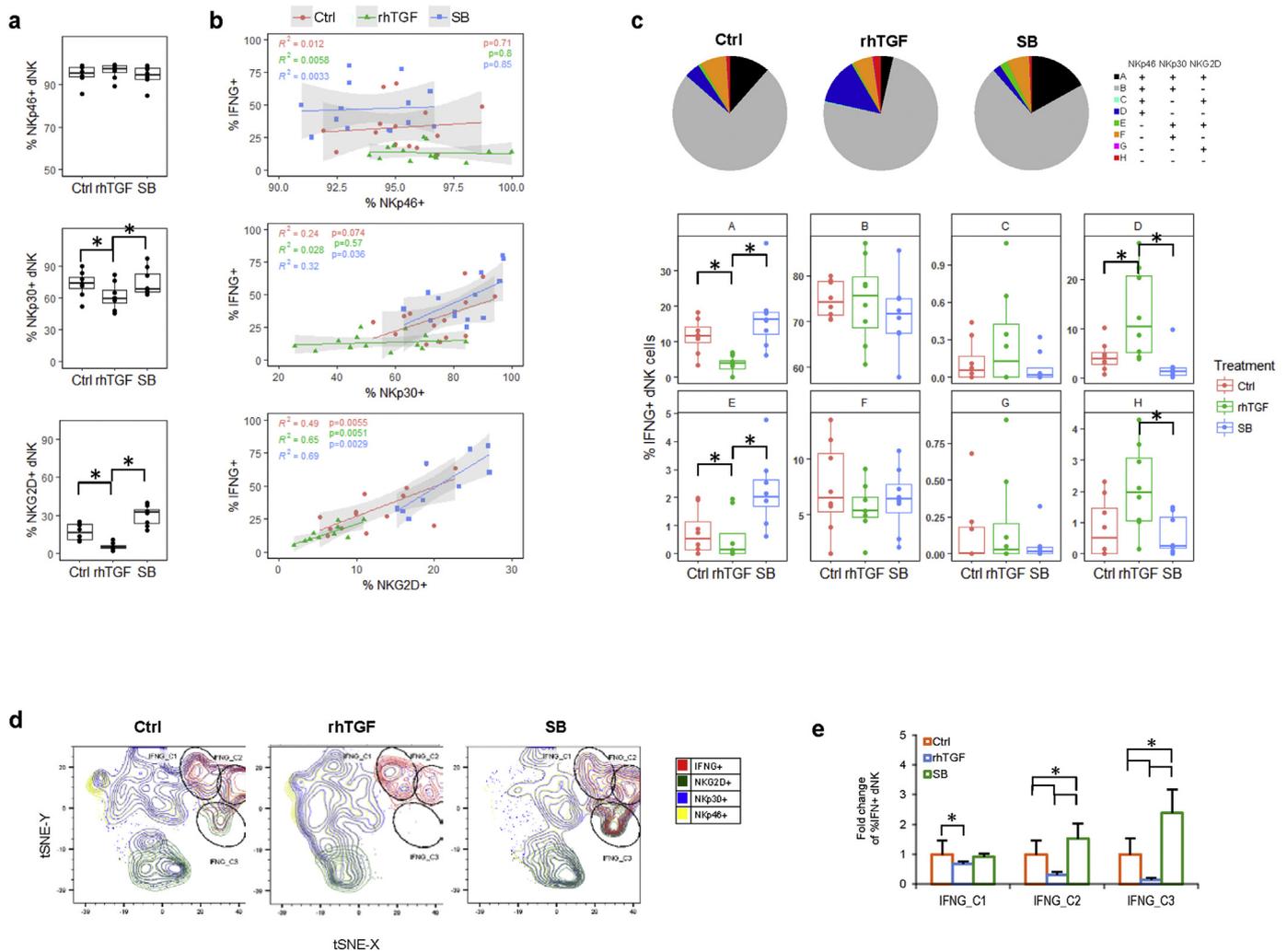
Fig. 4. TGFb conditioned IFNG expression by dNK cells is associated with NK receptors. a) The expression of surface markers NKp46, NKp30 and NKG2D on dNK cells was analyzed after rhTGFb1 and SB431542 (SB) treatment. *p < 0.05 when using Kruskal–Wallis test followed by Dunn's test. b) The linear regression of IFNG expression to dNK receptor NKp46, NKp30 and NKG2D was evaluated. P values were determined by Pearson correlation. c) IFNG expression by dNK subsets was assessed according to their NKp46, NKp30 and NKG2D combinations using logic gating (i.e. positive or negative subsets). n = 9–18 per group. d) The distribution patterns of IFNG⁺ dNK clusters in response to TGFb stimulation. Three major IFNG clusters (red) can be identified by manual gating according to tSNE maps and their fold changes were calculated (e). n = 6. *p < 0.05 when using Kruskal–Wallis test followed by Dunn's test.

and IFNG/IL-8/CD107a was greatly improved when TGFb signalling was inhibited (p < 0.05, Kruskal-Wallis test; Fig. 3e). Interestingly, tSNE mapping demonstrated that the functional coordination of dNK cells can be greatly reshaped by TGFb (Fig. 3f). When TGFb was blocked, IFNG⁺, IL-8⁺, VEGF⁺ and CD107a⁺ dNK subsets were more likely to cluster together in comparison to normal control status, suggesting multifunctional transformation of dNK cells; while in response to rhTGFb1 treatment they tended to form dispersed clusters, indicating that TGFb can downregulate the functional diversity of IFNG⁺, IL-8⁺, VEGF⁺ and CD107a⁺ dNK subsets.

3.3. NK receptor-associated IFNG expression is inhibited by TGFb1

Next, we set to address the association between IFN expression of dNK and their phenotypic characteristics. As seen in Fig. 4a, rhTGFb1 significantly downregulated the expression of NKp30 and NKG2D, but not NKp46, on dNK cells in comparison to control or TGFb blockade (SB), indicating that TGFb can selectively modulate dNK surface markers. Subsequent regression analysis found negligible correlation between NKp46 (%) and IFNG (%) expression on dNK cells either with or without TGFb1 stimulation (Fig. 4b). However, proportion of IFNG⁺ dNK cells had a significant positive correlation with their NKp30

expression (%) when TGFb signal pathway was inhibited by SB (R² = 0.32, p < 0.05, Pearson correlation) and this relationship was absent when exogenous rhTGFb1 was supplemented (R² = 0.026, p > 0.05, Pearson correlation; Fig. 4b). Strong and positive correlations were found between the percentages of IFNG⁺ and NKG2D⁺ dNK cells either with or without TGFb (Fig. 4b). These results suggest that the function of specific dNK subsets could be conditionally regulated by decidual TGFb signalling, depending on their phenotype.

We also quantified TGFb-regulated IFNG expression in different dNK subsets. In normal pregnancy, NKp46⁺ and NKp46⁻dNK cells had similar expression levels of IFNG while NKp30⁺, NKG2D⁺ dNK cells had significantly higher IFNG expression than NKp30⁻ or NKG2D⁻ dNK cells (Supplementary Fig. 2b). Furthermore, Boolean gating analysis revealed that for all IFNG⁺ dNK cells, the portions of NKp46⁺/⁻NKp30⁺NKG2D⁺ dNK subsets were significantly decreased by rhTGFb1 in comparison to control and SB groups (Fig. 4c). Alternatively, rhTGFb1 increased IFNG expression by NKp30⁻NKG2D⁻ dNK subsets (p < 0.05, Kruskal–Wallis test; Fig. 4c).

A high dimensional analysis of tSNE mapping further revealed the cellular diversity of IFNG⁺ dNK cells which were composed of three major clusters (C1-3; Fig. 4d & e). In cluster 3 (C3), dNK cells were the most sensitive subset affected by TGFb, while in cluster 1 (C1) IFNG
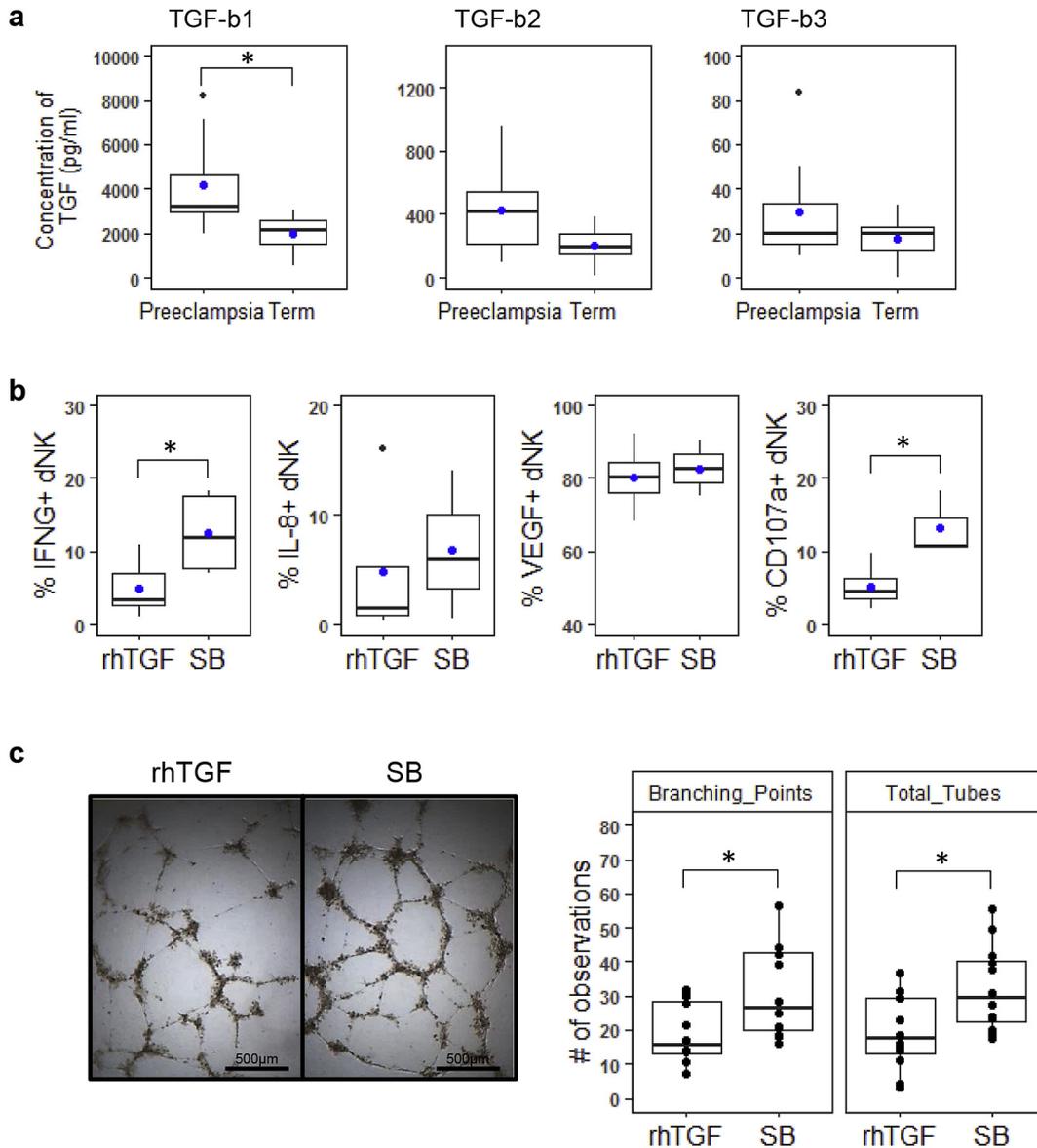
Fig. 5. The responsiveness of preeclamptic dNK to rhTGFb1 stimulation. a) The TGFb1protein secretion in decidual homogenates collected from preeclampsia and normal term pregnancy were measured by Bio-plex. n = 9 for each group. b) Functional changes of freshly isolated preeclamptic dNK cells stimulated by rhTGFb1 or SB431542 (SB). n = 6. c) Angiogenic function of dNK cells was changed by TGFb. Early gestation dNK cells were treated with rhTGFb1 or SB for 24 h, and then conditioned medium was collected for tube formation assay using HUtMEC. The representative images of 12 independent experiments were shown. *p < 0.05 when using Mann-Whitney *U* test.

expression by dNK was relatively stable. Furthermore, principal components analysis demonstrated that exogenous rhTGFb1 stimulation has a distinct partition in comparison to control and SB groups, both displaying similar clustering features (Supplementary Fig. 3). NKG2D, NKp30 and IFNG (identified by horizontal component PC1) together can explain 82.3% changes of dNK activity. The expression of IFNG is highly correlated with NKG2D and NKp30 rather than NKp46 (Supplementary Fig. 3).

3.4. TGFb1 blockade improved angiogenic function of dNK cells

Preeclamptic deciduae had a significant higher level of TGFb1 than normal term samples (4557 pg/ml vs 1974 pg/ml; p < 0.05, Mann-Whitney *U* test; Fig. 5a). To further verify the TGFb responsiveness of freshly isolated dNK cells from preeclamptic decidua, we incubated these dNK cells with rhTGFb1 or TGFb inhibitor. As shown in Fig. 5b, the expression of IFNG and CD107a by dNK cells was significantly increased by SB (p < 0.05, Mann-Whitney *U* test), suggesting that in preeclampsia, dNK function was subjected to the TGFb signalling regulation.

We further conducted a tube formation assay to assess dNK cell mediated angiogenesis regulated by TGFb (Fig. 5c). The number of branching points and total formed tubes of HUtMEC was significantly decreased by the conditioned medium prepared by dNK cells in the presence of rhTGFb1, in comparison to that of SB (p < 0.05, Mann-Whitney *U* test; Fig. 5c), indicating that inhibition of decidual TGFb1 signalling could improve dNK-mediated angiogenesis, a key event to support decidualization and placentation during pregnancy (Fig. 6).

4. Discussion

Complications of pregnancy are often associated with abnormal immune activity at the maternal-fetal interface, including impaired interactions between decidual stromal cells and leukocytes [26–28]. Our results provide deeper understanding regarding functional heterogeneity and defective responsiveness of dNK subsets in preeclamptic decidua. Essentially, we found that decidual TGFb1 play a critical role in modulating dNK phenotype and function, which may have a direct effect on the pathogenesis of preeclampsia (Fig. 6). Our data
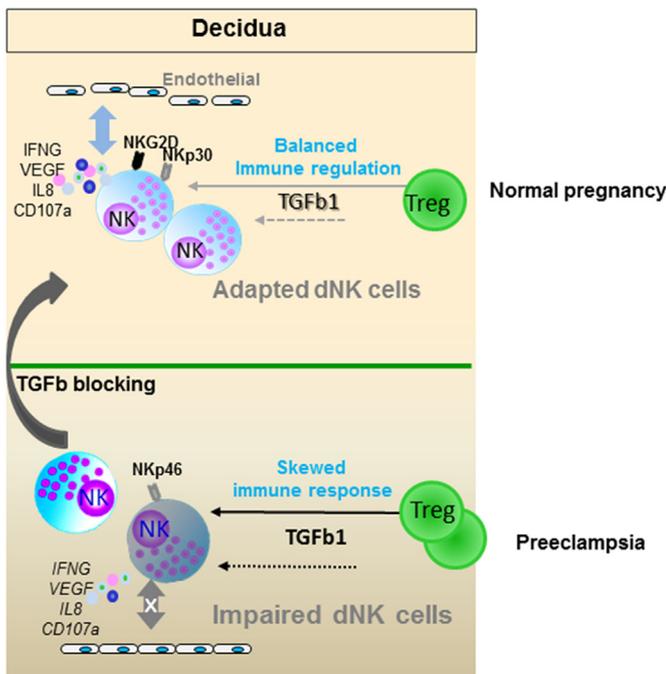
Fig. 6. Immune regulation in decidual area during preeclampsia development. In the decidual zone of normal pregnancy, dNK cell mediated immune response and angiogenesis was delicately regulated by Treg cells via soluble TGFb1. However in preeclamptic decidua, higher amounts of TGFb produced by Treg cells could greatly impair the phenotypic and functional diversity of dNK subpopulations. This skewed immune response may further damage decidual angiogenesis and cause pathological pregnancy.

show that elevated levels of TGFb1, produced by decidual Treg cells, may suppress the functional coordination of dNK cells and lead to the development of preeclampsia. We proposed that NKp46$^{+/-}$NKp30^{+-}NKG2D$^+$IFNG$^+$ dNK subsets are a pivotal population associated with preeclampsia due to their susceptibility to changes in TGFb signalling. In contrast, TGFb blockade may increase the function and angiogenic properties of dNK cells.

Similar to pbNK or other tissue resident NK, dNK cells express many NK receptors; however, they have very specific phenotype and function which reflects their decidual adaptation [28,29]. Our data found that preeclamptic deciduae retained a large number of dNK cells with higher CD56 molecular density, indicating that a majority of dNK cells in preeclampsia are arrested in an immature state. The unexpected confinement of CD56$^+$ dNK suggests that in preeclampsia, the decidua forms a specific microenvironment to trap dNK cells by modifying their transformation and trafficking. Our in vitro studies confirmed that the percentage and density of CD56 expression by dNK cells was greatly increased by TGFb1 (Supplementary Fig. 4). Thus, in agreement with the TGFb reactivity of other NK cells [30,31], we conclude that increased decidual TGFb1 is a critical factor to retain the dNK population at this site.

Wallace et al. reported that abnormal dNK cells in early pregnancy were related to a high risk of preeclampsia [32,33]. In preeclamptic deciduae, we found dysfunctional dNK cells with a high level of expression of NKp46 and NKp30. Surprisingly, TGFb did not change the expression of dNK-bound NKp46 expression, indicating that NKp46 is a conserved activating receptor to control decidualization and vasculature remodelling in pregnancy. Furthermore, the functional heterogeneity of dNK cells (the expression of IFNG/IL8/VEGF/CD107a) was differentially modulated by microenvironmental TGFb1. In normal term pregnancy, positive correlations between angiogenic and cytotoxic function of dNK cells were apparent (Fig. 2b). However, this relationship can be reversed under pathological condition in which a significant negative correlation between VEGF and CD107a expression is found in dNK cells from preeclamptic deciduae exposed to higher levels of TGFb. This discrepancy indicates that the angiogenic and cytotoxic function of individual dNK cells are regulated independently. Moreover, our data suggest that TGFb1 may serve as an immunological switch to modulate the functional differentiation of the dNK population. Although we showed that TGFb1 and Treg cells can downregulate dNK cell function (IFNG/IL-8/CD107a expression), it remains unclear why these dNK cells can preferentially adjust angiogenic function while sustaining their cellular identity (NKp46 expression). We speculate that such adaptive changes to decidual immunity may represent a hierarchical regulation of dNK cells required for decidual angiogenesis.

Abnormal placental TGFb response is related to pathological development of preeclampsia [23,34]. Since the pro- and anti-angiogenic effects of different TGFb isoforms depend on the tissue context and cellular composition [35], it is plausible to speculate that optimal decidual TGFb1 composition and content are necessary to facilitate dNK mediated vasculature remodelling. In human decidua, NKp30$^+$/NKp30$^-$, NKG2D$^+$/NKG2D- and NKp46$^+$/NKp46$^-$ dNK subsets have different IFNG reactivity in response to microenvironmental TGFb. These receptor-defined effects further highlight the complexity and intricacy of hierarchical function of dNK lineages with specific TGFb exposures [36,37]. Moreover, NKp46$^{+/-}$NKp30$^+$NKG2D$^+$IFNG$^+$ dNK subsets appeared as the most sensitive population in response to changing TGFb signalling. Thus, dNK cells in preeclampsia are more vulnerable to TGFb stimulation as they expressed higher levels of NKp46 and NKp30. Since NKp46$^+$ NK cells, via IFNG secretion, are involved in the regulation of tumor angiogenesis [38], we propose that decidual TGFb1 could selectively suppress the functional coordination of individual dNK cells, and abnormally high TGFb1 may impair dNK function and lead to reduced decidualization and placentation.

Our data suggest that preeclampsia is associated with the presence of hypoactive dNK subpopulations as a result of high levels of TGFb1 in the decidua microenvironment. Treg cells appear to play a key role suppressing activity of specific dNK subpopulations. However, we do not exclude the contribution of other cells at the maternal-fetal interface, including various immune and non-immune cells, necessary for normal dNK activity [39–42]. In addition, distinct regulatory mechanisms may underlie decidual immunosuppression since dNK subsets responded differently to TGFb1 signalling. The heterogeneity of dNK cellular responsiveness, together with the increasing recognition that preeclampsia itself represents multiple distinct pathologies, suggests that transcriptional analyses at the single cell level may be required to fully define the role of dNK in this disease.

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Declaration of interests

The authors declare no conflict of interest.

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Author contributions

J.H.Z. performed all experiments, acquired data, analyzed results, and drafted the manuscript. C.E.D., O.S. and I.C. interpreted the experimental results and edited the manuscript. S.J.L supervised the project, interpreted the data, and edited the manuscript.

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