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Maternal Decidual Macrophages Inhibit NK Cell Killing of Invasive Cytotrophoblasts During Human Pregnancy¹

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ABSTRACT

Human pregnancy is an immunological paradox. Semiallogeneic (fetal) placental cells (extravillous cytotrophoblasts [CTBs]) invade the uterine lining (decidua), which contains a unique decidual natural killer (dNK) cell population, identified by the cell surface phenotype CD56^{bright} CD16[−] CD3[−] and CD14⁺ CD206⁺ macrophages (dMac). Previous reports suggested that human dNK cells are not a threat to the fetoplacental unit because they are anergic. In contrast, here we showed that purified and exogenously stimulated dNK cells are capable killers of cellular targets, including semiallogeneic CTBs. However, dMacs in the decidual leukocyte (DL) population restrained dNK killing through a transforming growth factor beta₁ (TGF-beta₁)-dependent mechanism. Our findings support a new model whereby dNK cells, capable of killing CTBs, are prevented from doing so by neighboring macrophages, thus protecting the fetal cells from NK cell attack. We speculate that this mechanism would inhibit dNK cell-mediated killing, even under conditions where high levels of cytokines may stimulate dNK cells, which could pose a threat to the developing placenta.

decidua, macrophage, natural killer cells, placenta, pregnancy, trophoblast

INTRODUCTION

Natural killer (NK) cells are bone marrow-derived lymphocytes that function in the destruction of foreign, infected, or malignant cells and in the promotion of immune responses. A diverse repertoire of cell surface receptors controls their activation, killing, cytokine production, and proliferation [1]. These receptors are encoded by genes that do not undergo somatic recombination in the germline; therefore, NK cells are innate immune cells [2]. Phenotypically distinct NK cell subsets vary in their effector functions and specific roles. In humans, CD56^{dim} CD16⁺ CD3[−] NK cells in peripheral blood are highly cytotoxic, mediating both natural and antibody-

dependent killing [3]. Another subset of NK cells, the CD56^{bright} CD16[−] CD3[−] NK cells, are less lytic but potent producers of immunoregulatory cytokines [4]. Whether these NK cell subsets represent a differentiation continuum within a single lineage or independent sublineages is an open question [5, 6].

NK cells play a critical role in the establishment and maintenance of pregnancy and placental development [7]. The maternal–fetal interface is a rare example of a naturally occurring situation in which cells with two distinct genomes commingle without negative immunological consequences. Early in pregnancy, a specialized population of NK cells infiltrates the endometrium [8]. CTBs actively recruit and retain decidual NK (dNK) cells at the maternal–fetal interface by secreting the chemokine chemokine ligand 3 (CCL3) [9]. This unique subset comprises the majority of decidual leukocyte (DLs) during the first trimester, dwindles as the second trimester progresses, and is nearly absent at term [8].

Development of the placenta involves the generation of chorionic villi that either float in the blood-filled intervillous space (floating villi) or attach to the uterine lining (anchoring villi). Anchoring villi contain the specialized subpopulation of cytotrophoblasts (CTBs) that invades the decidua, the inner third of the uterine muscular wall, and the organ's blood vessels. Together these components of CTB invasion enable uterine attachment and incorporate the placenta into the maternal circulation, exposing fetal cells and antigens to the resident maternal DLs. The role of dNK cells in the maintenance of tolerance toward the semiallogeneic fetal CTBs at the maternal–fetal interface remains unclear.

The phenotype of the dNK cell population resembles that of the CD56^{bright} CD16[−] CD3[−] NK subset in adult peripheral blood. These cells express the same levels of granzyme B and more than 10 times the amount of granzyme A than the CD56^{dim} CD16⁺ CD3[−] NK cell subpopulation [3]. Further studies indicate that decidual granzysin and perforin transcription increase in the first and second trimesters of pregnancy compared to the nonpregnant endometrium [10]. In contrast to peripheral CD56^{bright} CD16[−] CD3[−] NK cells that are largely devoid of granules, dNK cells are highly granular [3]. Thus, they might have potent cytolytic potential. Studies in mice suggest that these cells are active cytokine producers [11]. Functional analyses demonstrate *in vivo* killing of transplanted trophoblast cell lines or primary trophoblasts, and additional *in vitro* experiments confirm that the mechanism is NK cell-mediated lysis [12]. However, the question of whether cultured human dNK cells, which possess cytolytic machinery, are capable killers remains controversial. Some reports indicate that dNK cells kill cell targets [13, 14], whereas others indicate that dNK cells fail to polarize granules to the target cell and, thus, are ineffective

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killers [15]. Currently, it is unknown whether human dNK cells are capable of inducing CTB apoptosis.

NK cell activation involves adhesion of an NK cell to its target. This association allows activating and inhibitory NK receptors to interact with ligands expressed by potential targets. If the strength of the activating signals outweighs the input of inhibitory pathways, the cell responds by cytoskeletal reorganization, which enables polarization and release of cytolytic granules directed again the target cell and, possibly, cytokine secretion [1].

The local cytokine environment created by surrounding cells also modulates NK cell functions. Interleukin 15 (IL-15; and IL-2, which signals through the same receptor) provides growth and survival signals for NK cells. Before implantation, endometrial NK cells express the IL-15 receptor, and the levels change in pregnancy and in pregnancy complications [16]. Although endometrial levels increase during the window of implantation [17], the IL-15 or IL-2 levels that are associated with normal pregnancy are as yet unknown. Additionally, there appears to be a role for IL-15 at the maternal–fetal interface because its receptor is expressed by CTBs as they differentiate into the invasive phenotype [18]. It is well understood that in mice, IL-15 is an important regulator of decidualization and maintenance of pregnancy [19, 20]. However there is ample evidence [8] that NK cells may play different roles in human pregnancies.

In addition, other cytokines can influence the behavior of NK cells. For example, activation by inflammatory cytokines (e.g., IL-12 and IL-18) can overcome inhibitory signals [21]. Conversely, IL-10 [22], reactive oxygen species [23], and transforming growth factor β (TGF- β) [24] inhibit NK cell activation. As to mechanisms, the latter growth factor operates on many levels to suppress NK cell effector functions [25, 26]. For example, TGF- β can downregulate NK cell expression of a key activating receptor, rendering these potential killers unresponsive to targets that express the corresponding ligands [27].

The other dominant DL subtype, CD14⁺ CD206⁺ macrophages (dMacs), constitutes 20%–30% of maternal immune cells at the implantation site [28, 29]. In contrast to dNK cells, their numbers remain constant throughout pregnancy [30]. Microarray analyses showed that they have special properties. Thus, they do not fit the typical M1/M2 paradigm [31]. The explanation may be the specialized cytokine environment in which they reside, which includes M-CSF and IL-10 [32], and their exposure to hormones of pregnancy such as relaxin [33]. In cases of abnormal placentation, e.g., trisomy 21 and preeclampsia, there is widespread apoptosis of CTBs within the uterine wall [34, 35], creating cellular debris, which is presumably phagocytosed by dMacs [30]. Additionally, macrophages play a major role in the induction and the resolution of inflammatory processes [36], and they can switch between pro- and anti-inflammatory states [37]. Currently, two major phenotypes are recognized: classically activated or type-I macrophages (CAM) and alternatively activated or type-II macrophages (AAM) [37]. CAMs exhibit an inflammatory phenotype [38]. AAMs have high phagocytic capacity and secrete a variety of anti-inflammatory cytokines, including TGF- β [37]. In fact, recent gene expression studies revealed that dMacs have a phenotype similar to that of AAM [39].

The role that dNK cells and dMacs play in the regulatory immune network during pregnancy is poorly understood. Here we asked whether IL-2-activated dNK cells had cytolytic activity. The results showed that maternal dNK cells effectively killed CTBs and that dMacs prevented lytic activity by a TGF- β -mediated mechanism. These findings may explain the

mechanisms underlying maternal tolerance to the semiallogeneic placenta.

MATERIALS AND METHODS

Human Tissue Collection

This study was approved by the University of California San Francisco Committee on Human Research. Informed consent was obtained from all donors. Placental (5–22 wks) and decidual tissues (5–13 wks) from patients undergoing elective terminations of pregnancy were obtained immediately, washed repeatedly with antibiotic-containing PBS to remove blood clots, and kept on ice until subsequent processing.

Isolation and Culture of Decidual Leukocytes

Decidua was isolated by manual dissection and suspended (10 ml/g tissue) in 181 U/ml collagenase I-A (Sigma), 0.12 mg/ml DNase I (Sigma), 0.70 mg/ml hyaluronidase type I-S (Sigma), and 1 mg/ml bovine serum albumin (BSA) in PBS. Enzymatic dissociation was carried out at 37°C with gentle shaking in a water bath. Cells were harvested at 50 and 75 min and washed by centrifugation in Hanks basic solution (HBS) supplemented with 10% fetal bovine serum (FBS; Hyclone). Cells were strained through sterile gauze (Kendall) and filtered through a 70- μ m nylon strainer (BD Falcon) to remove debris. Then they were washed in PBS and separated over a Ficoll-Hypaque 1077 (Sigma) density gradient. In some experiments, total DL cells were used. For further purification of individual DL subpopulations, CD56⁺, CD14⁺, and CD206⁺ cells were isolated by using the MACS cell separation system (Miltenyi Biotec) following the manufacturer's instructions. The purity of the isolated cell fractions, which was checked periodically by flow cytometry, was consistently 90% or higher. DLs were plated in Iscove medium (Gibco) with 10% FBS (Hyclone), 1% L-glutamine, 1% penicillin/streptomycin, and 1000 U/ml recombinant IL-2 (rIL-2; R&D Systems) and used in assays immediately following isolation. rIL-2 was also added to cytotoxicity assays at the indicated concentration. For experiments involving conditioned medium, DLs were cultured as described above, and the supernatant was collected at 18 h.

CTB Isolation

The methods were previously described [40]. Briefly, manually dissected chorionic villi were minced and then treated with a series of enzymes to release the CTBs. The cells were further purified by using Percoll gradient centrifugation (GE Healthsciences). Purity, based on cytokeratin-7 (CK7) staining, averaged 90% (n = 60). Occasionally staining of human leukocyte antigen G (HLA-G) was also used to determine the proportion of extravillous CTBs, which averaged 35%. The cells were cultured at 37°C in 5% CO₂ on Matrigel (BD Biosciences) diluted 2:1 in serum-free medium: Dulbecco modified Eagle medium (DMEM), 4.5 g/L glucose (Sigma-Aldrich) with 2% Nutridoma-SP 100x (Roche Diagnostics), 1% antibiotics (penicillin/streptomycin/gentamicin), 1% sodium pyruvate, and 1% HEPES. Conditioned medium was collected at 18 h. Our published work shows that under these conditions, the cells rapidly differentiated to the extravillous subtype [41].

Propagation of Cell Lines

NK92 cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Hyclone), 1% sodium pyruvate, 1% nonessential amino acids, 1% L-glutamine, 1% penicillin/streptomycin, and 200 U/ml recombinant human IL-2 (R&D Systems). K562 cells were grown in Iscove medium (Gibco) supplemented with 10% FBS (Hyclone), 1% L-glutamine, 1% penicillin/streptomycin, and 1.5 g/L sodium bicarbonate. Transfected mink lung cell (TMLC) reporters were cultured in DMEM (Gibco) supplemented with 10% FBS (Hyclone) and 250 μ g/ml G-418 (Roche). All cells were maintained at 37°C in 5% CO₂.

⁵¹Cr-Release Assay

We used a published method for the ⁵¹Cr-release assay [42]. K562 target cells (1 \times 10⁶) were labeled by incubation for 1 h with 50 μ Ci of ⁵¹Cr (Perkin-Elmer) in 1 ml of RPMI-1640 medium at 37°C in 5% CO₂. Effector (E) and target (T) cells were combined at the indicated ratios (see Figs. 1, 2, 3, and 5, n = 3) in 96-well V-bottom plates and incubated for 4 h at 37°C in 5% CO₂.

CTB targets, which preliminary experiments showed incorporated radioactivity poorly in suspension, were labeled as adherent cells. Briefly, CTBs were distributed at a concentration of 1 \times 10⁵/well of a 48-well plate or 1.5 \times 10⁵/well of a 24-well plate coated with Matrigel (BD Biosciences). CTBs

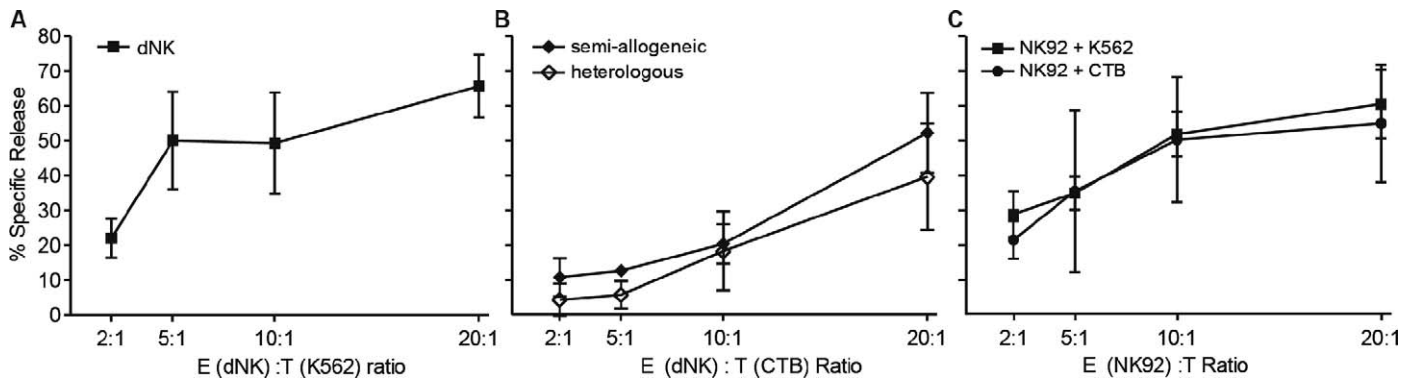


FIG. 1. Decidual NK cells lysed K562 targets and CTBs. **A**) In ^{51}Cr -release assays, purified CD56^+ dNK cells were potent killers of K562 targets in the presence of IL-2 (n = 3). **B**) dNK cells killed CTBs isolated from the same pregnancy (semiallogeneic) or an unrelated pregnancy (heterologous). There were no significant differences between the groups (n = 3). **C**) NK92 cells lysed K562 and primary CTB targets equally well (n = 6; no significant differences).

adhered to the substrate for 1.5 h at 37°C in 5% CO_2 , and then $100 \mu\text{Ci}$ of ^{51}Cr dissolved in PBS was added. Plates were rotated at 37°C in 5% CO_2 for 2 h and washed three times in PBS before medium and E cells were added at the indicated E:T ratios (n = 3 technical replicates/condition). The assay samples were incubated at 37°C in 5% CO_2 for 10 h.

At the conclusion of the assays, a portion of the culture medium was collected and added to scintillation fluid (Wallac/Perkin Elmer) for quantification of radioactivity (1450 Microbeta plate reader; Wallac). Spontaneous ^{51}Cr release was measured in wells containing only target cells. Maximum release was determined by the addition of 10% Triton-X-100 (Sigma). The results were expressed as a percentage of specific lysis: $[\text{experimental cpm} - \text{spontaneous cpm}] \times 100 / [\text{maximum cpm} - \text{spontaneous cpm}]$. For function blocking experiments, anti-TGF- β_1 (clone 2E6; Abcam) or a nonspecific control immunoglobulin G (IgG) antibody (clone 3k1; Abcam) was used at $10 \mu\text{g}/$

ml. The reagents were mixed with DLs 30 min before the addition of E cells. TGF- β_1 (R&D Systems) was added at a concentration of 20 ng/ml 30 min prior to the addition of dNK cells. For Transwell (Corning) assays, cells were plated in 96- or 24-well Transwell plates. The upper and lower chambers were separated by a $0.4\text{-}\mu\text{m}$ polycarbonate membrane.

TMLC Reporter Assay

TMLCs [43] were incubated for 24 h in 96-well flat-bottom plates (Falcon) in dMac- or CTB-conditioned medium; control TMLC cultures were incubated in basal medium alone. Cells were lysed and activity was assessed by using the Steady-glo luciferase assay (Promega). Samples were transferred to opaque polystyrene plates (Corning), and fluorescence was quantified by using a luminescence counter (Topcount NXT model; Packard).

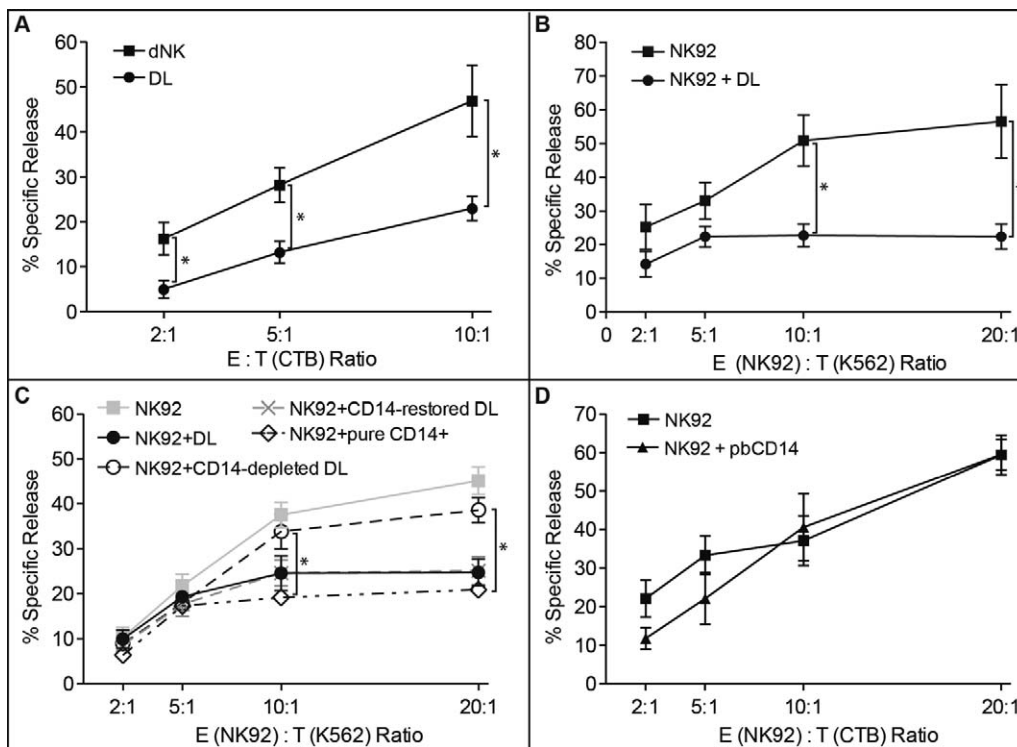


FIG. 2. Total decidual leukocytes and decidual CD14^+ cells were potent inhibitors of cytotoxicity. **A**) With CTB targets, purified IL-2-treated dNK cells had higher cytotoxic activity than unfractionated DLs, which contained other leukocytes types ($*P \leq 0.05$; n = 5). **B**) NK92 killing of K562 targets was inhibited by the addition of DLs (n = 9). **C**) NK92 cells lysed K562 targets in the absence or in the presence of DLs depleted of CD14^+ cells. Unfractionated DLs or CD14 -depleted DLs reconstituted with CD14^+ cells inhibited NK92 cell killing. Purified CD14^+ cells inhibited NK92-mediated killing to the greatest extent ($*P \leq 0.05$; n = 4). **D**) Unlike DLs, adult peripheral blood (pb) CD14^+ cells failed to inhibit CTB lysis (n = 5; no significant differences).

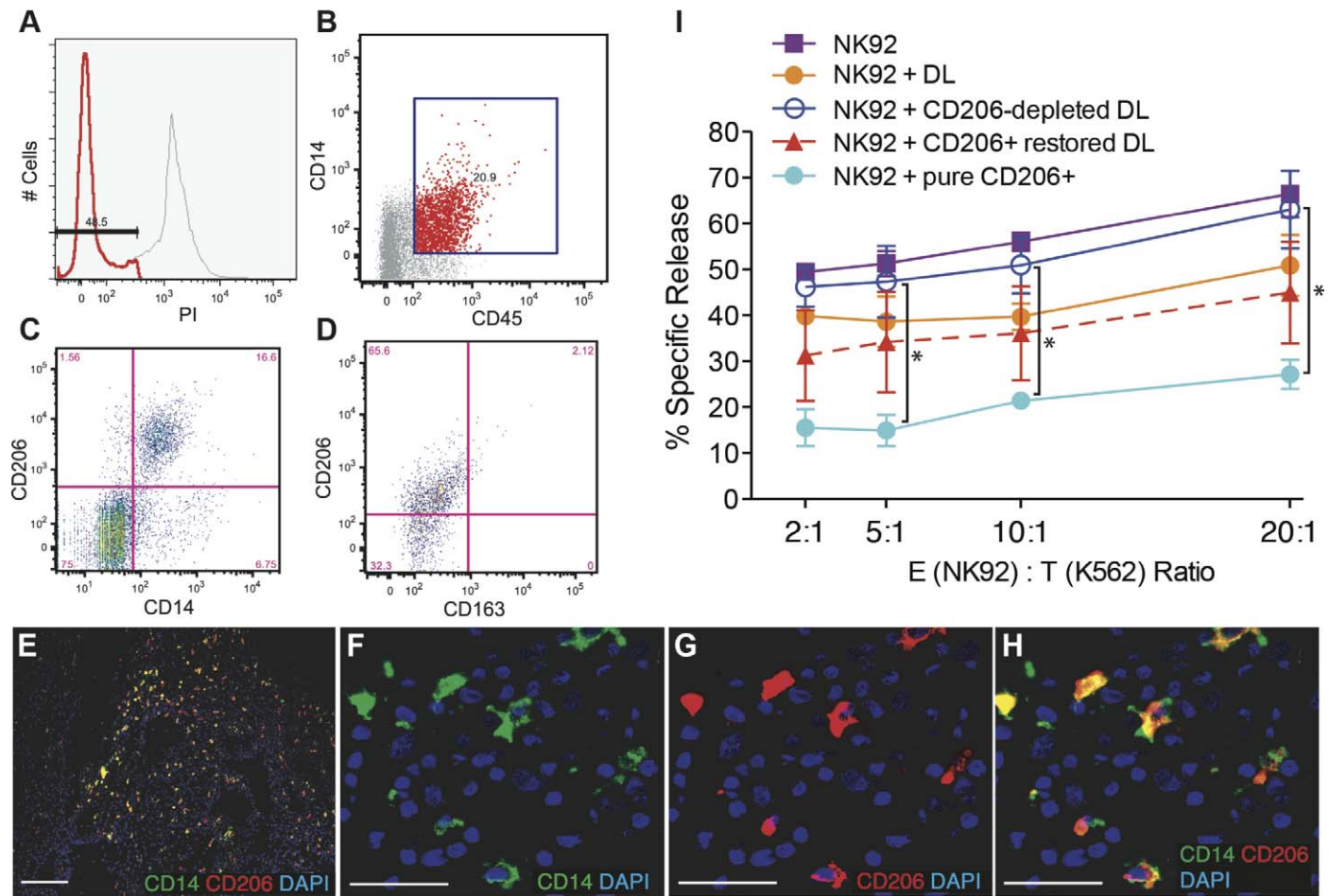


FIG. 3. Decidual macrophages, which expressed CD14 and CD206, were abundant at the maternal–fetal interface and inhibited cytotoxicity. Viable DLs were gated based on PI exclusion (A) and expression of CD45 and CD14 (B). dMacs also expressed CD206 (C), but few stained with anti-CD163 (D). Data are representative of 5 experiments. CD206⁺ macrophages were localized in tissue sections of human decidua; DAPI staining identified nuclei. E) At lower magnification, CD14⁺ (green) and CD206⁺ (red) cells were abundant. F–H) At higher magnification, many of these macrophages coexpressed CD14 (green) and CD206 (red). Bars = 100 μ m. I) NK92 cells killed K562 targets in the absence or presence of DLs depleted of CD206⁺ macrophages. Unfractionated DLs or CD206-depleted DLs reconstituted with CD206⁺ cells inhibited NK92 lysis of K562 targets. The addition of purified CD206⁺ macrophages inhibited cytotoxicity to the greatest degree (* $P \leq 0.05$; $n = 5$).

Immunofluorescence and Flow Cytometry

DLs were washed in Ca²⁺- and Mg²⁺-free PBS containing 1% BSA (fluorescence-activated cell sorting [FACS] buffer; Sigma) and 0.05% sodium azide. Nonspecific reactivity was blocked by incubating the samples for 20 min in 10% normal mouse serum (Jackson ImmunoResearch). DLs were labeled with fluorochrome-conjugated antibodies on ice (20 min), washed three times in FACS buffer, and analyzed using a FACSCalibur instrument (BD Biosciences) and FlowJo software (Treestar, Inc.). The cells were stained for CD163 (clone GHI/61; BD Pharmingen), CD206 (clone 19.2; BD Pharmingen), CD14 (clone MphiP9; BD Pharmingen), and CD45 (clone IVN816; BD Pharmingen). Nonviable cells were identified by staining with propidium iodide (PI; 1 μ g/ml; BD Pharmingen).

Immunolocalization and Confocal Microscopy

Freshly isolated placental and decidual tissues were fixed in 3% paraformaldehyde for 90 min and processed as previously described [44]. Tissue sections (5 μ m for fluorescence microscopy and 30 μ m for confocal microscopy) were prepared by using a cryostat (Leica CM 3050) and collected on charged slides (SuperFrost Plus; Fisherbrand).

Antibodies that recognized the following antigens were diluted 1:100 in PBS/3% BSA: CD206 (clone 309210; R&D Systems), CD14 (M5E2; Biolegend), CK7 (clone OV-TL; Dako Cytomation), and CD56 (clone AF2408; R&D Systems). Slides were treated with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI; Vector Labs) and viewed immediately

after preparation using a fluorescence microscope (Leica DM 5000B; confocal microscope; Leica TCS SP5).

Statistics

Data were analyzed using a paired Student *t*-test (Excel software version 12.1.0; Microsoft) and *P* values ≤ 0.05 were considered significant.

RESULTS

Decidual NK Cells Can Be Competent Killers and CTBs Are Susceptible to Lysis

To begin evaluating the functional status of dNK cells, we cocultured total DLs or dNK cells isolated from decidual samples with CTBs in the presence of IL-2. We found that IL-2 was necessary for dNK cell survival *in vitro*. As CTBs were much larger (~20–30 μ m) than dNK cells (~5–10 μ m), the two cell types were easily differentiated under the microscope. After 12 h, CTBs and dNK cell cocultures contained many placental cells that were undergoing apoptosis. In wells containing CTBs alone, there was very little apoptosis (data not shown).

These preliminary findings suggested that purified dNK cells were effective killers of CTBs. We asked whether the

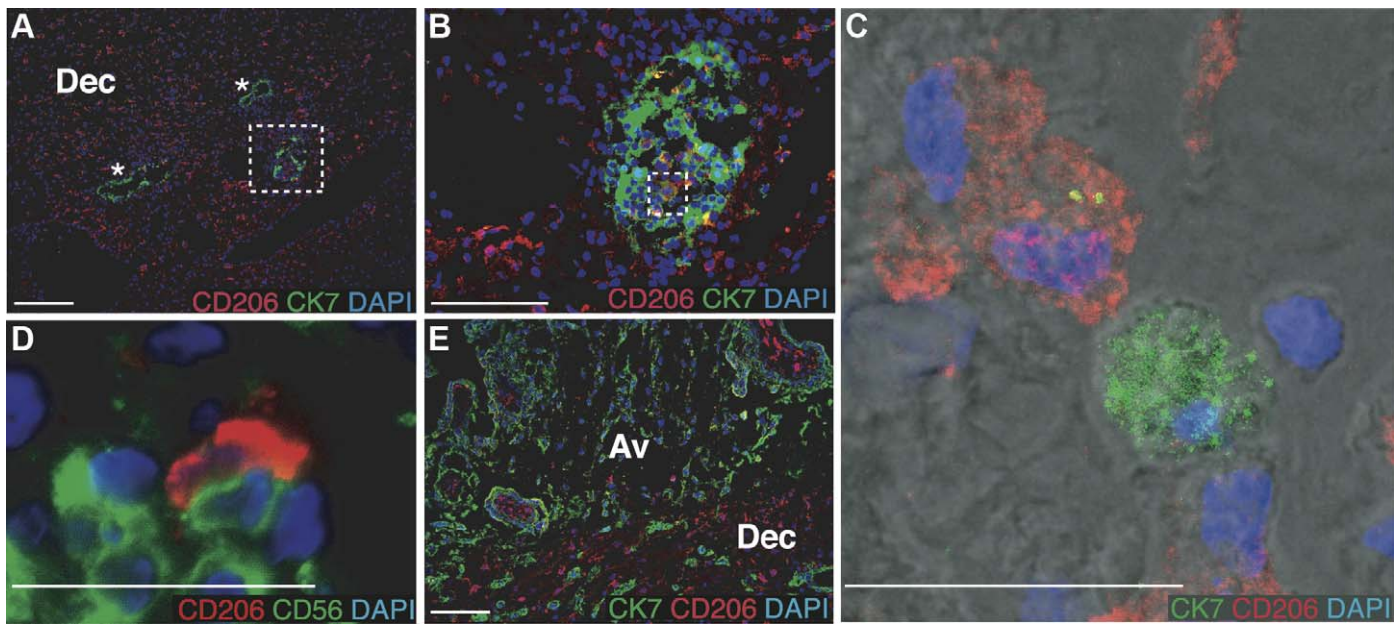


FIG. 4. Confocal immunolocalization demonstrated close interactions among dNK cells, dMacs, and CTBs within the human uterine wall and at the maternal–fetal interface. **A**) At low magnification, CD206⁺ macrophages (red) were detected throughout the interstitial compartment of the pregnant human uterus at 10 wks of gestation. CTBs, identified by CK7 staining (green), occupied the interstitium (box) and lined maternal blood vessels (*). **B**) Close interactions between CTBs and CD206⁺ macrophages were evident. **C**) At higher magnification of the box shown in **(B)**, an overlay of the phase contrast and fluorescent images showed contact between the plasma membranes of the two cell types. **D**) In 8-wk decidua, CD206⁺ dMacs formed close cell-cell contacts with CD56⁺ dNK cells. A representative image from one of four samples tested is shown. **E**) Immunolocalization of CK7⁺ CTBs (green) and CD206⁺ dMacs (red) at 16 wks of gestation. An anchoring villus (Av) is shown attached to the decidua (Dec). CTBs that invaded the uterine wall were found in close proximity to numerous dMacs. A representative image from one of four samples is shown. Bars = 100 μ m.

dNK cells could also kill the standard NK cell-target, K562. These data showed that dNK cells lysed K562 cells in a dose-dependent manner (Fig. 1A). Then we investigated whether dNK cells killed allogeneic CTBs from MHC-heterologous sources or semiallogeneic CTBs isolated from the placentas of the dNK donors. The results of these experiments showed that dNK cell-mediated lysis was independent of the CTB source, as the percentage lysis at all E:T ratios was not statistically different (Fig. 1B).

These data demonstrated that CTBs were susceptible to dNK cell-mediated lysis. Next, we compared the killing ability of an NK cell line, NK92, to lyse K562 and CTB targets. Our results showed that they were killed with equal efficiency (Fig. 1C). Together these data revealed that CTBs can be targeted for lysis by NK cells and that this phenomenon was independent of the level of allogenicity.

Decidual Leukocytes Regulate NK Cell Cytotoxic Activity

Next we tested our hypothesis that components of the DL population could inhibit dNK cell cytotoxicity. In these experiments, equal numbers of NK92 cells and unfractionated DLs (containing a mixture of dNK and myeloid cells) were cocultured with ⁵¹Cr-labeled primary CTBs. To equalize the number of NK cells in the two conditions, the number of DLs added to the wells was increased based on our flow cytometry data that showed ~70% of the DLs were dNK cells. The results showed that DL cytotoxicity was significantly lower than that of purified dNKs (Fig. 2A). To determine whether DL inhibition of NK-mediated lysis was specific to placental cells, we added this mixed population to cytotoxic assays that used K562 targets and NK92 effectors. Thus, they contained twice the number of NK cells as the experiments shown in Panel A. As before, the NK92 cells lysed K562 in a dose-dependent

manner and this activity was significantly inhibited by the addition of total DLs even though the cultures contained higher numbers of NK cells (Fig. 2B).

To identify the inhibitory cell type(s) in the DL population, we performed a series of preliminary experiments in which we systematically depleted and added back specific subpopulations, including CD3⁺ T cells, BDCA-1, -3, or -4⁺ dendritic cells or CD14⁺ myeloid cells (data not shown). Only removal of CD14⁺ cells restored control levels of NK92 cytotoxicity (Fig. 2C). Furthermore, the addition of these cells to the DL population from which they were removed restored inhibition to the level observed prior to depletion.

We also determined whether CD14⁺ cells from the decidua were unique in their ability to suppress NK cell activity. We isolated CD14⁺ cells from adult peripheral blood (pbCD14) and added them to a killing assay with NK92 cells as the effectors and CTBs as the targets. The pbCD14 cells had no effect on cytolysis (Fig. 2D). These data suggested that decidual CD14⁺ cells were a unique population with regard to their ability to inhibit NK cell-mediated cytotoxicity.

We conducted additional experiments to determine whether the decidual CD14⁺ cells were classically or alternatively activated macrophages. First, we used flow cytometric analyses to investigate their expression of CD206 and CD163, which are markers of alternative activation [36, 37] (Fig. 3). We identified live cells by PI exclusion (Fig. 3A) and myeloid cells by CD45 and CD14 coexpression (Fig. 3B). Most decidual CD14⁺ cells coexpressed CD206 (Fig. 3C) in the absence of CD163 (Fig. 3D). We confirmed these findings by staining tissue sections of the human decidua to identify this myeloid population in situ (Fig. 3, E–H). To understand the function of these cells, we isolated the CD206⁺ subset of DLs and investigated their inhibitory activity. As in the experiments shown in Figure 2C, depleting DLs of CD206⁺ cells resulted in

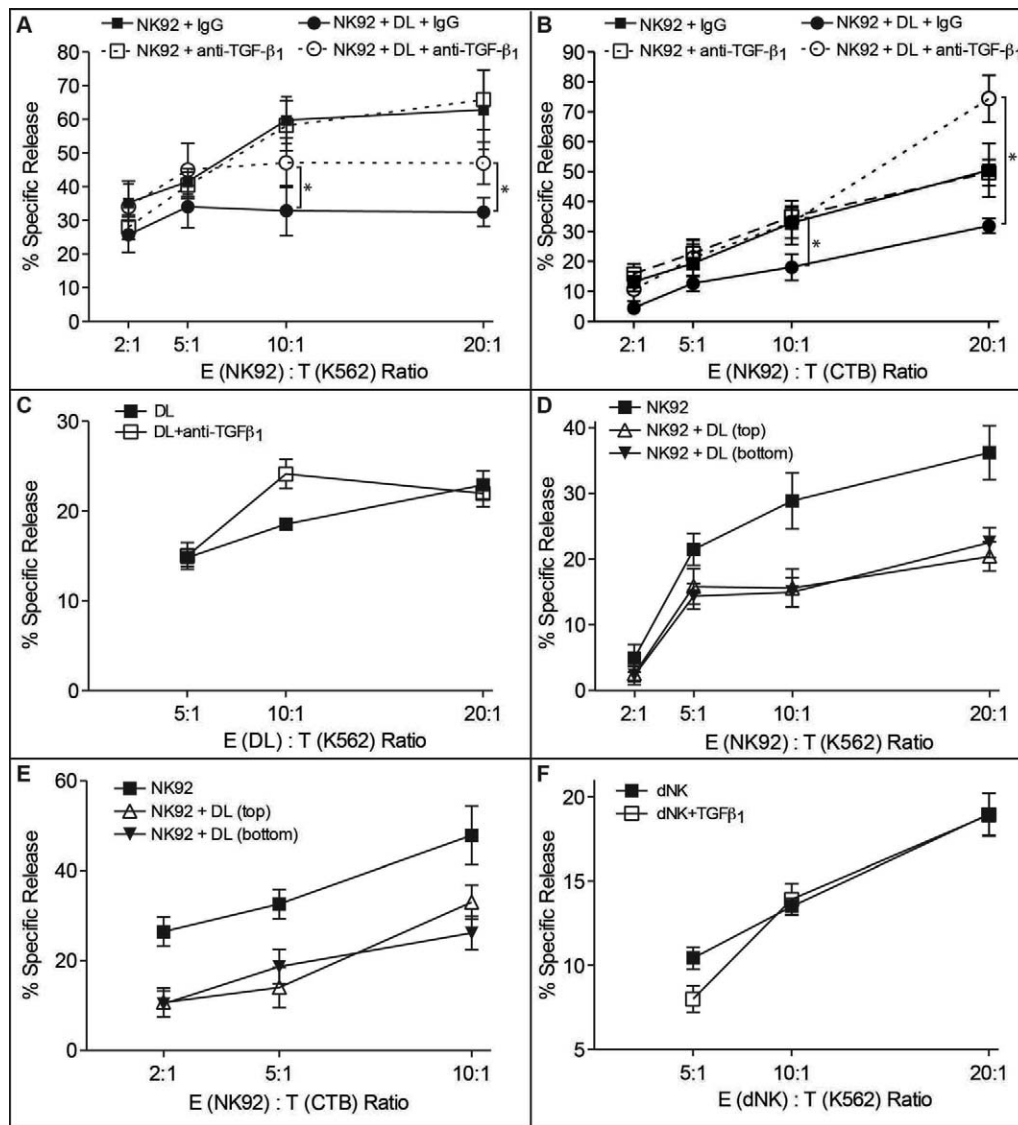


FIG. 5. TGF- β_1 was a potent inhibitor of dNK cell-mediated cytotoxicity. **A)** In ^{51}Cr -release assays, NK92 cells lysed K562 targets in the presence of anti-TGF- β_1 or control IgG antibodies. The addition of DLs and control antibody inhibited killing and this suppression was partially relieved by anti-TGF- β_1 ($n = 6$; $*P \leq 0.05$). **B)** NK92 cells killed CTBs in the presence of control antibodies and DL-mediated inhibition of cytotoxicity was relieved by the addition of neutralizing anti-TGF- β_1 ($n = 5$; $*P \leq 0.05$). **C)** The cytotoxic activity of purified DLs was increased by the addition of anti-TGF- β_1 antibody at the E:T ratio of 10:1 ($n = 2$; error bars reflect SEM of three technical replicates). **D)** In Transwell assays, NK92 cell lysis of K562 targets was inhibited by DLs whether or not they were cultured in the same or separate wells ($n = 4$). **E)** Identical results were obtained when CTBs were used as targets ($n = 4$). **F)** Exogenous TGF- β_1 decreased purified dNK cell-mediated lysis of K562 cells at the E:T ratio of 5:1 ($n = 2$; error bars reflect SEM of 3 technical replicates).

target lysis, which was inhibited when these cells were added back to the wells that contained DLs (Fig. 3I). Adding purified CD206 $^+$ cells to NK92 effectors inhibited target lysis to the greatest degree.

Given these results, we investigated the spatial relationships among CD206 $^+$ macrophages, dNK cells, and CTBs in tissue sections of the maternal-fetal interface. CD206 is expressed exclusively on macrophages, cultured dendritic cells, and by hepatic sinusoidal endothelium [45]. CD56 was used as a marker of NK cells and CK7 expression identified CTBs. In the first trimester, confocal microscopy revealed invasive CTBs and CD206 $^+$ dMacs were abundant in the same region (Fig. 4, A and B). At higher magnification, close associations between these two cell types were evident (Fig. 4C). During the first trimester, CD206 $^+$ cells were in close proximity to NK cells (Fig. 4D), while in early second trimester, CD206 $^+$ dMacs were found surrounding invasive CTBs (Fig. 4E). These

findings suggested that CD206 $^+$ dMacs interact with dNK cells and invasive CTBs within the uterine wall.

Decidual Leukocyte Secretion of TGF- β_1 Inhibited NK Cell-Mediated Lysis

We studied the role of TGF- β_1 in the regulation of dNK activity, as this cytokine is a well-characterized inhibitor of NK cells [46, 47]. Addition of an anti-TGF- β_1 neutralizing antibody to the DL inhibition assay with NK92 effectors and K562 targets (the design shown in Fig. 2B) restored cytotoxicity (Fig. 5A). To determine whether TGF- β_1 produced by DLs also inhibited NK cell-mediated killing of CTBs, we used placental cells as the targets (Fig. 5B). In this case, the greatest increase in cytotoxicity was observed in cultures containing DLs and anti-TGF- β_1 , likely due to dNK cells within the DL population that became cytolytic. Similar results were obtained in

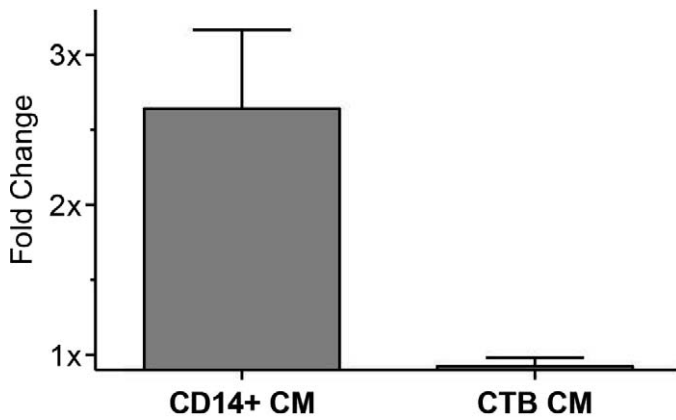


FIG. 6. Decidual macrophages produced TGF- β . TMLC reporter assays showed that conditioned medium (CM) from decidual CD14⁺ cells contained larger amounts of active TGF- β than CTB-conditioned medium (n = 2; error bars reflect SEM of three technical replicates).

experiments performed with purified dNK cells as effectors and K562 as targets (Fig. 5C). Killing was significantly increased by blocking TGF- β_1 at the E:T ratio of 10:1.

We also addressed the question of whether active TGF- β_1 was secreted or membrane-bound, as both forms inhibit NK cell function [46, 47]. Targets and effectors were plated on the bottom of Transwell culture dishes, and DLs were added to the upper or lower chamber. Targets were either K562 cells (Fig. 5D) or CTBs (Fig. 5E). DLs inhibited NK92-mediated lysis equally well under both conditions, suggesting that the secreted form of TGF- β_1 mediated the observed suppression. The addition of soluble TGF- β_1 to cytotoxicity assays had an inhibitory effect on purified dNK-mediated cytotoxicity at a low E:T ratio (Fig. 5F). However, the concentration of TGF- β_1 we used was not sufficient to inhibit lysis when higher numbers of dNK cells were added.

Decidual CD14⁺ Cells Produced Active TGF- β in Culture

To confirm that DLs secreted active TGF- β , we used the TMLC line reporter system. In response to TGF- β , the TMLCs activate the PAI-1 promoter, which drives luciferase production [43]. We cultured the TMLCs with CM from either CD14⁺ DLs or CTBs. The results, expressed as fold change over medium alone, showed that CD14⁺ DL-CM contained significantly more TGF- β than medium conditioned by CTBs (Fig. 6). This finding is in accord with our observation that CTBs cannot suppress NK cell-mediated lysis. Together, our data support a new model in which decidual CD14⁺ leukocyte secretes TGF- β , which inhibits NK cytotoxicity, thereby inducing CTB tolerance.

DISCUSSION

The abundance of NK cells at the maternal–fetal interface has long been enigmatic to researchers studying immune interactions during pregnancy. Although controversial [13], many investigators have proposed that dNK cells are incapable of mediating target cell lysis [15, 48, 49]. Prior studies proposed that CTBs in this location upregulate expression of an inhibitory ligand that prevents NK cell-mediated lysis. The nonclassical major histocompatibility class I molecule human leukocyte antigen G (HLA-G), which is expressed by CTBs, is one such candidate [50, 51]. Whether suppression of dNK cell-mediated cytotoxicity is caused by HLA-G expressed by CTBs has been controversial [52]. A recent study showed that HLA-

G failed to trigger dNK cell degranulation [53]. Here we report the unexpected finding that purified dNK cells and the NK92 line efficiently killed CTBs. This process was regulated by decidual macrophages that secrete TGF- β , thus inhibiting NK cell effector functions. Thus, we propose that the dialog among dNK cells, dMacs and CTBs, which involves TGF- β_1 , plays a role in controlling local maternal immune responses to invading CTBs.

The role of dMacs in pregnancy is likely multifaceted. In combination with dNK cells, they may induce regulatory T cells [54]. Recently published evidence suggests that the latter cells play a role in modulating maternal immune responses to the hemi-allogeneic fetus [55–57]. Our observations suggest an additional role for maternal macrophages in balancing interactions between the uterine immune system and invading embryonic/fetal CTBs. The finding that dMacs can inhibit NK cell-mediated lysis of human CTBs underscores the importance of this TGF- β_1 -mediated mechanism. dMacs suppressed the cytolytic activity of purified, activated dNK cells and the NK92 cell line. TGF- β is secreted in either latent or active form; functional assays detect only the latter. Therefore, our observation that maternal macrophages secreted active TGF- β , which suppressed NK cell-mediated killing, advances our understanding of this molecule's role at the maternal–fetal interface.

Previous reports described the detection, by various methods, of TGF- β family members in human and mouse decidua [21, 35, 51–54]. Interestingly, secretory phase human endometrium expresses a unique repertoire of TGF- β family members, which are likely to play an important role in governing trophoblast interactions at implantation and beyond [58]. Somewhat surprisingly, we found that extravillous CTBs secreted almost no active TGF- β . Therefore, we concluded that in addition to dMacs, the decidua could be an important source of TGF- β family members that dampen NK cytotoxicity.

The finding that TGF- β has immune functions is well supported by work over the last few decades [59]. This cytokine has long been recognized as an inhibitor of a variety of immune responses [59], especially NK activation [60]. TGF- β produced by myeloid-derived suppressor cells induces NK cell anergy in mouse tumor models [59]. This suggests the possibility that some tumors have adapted an ancient survival strategy, mimicking the placenta's ability to invade and survive in the potentially hostile maternal uterine environment. Parallels between tumor formation and placentation have been drawn previously [61–65]. Like tumors, CTBs invade while forming cell–cell contacts and penetrate blood vessels where they mimic many properties of the vascular cells they replace. Additionally, CTBs and malignant cells of many cancer types express a wide array of proto-oncogenes, such as members of the c-ras, c-met, and c-myc families, as well as HER1, HER2, and ABL1. CTBs and malignant tumor cells also express an overlapping repertoire of growth factors and their receptors as well as proteinases [65]. In line with the data reported here, many solid tumors (colon, breast, lung, and prostate) contain inhibitory macrophages, designated tumor-associated macrophages (TAMs) [66], which have functional relevance to tumor progression. For example, in a mouse model of breast cancer, genetic evidence suggests that the absence of macrophages does not change the incidence or growth of the primary tumor, but instead, decreases the rate of progression and metastases [67]. TAMs likely play important functions in human tumors as their number and density positively correlate with a poor prognosis [66].

Interestingly, placental and trophoblast proteins, exemplified by HLA-G, carry unusual poly-lactosaminylated carbohy-

drate structures, chains of galactose-*N*-acetylglucosamine disaccharides [68]. In this context, the observation that CD206⁺ macrophages in the decidua are the relevant inhibitory cell type is of particular interest. Specifically, CD206 recognizes *N*-acetylglucosamine glycoconjugates [45]. We speculate that these poly-lactosamine-containing oligosaccharide chains could be part of a system of signals that CTBs use to induce macrophage secretion of active TGF- β . In support of this theory, confocal microscopy showed frequent cell–cell contacts between CTBs and macrophages in the decidua. It is similarly possible that undifferentiated myeloid cells migrate to the decidua where they come into contact with invading CTBs, which alone or in combination with decidual and other resident cells, secrete factors that trigger their differentiation into alternatively activated macrophages that express CD206 and secrete TGF- β . In humans, it will be extremely difficult to distinguish between these two possibilities because the experiments require substantial amounts of endometrial tissue from the latter half of pregnancy.

Clinical entities that are associated with overactive CTB invasion further underscore the importance of the decidua in maintaining normal placental-uterine interactions. These conditions occur when the placenta attaches to the lower uterine segment where the endometrium thins (e.g., placenta accreta) or to extra-uterine sites (e.g., ectopic pregnancy). In these cases, CTB invasion, which escapes the usual bounds, may involve surrounding organs imperiling the life of the mother. Thus, the decidua acts as a containment device for the invasive cancer-like cells of the placenta. Our observations suggest that dNK cells could be part of the protective force exerted by the decidua.

In summary, we propose a model whereby CTBs, via secreted or surface molecules, trigger macrophage secretion of active TGF- β ₁, which in turn inhibits dNK cell killing of the placental cells that reside within the uterine wall. In this study, we did not investigate this paradigm in the context of decidual cells. However, due to their numbers and location within the uterus it is highly likely that they are an equally, if not a more important source of TGF- β ₁ with immunomodulatory functions. The results presented here further the current understanding of the role of dNK cells at the maternal–fetal interface and suggest new directions this work could take. For example, it would be interesting to reexamine the finding that dNK cells fail to polarize their granules toward the immunological synapse [15] in the context of our new data. Perhaps the presence of active TGF- β can explain this finding. Perturbations or disruptions of this system may be associated with a subset of fertility disorders and/or pregnancy complications. For example, inflammatory situations, such as preterm labor in the setting of infection, could overwhelm dMac inhibition. Whether or not alterations in these cells are causally related to abnormal placentation, such as the defects observed in preeclampsia, is another interesting question. Thus, the new insights presented here might be exploited for diagnostic or interventional purposes.

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