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# Mixed signature of activation and dysfunction allows human decidual CD8$^+$ T cells to provide both tolerance and immunity

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**Understanding how decidual CD8$^+$ T cell (CD8$^+$ dT) cytotoxicity is regulated and how these cells integrate the competing needs for maternal–fetal tolerance and immunity to infection is an important research and clinical goal. Gene-expression analysis of effector-memory CD8$^+$ dT demonstrated a mixed transcriptional signature of T cell dysfunction, activation, and effector function. High protein expression of coinhibitory molecules PD1, CTLA4, and LAG3, accompanied by low expression of cytolytic molecules suggests that the decidual microenvironment reduces CD8$^+$ dT effector responses to maintain tolerance to fetal antigens. However, CD8$^+$ dT degranulated, proliferated, and produced IFN-γ, TNF-α, perforin, and granzymes upon in vitro stimulation, demonstrating that CD8$^+$ dT are not permanently suppressed and retain the capacity to respond to proinflammatory events, such as infections. The balance between transient dysfunction of CD8$^+$ dT that are permissive of placental and fetal development, and reversal of this dysfunctional state, is crucial in understanding the etiology of pregnancy complications and prevention of congenital infections.**

pregnancy | T cell exhaustion | trophoblast | cytotoxicity | placenta

To establish a successful pregnancy, maternal decidual CD8$^+$ T cells (CD8$^+$ dT) at the maternal–fetal interface must integrate the antithetical demands of maternal–fetal tolerance and antiviral immunity (1). The key question is whether CD8$^+$ dT have the ability to elicit cytolytic responses to placental, fetal, or viral antigens or are rendered permanently dysfunctional and exhibit impaired effector functions. Among dysfunctional T cells are exhausted CD8$^+$ T cells that initially obtain effector functions and become dysfunctional during chronic exposure to antigen (2). Other dysfunctional cells include anergic T cells that fail to gain effector functions due to priming without costimulation and suppressed T cells that may be temporarily inhibited in their effector function after interaction with immune suppressive cells, such as regulatory T cells (Tregs) (2). T cell dysfunction is characterized by loss of IL-2, IFN-γ, and TNF-α production, diminished proliferative capacity, and low T cell cytotoxicity. A variety of markers have been implicated to identify dysfunctional T cells but expression of these coinhibitory molecules [e.g., programmed cell death-1 (PD1), T cell Ig mucin-3 (TIM3), and cytotoxic T-lymphocyte–associated protein 4 (CTLA4)] is not exclusive to dysfunctional T cells and is also observed in activated T cells (3–6). The significant overlap of gene-expression profiles and cell-surface markers between dysfunctional and activated T cells makes functional assessment (e.g., proliferation, cytokine secretion, cytotoxicity) necessary to separate these cell types.

T cell dysfunction was first described in chronic lymphocytic choriomeningitis virus (LCMV) infection in mice where LCMV-specific CD8$^+$ T cells were unable to control the infection (7, 8). However, infected mice retained antiviral CTL responses and applied selection pressure on the persisting virus (9). In both HIV and hepatitis-C virus infection, the emergence of viral escape mutants highlights the fact that T cell effector responses are retained regardless of the presence of phenotypically dysfunctional T cells (10, 11). Human cytomegalovirus (HCMV)-specific CD8$^+$ T cells have low proliferative capacity, low production of IL-2, and express PD1. Despite these signs of dysfunction, they are capable of producing ample amounts of IFN-γ and granzyme B (GZMB) when stimulated (12). In humans, T cell dysfunction has been demonstrated in a wide variety of cancers, including melanoma and colorectal cancer patients (6, 13) and during chronic infections (5). Furthermore, blockade of CTLA4 and PD1 pathways has been associated with improved effector T cell responses in these patients (14, 15). A recent study described gene signatures in dysfunctional tumor infiltrating T cells that can be uncoupled from activation signatures at the single-cell level. This study identified metallothionein-1 (MT1) and MT2 as specific markers for dysfunctional CD8$^+$ tumor infiltrating T cells (16). MTs are cysteine-rich zinc chaperones that are involved in zinc regulation and protection against oxidative stress (17). Deletion of MT1 resulted in increased T cell proliferation, loss of T cell dysfunction, and thus reduced tumor growth (16). These clinical and experimental observations provide evidence that immune surveillance

## Significance

Successful pregnancy requires establishment of immune tolerance for invading fetal trophoblasts, as well as immunity to a variety of pathogens that cause placental and congenital infections. Decidual CD8$^+$ T cells are key cells for recognition and response to foreign fetal, placental, and viral antigens at the maternal–fetal interface. Thus, regulation of decidual CD8$^+$ T cell activation and cytotoxicity is crucial for a healthy pregnancy. Here, we demonstrate that decidual CD8$^+$ T cells have a mixed profile of T cell dysfunction, activation, and effector function, which allows for both immune tolerance and immunity. This is of great relevance for understanding the development of pregnancy complications as well as prevention of congenital infections that occur as result of impaired placental immunity.

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Data deposition: Microarray data have been deposited in the ArrayExpress database, https://www.ebi.ac.uk/arrayexpress/ (accession no. E-MTAB-5890).

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remains active in chronic infections and cancer and that T cell dysfunction is incomplete.

Previous studies have shown that $CD8^+$ dT in first trimester pregnancy have an increased ability to produce cytokines, including IFN-γ (18, 19). Furthermore, a subset of first trimester $PD1^+TIM3^+$ $CD8^+$ dT showed increased proliferation potential and Th2 cytokine production (20). Despite this evidence suggesting increased activation, $CD8^+$ dT were also hypothesized to be dysfunctional/exhausted because of the high expression of the coinhibitory molecule PD1 and the ability of PDL1 to modify cytokine secretion (21), as well as the low expression levels of perforin (PRF) and GZMB in term pregnancy $CD8^+$ dT (22). Thus far, no comprehensive data has been presented on $CD8^+$ dT function throughout gestation and whether these cells are rendered dysfunctional or maintain the ability to generate proinflammatory responses. $CD8^+$ dT are exposed to allogeneic fetal minor and major histocompatibility antigens (mHag and MHC) expressed by fetal $HLA\text{-}G^+$ $HLA\text{-}C^+$ extravillous trophoblasts (EVT) throughout gestation (23, 24). $CD8^+$ dT make up 2–7% of leukocytes in first trimester decidua and their proportion increases to ∼30% at term pregnancy (25). $CD8^+$ dT are differentiated effector-memory (EM) cells that express reduced levels of PRF and GZMB (22). In mice, maternal $CD8^+$ T cells responded to viral and bacterial antigens, but were unable to completely clear the pathogens during pregnancy (26, 27). Activation and expansion of fetus-specific $CD4^+$ and $CD8^+$ T cells by seminal fluid in mice resulted in high levels of $CD4^+CD25^+$ (Treg) and activation of fetus-specific $CD8^+$ T cells did not have an influence on pregnancy outcome (28).

In humans, antibody and CTL responses to MHC and mHag (e.g., HY) were detected in maternal blood during and as a result of pregnancy (29, 30). While induction of HLA-A– and HLA-B–specific antibodies did not negatively impact pregnancy outcome, the presence of HLA-C–specific antibodies in women with recurrent miscarriage suggested that antibody-mediated rejection may be involved in the origin of unexplained recurrent miscarriages (31). HCMV seropositivity profoundly influenced the T cell repertoire during pregnancy and led to the accumulation of highly differentiated memory T cells (32). HLA-A– and HLA-B–restricted virus-specific $CD8^+$ T cells, as well as $CD8^+$ T cells specific for the HY antigen, are present in human decidual tissue (1, 33). Thus, both murine and human studies clearly demonstrate that maternal $CD8^+$ T cells respond to viral, fetal, and placental antigens during pregnancy. However, regulation of $CD8^+$ dT effector function prevents detrimental cytolytic responses to invading fetal EVT and maintains maternal–fetal immune tolerance. In this study, $CD8^+$ T cell function was investigated by transcriptome analysis of $CD8^+$ dT from human first trimester and term pregnancy. Moreover, gene-expression profiles were combined with phenotypic characterization and assessment of $CD8^+$ dT effector functions.

## Results

**$CD8^+$ EM dT Have a Mixed Gene-Expression Profile.** A significantly increased percentage of $CCR7^-CD45RA^-$ EM $CD8^+$ T cells and a decrease in $CCR7^+CD45RA^+$ naïve $CD8^+$ T cells was observed in first trimester (6–12 wk) and term (>37 wk) pregnancy decidual tissue when compared with peripheral blood $CD8^+$ T cells ($CD8^+$ pT), confirming previous studies (Fig. S1 *A–D*) (22, 34). Within the EM subsets, $T_{EM1}$ cells, defined as $CD28^+CD27^+$ EM cells, were significantly increased in the first trimester compared with term pregnancy decidua (Fig. S1*E*). Furthermore, a small but not significant increase in $CD28^-CD27^+$ $T_{EM2}$ and $CD28^-CD27^-$ $T_{EM3}$ cells was detected in term compared with first trimester pregnancy decidua. Analysis of the cytolytic molecule PRF also showed reduced expression in first trimester decidual $CD8^+$ effector (Eff) and $T_{EM3}$ cells compared with the same populations in blood, as has previously been described for term $CD8^+$ dT (Fig. S1*F*) (22). Gene-expression profiles were generated from RNA purified from $CD8^+CCR7^-CD45RA^-$ EM T cells in blood ($CD8^+$ EM pT) and decidua ($CD8^+$ EM dT; 6–12 wk and >37 wk). Unsupervised principle component analysis (PCA) separated $CD8^+$ EM dT from $CD8^+$ EM pT along the first principal component (35.9% of variance). PC2 separated first trimester from term pregnancy $CD8^+$ EM dT (18.0% of variance) (Fig. 1*A*). A transcriptional signature that uniquely defined $CD8^+$ EM dT and EM pT was identified (Fig. S2*A* and Dataset S1). Genes up-regulated in $CD8^+$ EM dT compared with EM pT included genes involved in chemotaxis (CCL3, CCL4, IL-8, XCL1), T cell activation (IFN-γ, TNF, FOS, ICOS, NFKB1), and coinhibitory receptors (FASLG, CTLA4, LAG3, TIGIT, CRTAM, and TIM3). An increase in mRNA for granzymes, but not the other cytolytic molecules PRF and granulysin, was observed in term $CD8^+$ EM dT (Fig. S3*A*).

**$CD8^+$ EM dT Have a Mixed Profile of Dysfunction, Activation, and Effector Function.** To further identify transcriptional differences between first trimester $CD8^+$ EM dT and EM pT, gene-set enrichment analysis (GSEA) was performed by comparing the $CD8^+$ EM dT gene set to existing immunological gene sets in the ImmSigDB database (35, 36). GSEA demonstrated a significant enrichment of effector vs. naïve, effector vs. memory, exhausted vs. naïve, and exhausted vs. effector gene sets in $CD8^+$ EM dT relative to $CD8^+$ EM pT (Fig. 1*B*). GSEA also revealed that expression of genes associated with both dysfunction and activation states of $CD8^+$ T cells are increased in first trimester $CD8^+$ EM dT compared with $CD8^+$ EM pT. For this analysis, our data were compared with a recently published gene signature where dysfunction and activation gene modules were uncoupled (Fig. 1*C*) (16). Flow cytometric analysis detecting protein expression of selected activation markers (CD69, HLA-DR, GITR, and CD25) and coinhibitory molecules (PD1, CTLA4, LAG3, and TIGIT) confirmed that expression of markers associated with both activation and dysfunction are increased on CD8+ dT compared with pT (Fig. S3 *C–J*).

**Term $CD8^+$ EM dT Express Methallothioneins, a Signature for Dysfunctional T Cells.** No significantly different gene sets were identified when gene-expression profiles of first trimester and term $CD8^+$ EM dT were compared. However, a striking enrichment of MT1 and MT2 genes, which have recently been associated with dysfunctional T cells, was observed in term $CD8^+$ EM dT (Fig. S2*B* and Dataset S2) (16). The presence of MT genes in term $CD8^+$ EM dT and not in first trimester suggests that antigenic stimulation throughout the 9 months of pregnancy may gradually increase $CD8^+$ EM dT dysfunction. Other differences between first trimester and term $CD8^+$ EM dT included increased expression of galectin-8 (LGALS8) and galectin-9 (LGALS9) in term $CD8^+$ EM dT. Galectins have a broad variety of functions including mediation of cell–cell interactions, apoptosis, and facilitating the differentiation of regulatory T cells (37). Thus, toward the end of pregnancy $CD8^+$ EM dT have also acquired gene signatures associated with immune suppression.

**$CD8^+$ EM dT Can Acquire Signatures of T Cell Activation.** To investigate if and how $CD8^+$ EM dT respond to T cell receptor stimulation, gene-expression profiles were generated from first trimester $CD8^+$ EM dT stimulated with anti-CD3/28 for 0, 12, and 72 h. Approximately 2,000 immunologically relevant genes were preselected based on the Immune System Process Gene Ontology (GO) terms (36). A MaSigPro time-course identified 470 genes that changed significantly over time (Fig. 2*A* and Dataset S3). K-cluster analysis divided these temporally sensitive genes into five clusters (Fig. 2). While gene clusters 1 and 2 identified genes rapidly decreasing upon stimulation (ICOS, FOS, CXCL16, CD28, PD1, TGF-β), cluster 3 identified genes with a slower decline in expression. Cluster 3 included genes involved in T cell activation (IL-7R) and signaling (IL-6ST, CXCL3), as well as IL-11 that is known to play a function in placentation and to some extent decidualization (38). Subsequently, genes regulating T cell receptor signaling (IL-2RA), cell cycle (CDK6), T cell differentiation and activation (BATF), IFN-β expression (PRDMI), and antiviral activity (PRDX1) were induced within 12 h after stimulation (Fig. 2*B*, cluster 4). Stimulation of 72 h resulted in up-regulation of genes involved in activation and maturation (GZMB, IL-9, RUNX1), regulation of T cell activation and antiinflammatory activity (IL-13, IL-10RB, TGFBR1), and inhibition (LAG3) (Fig. 2*B*, cluster 5). DROSHA,

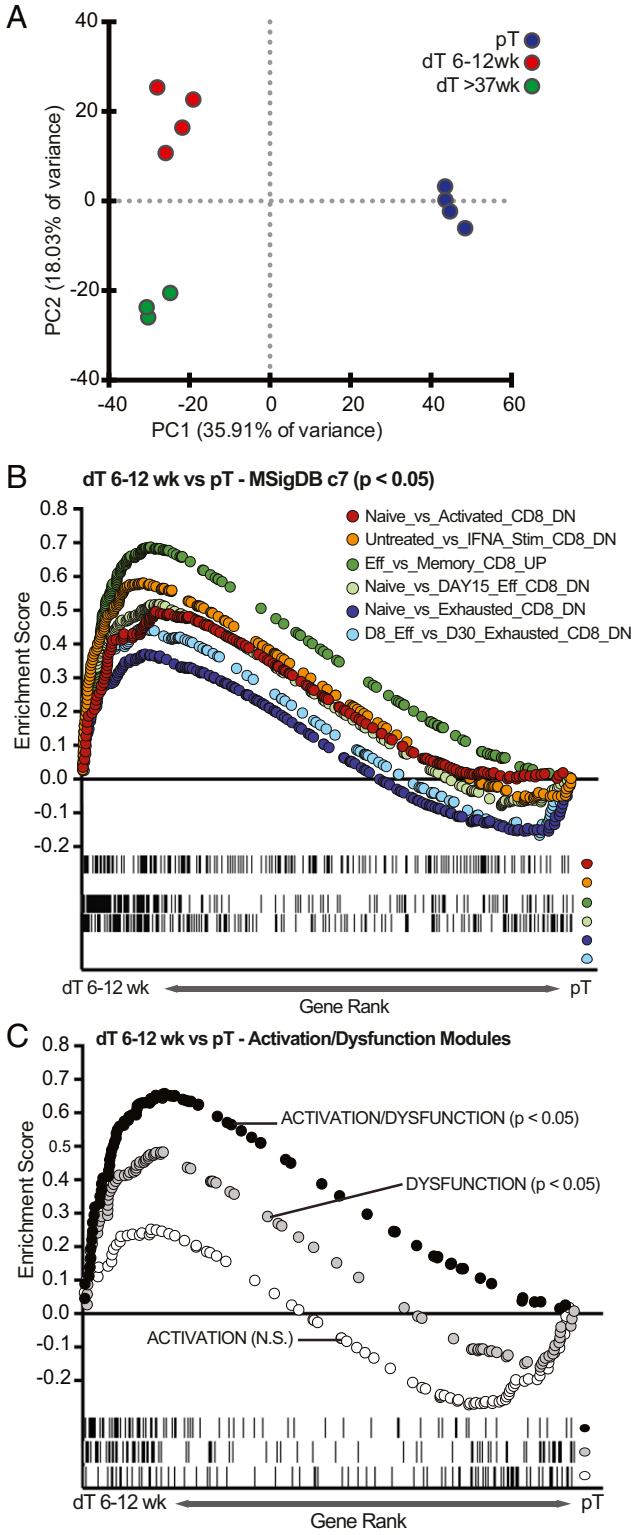


**Fig. 1.** Transcriptional signatures of CD8$^+$ EM dT dysfunction, activation, and effector function are coupled. (*A*) Unsupervised PCA analysis, using all 54,715 probes, of first trimester (dT 6–12 wk) and term pregnancy (dT > 37 wk) CD8$^+$ EM dT and EM pT. (*B*) GSEA performed with assorted immunological signatures, showing positive correlation for an in vitro CD8$^+$ activation signature, an effector signature, and a CD8$^+$ viral exhaustion signature (MSigDB). (*C*) GSEA comparing first trimester CD8$^+$ EM dT to gene modules of dysfunction, activation, and activation coupled to dysfunction (16).

a player in microRNA biogenesis, was induced after 72 h of stimulation. The proinflammatory cytokines IFN-γ and TNF revealed higher expression levels within 12 h after stimulation, whereas expression of the cytolytic molecules PRF, GZMA, GZMB, GZMH, and GNLY were induced within 72 h of stimulation (Fig. S3*B*). Taken together, stimulation of first trimester CD8$^+$ EM dT resulted in up-regulation of genes involved in T cell activation and cytotoxicity.

**CD8+ dT Are Functional Upon Activation.** T cell dysfunction is associated with a limited capacity to degranulate, proliferate, and secrete cytokines upon stimulation (5, 11). Although, previous studies have shown increased proliferation and cytokine production of first trimester CD8$^+$ dT (18–20), no comprehensive study has compared functional aspects of CD8$^+$ dT throughout gestation from first trimester to term. To determine whether activation of CD8$^+$ dT elicits these effector responses, total CD8$^+$ dT and pT were stimulated in vitro. A significantly increased percentage of first trimester CD8$^+$ dT degranulated upon PMA/Ionomycin stimulation compared with CD8$^+$ pT (Fig. 3*A*). Degranulation was predominantly observed in CD8$^+$ $dT_{EFF}$ (CD45RA$^+$CCR7$^-$) and $dT_{EM}$ (CCR7$^-$CD45RA$^-$). Term CD8$^+$ dT degranulated significantly less than first trimester CD8$^+$ dT, but at a level similar to CD8$^+$ pT (Fig. 3*A*).

Carboxyfluorescein diacetate succinimidyl ester (CFSE) -labeled CD8$^+$ pT and dT were stimulated with anti-CD3/28 and analyzed at days 3, 4, 5, and 6 for their capacity to proliferate. At day 3, significantly fewer CD8$^+$ dT had proliferated compared with CD8$^+$ pT. However, by days 5 and 6 virtually all first trimester CD8$^+$ dT and pT had lost CFSE expression (Fig. 3*B*). While the proliferation index for term CD8$^+$ dT was similar to CD8$^+$ pT on day 6 (Fig. S4*A*), there was a significantly lower percentage of term CD8$^+$ dT that had divided compared with CD8$^+$ pT (median of 65% vs. 89%) (Fig. 3*B*). No significant differences between the proliferation index of CD8$^+$ pT and dT were observed (Fig. S4*A*). These data demonstrated that CD8$^+$ dT required more time to initiate proliferation, yet once they started dividing, first trimester CD8$^+$ dT proliferated at a similar rate to CD8$^+$ pT.

The percentage of CD8$^+$ dT expressing IFN-γ, TNF-α, and IL-2 upon stimulation with PMA/Ionomycin was comparable to CD8$^+$ pT (Fig. 3*C*). All effector and EM CD8$^+$ pT and dT produced IFN-γ and TNF-α, whereas the production of these cytokines was in effect absent in naïve CD8$^+$ T cells. Among the EM subsets, EM-1 CD8$^+$ T cells were the main producers of IFN-γ and TNF-α (Fig. S4 *B* and *C*). No production of IL-10 and IL-17a was observed in CD8$^+$ pT or dT.

Next, the expression of PRF and GZMB in activated CD8$^+$ dT was analyzed to determine whether the reduced levels of PRF in CD8$^+$ dT is reversible or permanently suppressed. Activation with IL-12 or anti-CD3/28 was sufficient to increase PRF expression in first trimester and term pregnancy CD8$^+$CD28$^-$ and CD8$^+$CD28$^+$ dT by approximately twofold (Fig. 4*A* and Fig. S5). However, activation of CD8$^+$ dT did not increase the PRF content to levels observed in CD8$^+$ pT. Moreover, treatment of CD8$^+$ dT with either anti-CD3/28 or the combination of IL-12 and anti-CD3/28 increased GZMB in CD8$^+$ dT to levels comparable or higher than CD8$^+$ pT (Fig. 4*B* and Fig. S6). Thus, the majority of CD8$^+$ dT in both first trimester and term pregnancy degranulate, proliferate, secrete proinflammatory cytokines, and increase cytolytic molecules upon activation and do not reside in a permanently dysfunctional state.

**PRF, but Not GZMB, Is Suppressed in HCMV-Specific CD8$^+$ dT.** Next, the question whether virus-specific CD8$^+$ dT have suppressed expression of cytolytic molecules that could impair or delay their ability to respond to infections in the placenta was addressed. HCMV is the most common congenital infection and HCMV-specific CD8$^+$ dT are present at the maternal–fetal interface (33). First trimester CD8$^+$CD28$^-$ dT and pT were stained with HCMV-specific tetramers and analyzed for expression of PRF and GZMB (Fig. 5 and Fig. S7*A*). PRF expression was about twofold lower in HCMV-specific CD8$^+$ dT compared with HCMV-specific CD8$^+$ pT (Fig. 5 *A* and *B*). In contrast, HCMV-specific CD8$^+$ dT had

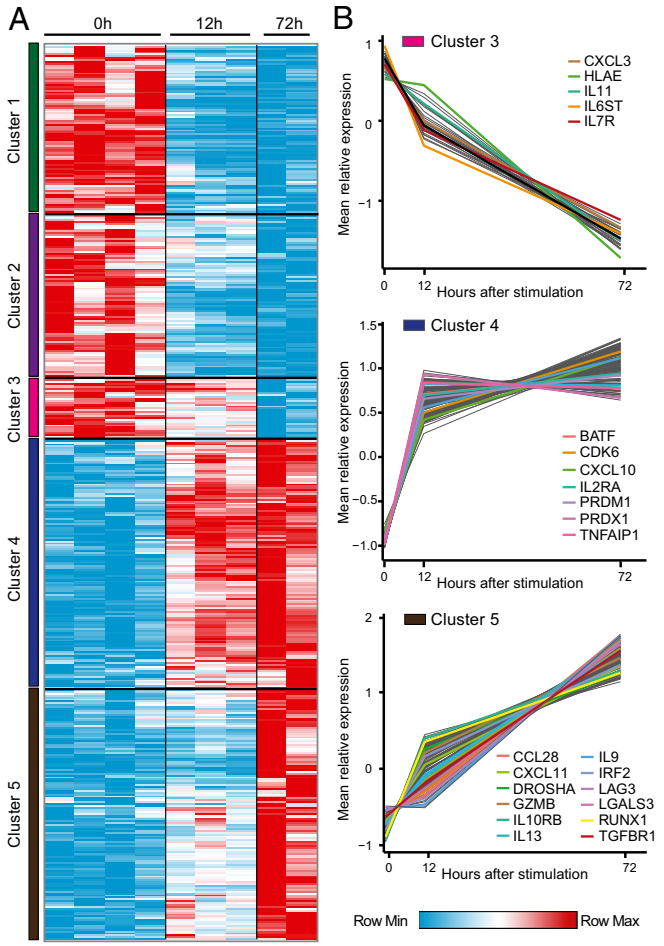


**Fig. 2.** Stimulation of CD8$^+$ EM dT generates signatures of T cell activation. (*A*) A MaSigPro time-course analysis of ∼2,000 immunological relevant genes preselected based on the Immune System Process GO terms expressed by first trimester CD8$^+$ EM dT stimulated with anti-CD3/CD28 in the presence of 50 U/mL IL-2 for 0, 12, or 72 h. Five clusters of temporally sensitive genes were identified. (*B*) K-Cluster analysis highlighting select genes in clusters 3–5.

comparable GZMB levels to CD8$^+$ pT (Fig. 5 *C* and *D*). A ratio of PRF and GZMB expression of each individual donor revealed a significant difference between HCMV-specific CD8$^+$ dT and pT (Fig. S7*B*). HCMV-specific CD8$^+$ dT and pT were expanded and PRF levels in the expanded CD8$^+$ dT did not increase, while PRF significantly decreased in CD8$^+$ pT after expansion (Fig. 5*B* and Fig. S7*C*). Expansion of HCMV-specific cells significantly increased GZMB content in both CD8$^+$ dT and pT, although GZMB levels in CD8$^+$ dT did not reach the same levels as in CD8$^+$ pT (Fig. 5*D*). Thus, suppression of cytolytic proteins in virus-specific CD8$^+$ dT can be partially overcome by T cell activation as may occur during a viral infection in placental tissues.

**CD8$^+$ dT Do Not Degranulate in Response to EVT.** The antigen-specificity of CD8$^+$ dT and their ability to recognize and respond to fetal antigens expressed by EVT is a key question that is yet to be answered. The potential of CD8$^+$ T cells to degranulate in response to EVT was determined by culturing CD8$^+$ T cells alone, or in the presence of EVT or anti-CD3/28 beads for 12 h. Co-culture of EVT with CD8$^+$ dT from the same pregnancy sample (dT sample-matched), a different pregnancy sample (dT nonmatched), or from unrelated blood donors (pT nonmatched) did not induce degranulation by any of the CD8$^+$ T cells (Fig. S8). Addition of anti-CD3/28 increased degranulation by all CD8$^+$ T cells, demonstrating T cell viability. Thus, similar to EVT and decidual NK cell (dNK) cocultures (39), EVT do not directly elicit degranulation by CD8$^+$ T cells.

## Discussion

Numerous gaps remain in our understanding of how pregnancy affects adaptive immunity and, in particular, how CD8$^+$ dT integrate protective immunity against pathogens with immune tolerance to invading EVT. In this study, expression analysis of decidual CD8$^+$ EM T cells demonstrated a mixed transcriptional signature of T cell dysfunction, activation, and effector function. The enriched gene signature for T cell dysfunction in CD8$^+$ dT together with the increased expression of coinhibitory molecules PD1, CTLA4, and LAG3, and low expression of the cytolytic molecule PRF suggests that the decidual microenvironment reduces CD8$^+$ dT effector responses, possibly to maintain immune tolerance to fetal antigens. However, the ability of CD8$^+$ dT to up-regulate PRF and GZMB expression, degranulate, proliferate, and secrete proinflammatory cytokines upon activation suggests that CD8$^+$ dT are not permanently suppressed and retain the capacity to respond to proinflammatory events, such as infections. This also confirms the uncoupling of coinhibitory receptors from the dysfunctional T cell phenotype (16).

RNA sequencing on single cells or small population level of CD8$^+$ dT combined with functional assessment of the CD8$^+$ T cell subpopulations will determine whether gene modules for activation and dysfunction are intertwined or uncoupled. It is of high clinical relevance to determine whether there are CD8$^+$ EM dT populations responsible for maternal immune tolerance that

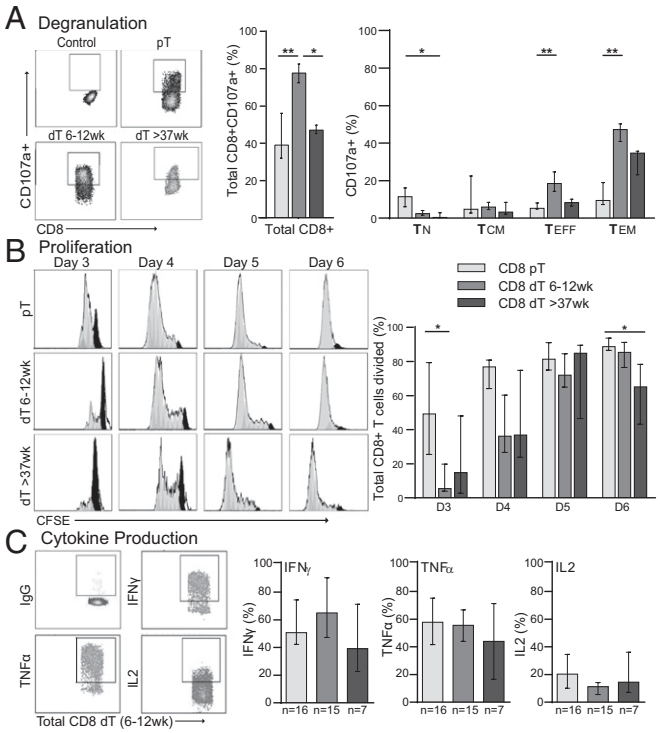


**Fig. 3.** CD8$^+$ dT are functional upon activation. (*A*) FACS plots (*Left*) and percentages (*Right*) of CD107a$^+$CD8$^+$ pT and dT after stimulation with PMA/Ionomycin (1 μg/mL) for 6 h. Percentage CD107a$^+$ cells are depicted within total CD8$^+$ T cells and the four CD8$^+$ T cell subpopulations (as percentage of total CD8). (*B*) Representative histograms (*Left*) and percentage divided cells (*Right*) of CFSE-labeled total CD8$^+$ T cells at days 3, 4, 5, and 6 after stimulation with anti-CD3/28 (2 μL/mL) and 50 U/mL IL-2. (*C*) FACS plots (*Left*) and percentages (*Right*) of expression of intracellular IFN-γ, TNF-α, and IL-2 in total CD8$^+$ pT and dT stimulated with PMA/Ionomycin (1 μg/mL) for 6 h. Bars represent the median with interquartile range; data are representative of three to six independent experiments; $*P \leq 0.05$, $**P \leq 0.01$.

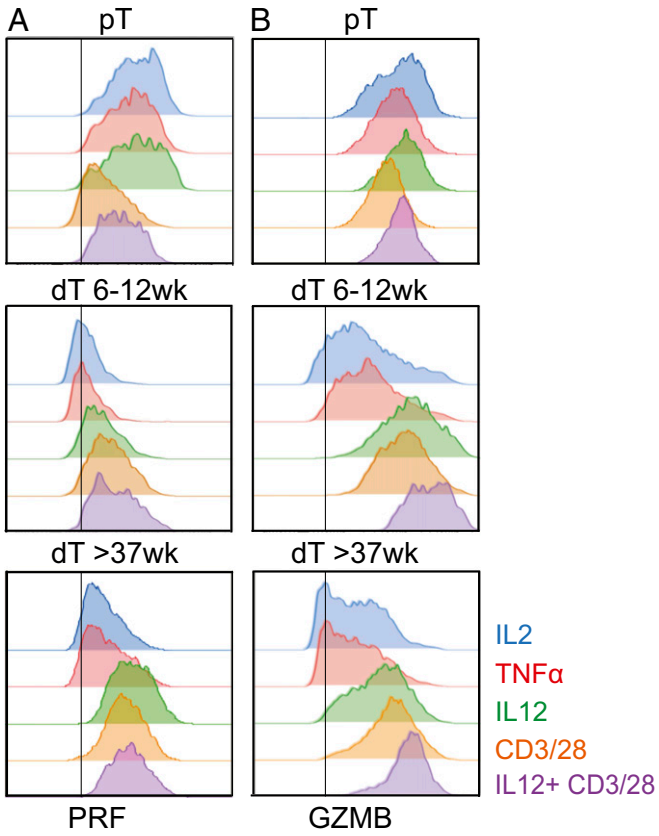


**Fig. 4.** Decidual $CD8^+CD28^-$ T cells increase PRF and GZMB upon activation. Histograms of intracellular PRF (*A*) and GZMB (*B*) expression in $CD8^+CD28^-$ pT and dT cultured for 6 d in the presence of IL-2 (50 U/mL), TNF-α (20 ng), IL-12 (20 ng), anti-CD3/28 (2 μL/mL), or a combination of anti-CD3/28 and IL-12. The vertical line signifies the PRF and GZMB mean fluorescence intensity (MFI) after addition of a nonactivating concentration of IL-2.

are distinct from a subset of activated $CD8^+$ EM dT with effector functions ready to confront incoming pathogens. A major challenge here is to connect gene-expression analysis at the single-cell level with accurate functional assessment of T cell function. This is particularly challenging for human T cell subtypes that proliferate in vivo but not in vitro. Further identification and validation of markers that can separate dysfunctional T cells from activated T cells such as the recently proposed MT1 and MT2 genes is required (16). Consistent with prolonged antigen stimulation of $CD8^+$ dT at term pregnancy, enrichment of MT1 and MT2 genes was observed in term $CD8^+$ EM dT compared with first trimester $CD8^+$ EM dT. However, phenotypic and functional assessment of first trimester and term $CD8^+$ EM dT revealed only minor differences. The reduced capacity of $CD8^+$ dT to initiate proliferation and the failure of term $CD8^+$ dT to fully degranulate and proliferate at day 6 suggests that a subset of $CD8^+$ dT gradually becomes dysfunctional through exhaustion or suppression over the course of pregnancy, while the majority retains the capacity to respond. A recent study in mice observed that the priming of maternal naïve T cells by fetal antigens resulted in the differentiation of long-lived $PD1^+$ $CD8^+$ T cells with selective silencing of effector function in a subsequent pregnancy. This dysfunction was reversed during skin transplantation (40). In the same manner, the integration of the competing needs for maternal immune tolerance in concert with immunity to placental infections may be achieved by the proficiency of the decidual microenvironment to dampen the activation state of $CD8^+$ dT while not exclusively inducing their dysfunctional state.

dNK biology and interactions with MHC molecules expressed by EVT has been a major focus of research for over two decades. Despite their abundance of cytolytic granules, dNK degranulate significantly less than blood NK cells in response to classic target cells (41, 42). However, when activated with a low dose of IL-15, dNK degranulate and produce proinflammatory cytokines to HCMV-infected decidual stromal cells (41, 43), yet are unable to respond to HCMV-infected EVT (39). The inability of dNK to kill infected EVT may make placental tissue more dependent on $CD8^+$ dT responses to clear infections. Activation of $CD8^+$ dT increased both PRF and GZMB mRNA and protein expression. The high levels of PRF mRNA in the absence of PRF protein in term $CD8^+$ dT (22) and the induction of DROSHA, as presented here, suggests that posttranscriptional regulation mediated by miRNAs may allow for a rapid increase in cytolytic proteins and cytolytic capacity upon stimulation (44). Thus, although dNK and $CD8^+$ dT both require additional activation by cytokines or receptor-ligand interactions to display their full cytotoxicity, the mechanisms that inhibit or delay their cytotoxic response are inherently different.

HLA-A– and HLA-B–restricted virus-specific $CD8^+$ T cells are enriched in decidual tissue at term pregnancy (33). Here, we demonstrated that HCMV-specific $CD8^+$ dT in first trimester pregnancy have reduced levels of PRF protein compared with HCMV-specific $CD8^+$ pT, whereas their GZMB protein levels are comparable. After expansion of HCMV-specific $CD8^+$ dT, PRF levels remained the same and GZMB levels significantly increased, although levels remained lower than in $CD8^+$ pT. HCMV-specific $CD8^+$ dT and pT are capable of cytotoxicity regardless of low PRF levels (33). Our data imply a general suppression of PRF translation in all $CD8^+$ dT, including virus-specific $CD8^+$ dT. However, this suppression can be overcome by T cell activation as might be the case during viral infections of the placenta. HLA-A and HLA-B are not expressed by EVT, and further investigation is needed to determine whether HLA-C–restricted pathogen-specific responses by $CD8^+$ dT can provide immunity when EVT are infected. Investigation into whether HLA-C–restricted T cells with specificity for placental or fetal antigens cause detrimental immune responses and contribute to the development of pregnancy complications is required. Here, we demonstrated that $CD8^+$ dT do not degranulate during coculture with healthy EVT. Additional research should determine whether the failure of $CD8^+$ dT to respond to EVT is due to their dysfunction or to a lack of antigen specificity to EVT antigens. Furthermore, investigation into how the interactions of EVT and other immune modulatory placental cells (e.g., decidual

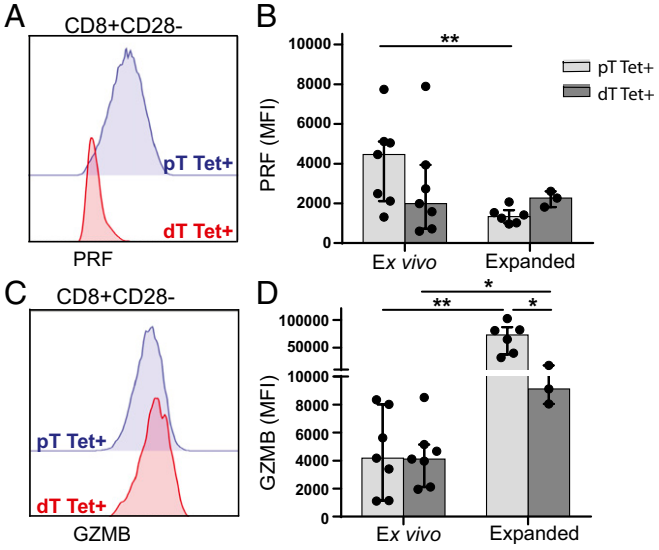


**Fig. 5.** PRF, but not GZMB, is suppressed in HCMV-specific $CD8^+CD28^-$ dT. Histograms and MFI of PRF (*A* and *B*) and GZMB (*C* and *D*) expression in tetramer-positive $CD8^+CD28^-$ pT and dT, directly ex vivo and after in vitro expansion. Bars depict the median with interquartile range; $*P \leq 0.05$, $**P \leq 0.01$.

Treg and decidual macrophages) with $CD8^+$ dT changes $CD8^+$ dT function is needed to determine if $CD8^+$ dT are rendered dysfunctional through exhaustion or suppression and what molecular pathways are involved. Another key question is to determine if immune tolerance by $CD8^+$ dT in response to EVT is maintained during placental infections as was recently shown for dNK (39).

Coordinated interaction between many different cell types present at the maternal–fetal interface establishes immune tolerance and allows allogeneic fetal trophoblasts to invade maternal tissues. How infections or other inflammatory responses destabilize the tolerogenic placental environment, increase dNK and $CD8^+$ dT cytotoxicity, and contribute to placental pathology is central to understanding the development of pregnancy complications, such as miscarriages and preterm birth.

## Materials and Methods

Decidua parietalis was obtained from healthy women after uncomplicated pregnancy at term (gestational age > 37 wk) delivered by elective cesarean section or uncomplicated spontaneous vaginal delivery at Tufts Medical Center. Discarded first trimester human placental and decidual materials (gestational age 6–12 wk) were obtained from women undergoing elective pregnancy termination at a local reproductive health clinic. Peripheral blood leukocytes were isolated from leuko packs from unrelated healthy blood donors at Massachusetts General Hospital in Boston. All human tissue used for this research was de-identified, discarded clinical material. The committee on the use of human subjects (Harvard Institutional Review Board) determined that this use of placental and decidual material is not human subject's research. Term placental tissue was collected under a protocol approved by Tufts Health Sciences Institutional Review Board. Decidual lymphocytes and peripheral blood leukocytes were isolated as previously described (19). In short, first trimester, villous, and decidual tissues were macroscopically identified and separated. Decidua parietalis from term pregnancy was collected by removing the amnion and delicately scraping the decidua parietalis from the chorion. Collected decidual tissues were washed, minced, and digested with 0.1% collagenase type IV and 0.01% DNase I (Sigma-Aldrich) for 60–75 min at 37 °C. After digestion, cells were washed and filtered through 100-, 70-, and 40-μm sieves (BD, Labware). Lymphocytes were dissolved in 20 mL 1.023 g/mL Percoll (GE Healthcare) and layered on a Percoll gradient (10 mL 1.080 g/mL; 15 mL 1.053 g/mL) for density gradient centrifugation (30 min, 800 × *g*). Lymphocytes were isolated from the 1.080–1.053 g/mL interface, washed twice, and directly stained for sorting on a BD FACS ARIA-II (Fig. 1). Peripheral $CD8^+$ T cells were isolated using RosetteSep (StemCell Technologies) followed by Ficoll (GE Healthcare) gradient centrifugation (20 min, 800 × *g*). Additional details on methods for RNA preparation and microarray hybridization; computational analysis; isolation of EVT; flow cytometry; cell culture; proliferation, degranulation, and cytokine assays; generation of HCMV-specific $CD8^+$ T cell lines and clones; and statistical analyses used are described in *SI Materials and Methods*.

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