Human Decidual Tissue Contains Differentiated CD8\(^+\) Effector-Memory T Cells with Unique Properties

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Human Decidual Tissue Contains Differentiated CD8⁺ Effector-Memory T Cells with Unique Properties

Tamara Tilburgs,⁎† Dorrith Schonkeren,‡ Michael Eikmans, † Nicole M. Nagtzaam, †
Gert Datema, † Godelieve M. Swings,‡ Frans Prins, † Jan M. van Lith, † Barbara J. van der Mast, †
Dave L. Roelen, † Sico A. Scherjon, † and Frans H. Claas †

During pregnancy, maternal lymphocytes at the fetal–maternal interface play a key role in the immune acceptance of the allogeneic fetus. Recently, CD4⁺CD25bright regulatory T cells have been shown to be concentrated in decidual tissue, where they are able to suppress fetus-specific and nonspecific immune responses. Decidual CD8⁺ T cells are the main candidates to recognize and respond to fetal HLA-C at the fetal–maternal interface, but data on the characteristics of these cells are limited. In this study we examined the decidual and peripheral CD8⁺ T cell pool for CD45RA, CCR7, CD28, and CD27 expression, using nine-color flow cytometry. Our data demonstrate that decidual CD8⁺ T cells mainly consist of differentiated CD45RA⁻CCR7⁺ effector-memory (EM) cells, whereas unprimed CD45RA⁺CCR7⁻ naive cells are almost absent. Compared with peripheral blood EM CD8⁺ T cells, the decidual EM CD8⁺ T cells display a significantly reduced expression of perforin and granzyme B, which was confirmed by immunohistochemistry of decidual tissue sections. Interestingly, quantitative PCR analysis demonstrates an increased perforin and granzyme B mRNA content in decidual EM CD8⁺ T cells in comparison with peripheral blood EM CD8⁺ T cells. The presence of high levels of perforin and granzyme B mRNA in decidual EM T cells suggests that decidual CD8⁺ T cells pursue alternative means of EM cell differentiation that may include a blockade of perforin and granzyme B mRNA translation into functional perforin and granzyme B proteins. Regulation of deciduial CD8⁺ T cell differentiation may play a crucial role in maternal immune tolerance to the allogeneic fetus. The Journal of Immunology, 2010, 185: 4470–4477.

Maternal lymphocytes at the fetal–maternal interface play a key role in the immune acceptance of the allogeneic fetus. Many studies have shown that decidual NK cells contain immune modulatory properties and facilitate trophoblast invasion into maternal tissue (1, 2). More recently, CD4⁺CD25bright regulatory T cells have been shown to be concentrated in human decidual tissue and are able to suppress fetus-specific and nonspecific responses (3). However, thus far, the mechanisms of fetus-specific immune recognition and the possible effector cell functions of decidual T cells remain poorly defined. Previous studies have shown that the maternal immune system is capable of generating Abs and CTLs specific to fetal HLA and fetal minor histocompatibility Ags in maternal peripheral blood during pregnancy (4–6). Recent data by our group indicate that a fetal–maternal HLA-C mismatch correlates with an increased deciduial CD4⁺ T cell activation and appearance of functional CD4⁺CD25bright regulatory T cells (7). However, deciduial CD8⁺ T cells form the largest fraction of T cells at the fetal–maternal interface and are the main candidates to recognize and respond to fetal HLA-C at the fetal–maternal interface (8). Thus far, data on the phenotype and function of decidual CD8⁺ T cells during human pregnancy are limited.

In healthy individuals and during viral infections, the CD8 effector T cell differentiation process has been studied extensively (9–11). These studies have identified particular CD8⁺ T cell subsets capable of eliciting a cytotoxic response and have identified many phenotypic markers to categorize these cells. In this study we use the cell surface markers CD45RA and CCR7 to discriminate naive (RA⁺CCR7⁺), effector (RA⁺CCR7⁻), effector-memory (EM) (RA⁻CCR7⁺), and central-memory (CM) (RA⁻CCR7⁻) CD8⁺ T cells (12–15) in decidual tissue. In addition, we analyzed expression of the costimulatory molecules CD28 and CD27 to determine additional heterogeneity in CD8⁺ EM cells: EM1 (28⁺27⁺), EM2 (28⁺27⁻), EM3 (28⁻27⁺), and EM4 (28⁻27⁻) (14). To examine the cytolytic capability of deciduial and peripheral blood CD8⁺ T cell subsets, expression of perforin and granzyme B was analyzed using flow cytometry, immunohistochemistry, and quantitative PCR (qPCR). Perforin is a membrane-perturbing protein that delivers granzymes into the target cell (16). Among all known granzymes, granzyme B is essential for inducing DNA fragmentation and apoptosis in target cells (16), whereas granzyme A and granzyme K may provide alternative mechanisms to kill target cells (17, 18). We examined the CD8⁺ T cell pool at two different sites of the fetal–maternal interface: the decidua basalis, the maternal part of the placenta at the implantation site, and the decidua parietalis, the maternal part of the membranes connected to the fetal trophoblasts of the chorion. We compared the CD8⁺ T cell subset distribution in deciduial tissue to the maternal peripheral blood (mPBLs). As a control, we analyzed peripheral blood CD8⁺ T cells of age-matched healthy volunteer donors.

⁎Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138; and 1Department of Immunohematology and Blood Transfusion, †Department of Obstetrics, and 2Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands

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Address correspondence and reprint requests to Dr. Tamara Tilburgs, Department of Molecular and Cellular Biology, Harvard University, 7 Divinity Avenue, Cambridge MA, 02138. E-mail address: Tilburgs@FAS.Harvard.edu

The online version of this article contains supplemental material.

Abbreviations used in this paper: CM, central-memory; cPBL, control PBL; DAB, diaminobenzidine; E, effector; EM, effector-memory; MFI, mean fluorescence intensity; miRNA, microRNA; mPBL, maternal PBL; N, naive; pE1, pre-effector-1; pE2, pre-effector 2; qPCR, quantitative PCR.
Materials and Methods

Blood and tissue samples

Paired samples of decidua basalis, decidua parietalis, and heparinized mPBLs were obtained from healthy women after uncomplicated term pregnancy (gestational age range: 37–42 wk). Tissue samples were obtained after delivery by elective cesarean section or uncomplicated spontaneous vaginal delivery. mPBL samples were taken either directly before or directly after delivery. Control PBL (ePBL) samples were taken from healthy nonpregnant volunteer female donors. Signed informed consent was obtained from all women, and the study received approval by the Leiden University Medical Center Ethics Committee (P02-200).

Lymphocyte isolation

Lymphocyte isolation from decidua was done as described previously (19). In brief, decidua basalis was macroscopically dissected from the maternal side of the placenta. Decidua parietalis was collected by removing the amnion and delicately scraping the decidua parietalis from the chorion. The obtained tissue was washed thoroughly with PBS and thereafter finely minced between two scalpel blades in PBS. Decidual fragments were incubated with 0.2% collagenase I (Life Technologies-BRL, Grand Island, NY) and 0.02% DNase I (Life Technologies) in RPMI 1640 medium, gently shaken in a water bath at 37 °C for 60 min, and thereafter washed once with RPMI 1640. The resultant suspensions were filtered through a 70-μm sieve (BD Biosciences, Franklin Lakes, NJ) and washed once in RPMI 1640. The decidual isolates were layered on a Percoll gradient (of 7.5 ml 1,080 g/ml; 12.5 ml 1,053 g/ml; 20 ml 1,023 g/ml) for density gradient centrifugation (30 min/800 g). Lymphocytes were isolated from the 1,080 g/ml–1,053 g/ml interface. PBL samples were directly layered on a Ficoll Hypaque gradient (Leiden University Medical Center Pharmacy, Leiden, The Netherlands) for density gradient centrifugation (20 min/800 g). Mononuclear cells were collected, washed twice with PBS containing 1% FCS, and stored at −80 °C until cell staining and flow cytometric analysis. Median lymphocyte yield in decidua basalis is 5.4 × 10^6 cells (range: 0.9–13.0 × 10^6) and in decidua parietalis, 2.8 × 10^6 cells (range: 0.8–9.0 × 10^6). The median percentage of CD45^+CD3^+ T cells is 51% (range 31–86%) and 65% (range 28–95%) within the CD45^+ lymphocyte fraction, and the median percentage of CD8^+ T cells is 46% (range 29–57%) and 44% (range 29–60%) within the total T cell fraction in the decidua basalis and decidua parietalis, respectively (8). All data were generated from independent placental specimens, and decidual isolates were not pooled for experiments.

As a control for the decidual lymphocyte isolation procedure, peripheral blood samples were treated with enzymes and Percoll gradient centrifugation, similar to the decidual lymphocyte isolation procedure. Subsequently, CD45^+CD14^+CD28^−CD27^−CD45RA^−CCR7^− (EM1), CD45^+CD14^+CD28^−CD27^−CD45RA^+CCR7^− (EM2), CD45^+CD14^+CD28^−CD27^+CD45RA^−CCR7^− (EM3), and CD45^+CD14^+CD28^−CD27^+CD45RA^+CCR7^− (EM4) were enumerated by flow cytometry. As a control for the decidual lymphocyte isolation procedure, peripheral blood samples were treated with enzymes and Percoll gradient centrifugation, similar to the decidual lymphocyte isolation procedure. Subsequently, CD95^+CD105^+CD45RA^+CD28^−CD27^−CD45RO^− (EM5), CD95^+CD105^−CD45RA^+CD28^−CD27^−CD45RO^− (EM6), CD95^+CD105^+CD45RA^−CD28^−CD27^−CD45RO^− (EM7), and CD95^+CD105^−CD45RA^−CD28^−CD27^−CD45RO^− (EM8) were enumerated by flow cytometry.

Flow cytometry

A nine-color FACS panel was analyzed on an LSR-II flow cytometer (BD Biosciences) using FACS DIVA software. The LSR-II configuration and mAbs used are listed in Table I. All mAbs were titrated to determine optimal dilutions. All CD8^+ T cells were analyzed within the lymphocyte gate and were selected within the CD45^−CD3^+CD4^+CD8^+ phenotype. The gating strategy to determine the proportion of CD8^+ T cell subsets was shown in Fig. 1A–C and depicted as a percentage within the CD8^+ T cell gate. The phenotypes and main functions of all CD8^+ T cell subsets are summarized in Table II. To determine intracellular expression of perforin, granzyme B, and granzyme K, the effects of the first stained for surface expression of CD45, CD3, CD14, CD8, CD45RA, CCR7, CD28, and CD27 and thereafter treated with permeabilizing solution buffer (containing 0.1% saponin, 5% FCS, and 0.05% sodium azide in PBS) for 10 min and stained with either perforin, granzyme B, granzyme K, granulysin, FasL, and all matching isotype controls in separate tubes. Perforin, granzyme B, and granzyme K expression is depicted as mean fluorescence intensity (MFI) within the naive, effector, CM, EM1, EM2, EM3, and EM4 CD8^+ T cell subsets. Granulysin (eBioscence, San Diego, CA) and FasL (Invitrogen, Carlsbad, CA) are depicted as MFIs within CD45^+CD3^+CD8^+ T cells. All matching isotype controls were analyzed in parallel and did not show positive MFIs. Data acquisition of decidua and PBL samples was accomplished using the same LSR-II settings, and data analysis was performed using the same FACS DIVA analysis template.

Immunohistochemistry

Tissue samples from placenta and membranes were fixed in 4% formalin and then routinely processed to paraffin blocks. Serial sections (4 μm) were cut on coated slides and dried overnight at 37 °C. Slides were deparaffinized and hydrated via graded alcohols to denaturation. After heat-induced Ag retrieval with Tris/EDTA (pH 9.0) for 20 min in a microwave oven, double immunohistochemistry staining was performed using the DAKO (Glostrup, Denmark) EnVision G2 Doublestain System (code K5361), according to the manufacturer’s protocol. Briefly, endogenous alkaline phosphatase and peroxidase activity was blocked for 5 min by dual endogenous enzyme block. The sections were incubated with primary Ab anti-perforin (Neo-markers 5B10) or anti-granzyme B (Monosan GrB-7), followed by incubation with polymer/AP reagent, using diaminobenzidine as chromogen. Next a blocking step with double-stain block reagent was performed. The specimens were incubated with the second primary Ab anti-CD8 β (F5; Santa Cruz Biotechnology, Santa Cruz, CA); afterward, a Rabbit/Mouse LINK was added, followed by incubation with polymer/AP reagent, using Permanent Red as chromogen (DAKO). As a control, one or both primary Abs were replaced with isotype control antibodies to obtain single-immunohistochemical staining. Double-stained specimens were counterstained with hematoxylin.

Tissue slides were observed with a Leica microscope (DM4000B; Leica Microsystems, Deerfield, IL) with a multispectral imaging system. Spectra were acquired from 460 to 660 nm at 10-nm intervals. Nuance software version 2.6.0 was used for analysis. The individual spectra of each Ab and hematoxylin counterstaining were obtained by scanning the single-stained slides and saved as a spectral library. The double-staining images were saved as a spectral image cube in which the images were unmixed, using the spectral library of each individual staining. With this technique, from every double-stained specimen three (DAB*, PR, and hematoxylin) grayscale images were acquired, each of which shows the exact distribution of the used chromogen. The images were observed and positive stained cells were counted by two independent observers.

qPCR

Decidual and peripheral blood cells were stained with CCR7 FITC, CD8 PE, CD45RA PE-PE-TrxRed, CD14 PE-Cy7, CD4 Pacific Blue, and CD28 APC (Supplemental Fig. 4). To avoid T cell activation, cells were not stained with an anti CD3 Ab. The CD8 T cell subsets were FACS sorted on a flow sorter, Aria. Supplemental Fig. 4 depicts the FACS sort scheme of one representative decidua basalis isolate. In short, CD45^+CD14^− lymphocytes were selected and depicted in a FSC-A/SSC-A plot, and the lymphocyte gate was selected. Using additional gates in the FSC-H/FLS-W and SSC-H/SSC-W plots avoids aggregates. All gates were combined and the CD4^−CD8^+CD38^+CD161^+CD28^+ (EM1), CD4^−CD8^+CD38^+CD161^+CD28^− (EM2), CD4^−CD8^+CD38^−CD161^+CD28^+ (EM3), and CD4^−CD8^+CD38^−CD161^+CD28^− (EM4) were selected for cell sorting. The cells were used for cDNA synthesis (Qiagen). The RNA yield was assessed with a spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA was synthesized by incubating 12.8 μl RNA solution with 7.2 μl cDNA mix containing 2'-deoxyxribonucleotide 5'-triphosphate (final concentration of 0.5 mM), 2 U RT-AMV, 20 μl RNase inhibitor, 100 ng oligo-dT primers, 500 ng random primers, and 1 reverse transcriptase buffer (all from Promega, Leiden, The Netherlands). Primer sets (Supplemental Table 1) for qPCR were selected using Beacon Designer Software (version 7.02, Premier BioSoft International, Palo Alto, CA) and were obtained from Eurogentec (Liège, Belgium). PCR mixes contained 1 μM forward and reverse primers, 3 mM MgCl2, and 1× iQ SYBR Green Supermix (Bio-Rad, Veennendaal, The Netherlands). PCR was performed using an iCycler MyQ (Bio-Rad). The PCR program consisted of 1 cycle of 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C, and was finalized with a melting curve analysis. Reactions were carried out in optical 96-well plates (Bio-Rad) covered with Microseal ‘B’ Film (Bio-Rad). The mean signal of the reference genes GAPDH and β-actin served as normalization factor.

Statistical analysis

To determine differences between two groups, a Student t test was performed. In the case of analyses including more than two groups, a non-parametric Kruskal–Wallis one-way ANOVA was performed. If p < 0.05, a Dunn multiple comparison posttest was done to compare all pairs of columns. The p values < 0.05 are considered to reflect significant differences.
Results

Decidual CD8+ T cells mainly consist of highly differentiated EM cells

Human peripheral blood CD8+ T cells can be separated into four functionally distinct cell populations based on CD45RA and CCR7 expression: naive (RA+CCR7+), effector (RA+CCR7-), CM (RA-CCR7+), and EM (RA-CCR7-) cells (Fig. 1A,1B). Subsequent analysis of CD28 and CD27 identifies four subsets of EM cells: EM1 (CD28+CD27+), EM2 (CD28+CD27+), EM3 (CD28+CD27+), and EM4 (CD28+CD27+); and three subsets of effector cells: pre-effector-1 (CD28+CD27+), pre-effector-2 (CD28+CD27+), and effector cells (CD28+CD27-) (Fig. 1A,1B). The main functions of the CD8 subtypes are summarized in Table I. FACS analysis of peripheral blood of nonpregnant controls (cPBLs) and maternal peripheral blood (mPBLs) shows that naive, effector, and EM1 cells are the most abundant cell types in peripheral blood, whereas CM, EM2, EM3, and EM4 cells are minor cell populations (Fig. 1D). FACS configuration and mAbs are depicted in Table II. In contrast, analysis of lymphocyte isolates from decidua basalis and decidua parietalis shows that EM1, EM2, and EM3 cells form the largest fractions of CD8+ T cells in the decidua (Fig. 1C,1D). Therefore, the EM2 and EM3 cell fractions in both decidua basalis and decidua parietalis are significantly increased compared with the PBL samples, whereas the proportion of naive cells in both decidua basalis and decidua parietalis is significantly reduced in comparison with the PBL samples (Fig. 1C,1D).

Significantly reduced expression of perforin in decidual CD8+ T cells

CD8+ T cell subsets from peripheral blood and decidua were analyzed for intracellular expression of perforin. Consistent with a previous report by Romero et al. (14), peripheral blood CD8+ effector and EM3 cells express high levels of perforin, whereas the

<table>
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<th>Table I. Phenotype and functions of CD8+ T cell subsets</th>
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E, effector; N, naive.
expression of perforin in EM2 cells is detectable but reduced in comparison with that in effector and EM3 cells. Naive, CM, and EM1 CD8+ T cells in peripheral blood do not express perforin (Fig. 2A, 2B). Analysis of decidual T cell subsets shows significantly reduced levels of perforin in the CD8+ effector, EM2, and EM3 cell populations, in comparison with the peripheral blood CD8+ T cell subsets with the matching phenotype (Fig. 2A, 2B). Like peripheral blood naive and CM CD8+ T cells, decidual naive and CM CD8+ T cells do not express perforin. As a positive control for the lymphocyte isolation procedure and flow cytometric analysis, decidual and peripheral blood CD45+CD3+CD56+ NK cells were analyzed for expression of perforin. High proportions of CD45+CD3+CD56+ NK cells from decidua and peripheral blood do express perforin (Fig. 2C).

### Table II. LSR-II configuration and used mAbs

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<td>530/30</td>
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<td>730/45</td>
<td>Alexa 700</td>
<td>CD27f, Granzyme B+, IgG1e</td>
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*aCaltag Laboratories (Burlingame, CA). 
*bImmunoTools (Friesoythe, Germany). 
*cBD Biosciences. 
*dBD Pharmingen (San Diego, CA). 
*eDAKO. 
*fEbioscience.

**Significantly reduced expression of granzyme B in decidual CD8+ T cells**

Confirming previous studies, our data show that peripheral blood CD8+ effector and EM3 T cells express high levels of granzyme B, whereas EM2 cells contain intermediate levels of granzyme B (Fig. 3A, 3B). Comparison of cPBLs and mPBLs reveals a slight but not significantly increased MFI of granzyme B in effector and EM3 cells in mPBLs compared with cPBLs. In contrast, effector, EM2, and EM3 CD8+ T cells from decidua basalis display significantly reduced levels of granzyme B compared with the maternal peripheral blood CD8+ T cell subsets with the matching phenotype (Fig. 3A, 3B). In decidua parietalis, the EM3 cell fraction contains significantly reduced levels of granzyme B, whereas the effector and EM2 cells contain similar levels of granzyme.

**FIGURE 2.** Perforin expression in CD8+ T cell subsets in decidua and PBLs. A, Representative histograms of intracellular perforin expression in CD8+ T cell subsets from PBLs and decidua. B shows the average MFI of perforin and SD within cPBLs (n = 9), mPBLs (n = 10), decidua basalis (n = 6), and decidua parietalis (n = 6) CD8+ T cell subsets. Bars indicate average percentage and SD. *p < 0.05; **p < 0.01; ***p < 0.001. C, Representative histograms of perforin expression in CD45+CD3+CD56+ NK cells from control PBLs and decidua parietalis.
B compared with their matching phenotype from maternal peripheral blood. As a positive control for the lymphocyte isolation procedure and flow cytometric analysis, CD45+CD3⁺CD56⁺ NK cells from decidua and peripheral blood were analyzed for expression of granzyme B. High proportions of CD45+CD3⁺CD56⁺ NK cells from decidua and peripheral blood express granzyme B (Fig. 3C).

Expression of granzyme K, granulysin, and FasL

Besides perforin and granzyme B, which are the most important mediators of T cell cytotoxicity, we also assessed the expression of granzyme K, granulysin, and FasL, molecules that can provide alternative means of cytotoxicity. Granzyme K expression in cPBLs, mPBLs, decidua basalis, and decidua parietalis samples showed that EM1 and EM4 cells express the highest levels of granzyme K, whereas effector cells express intermediate levels of granzyme K. No significant difference in granzyme K expression in all CD8⁺ T cell subsets is observed between peripheral blood and decidual samples (Supplemental Fig. 1A, 1B). Granulysin is highly expressed in decidual and peripheral blood NK cells (data not shown); however, peripheral blood CD8⁺ T cells do not express granulysin, whereas decidual CD8⁺ T cells express reduced levels of granulysin, in comparison with decidual NK cells (Supplemental Fig. 2A–C). FasL expression can be detected in decidual CD8⁺ T cells, but not decidual NK cells (Supplemental Fig. 2D–F).

Immunohistochemistry confirms reduced perforin and granzyme B expression in decidual CD8⁺ T cells

Decidual lymphocyte isolation procedures may alter the lymphocyte phenotype and functional activity of the isolated cells. To confirm the reduced expression of perforin and granzyme B in CD8⁺ T cells in vivo, we performed immunohistochemical staining on tissue sections from decidua basalis and decidua parietalis. CD8⁺ T cells express CD8 in the CD8αβ isoform, whereas NK cell subsets have been shown to express CD8 on the cell surface in the CD8αα isoform. FACS analysis confirmed that a proportion of decidual NK cells express CD8 but were all negative for CD8β, whereas of decidual CD3⁺CD8⁺ T cells >93% expressed CD8β (Supplemental Fig. 3). Double immunohistochemical staining for CD8β was combined with either perforin or granzyme B and analyzed using the multispectral imaging technique (Fig. 4A, 4B). The single-scan grayscale images and composite images (Fig. 4A, 4B) were used to count total CD8β⁺, perforin⁺, and CD8β⁺perforin⁺ double-positive cells per high magnification field (Fig. 4C). Consistent with the flow cytometry results, only a small fraction of CD8β⁺ T cells in decidua basalis and decidua parietalis stained positive for perforin. The majority of perforin⁺ cells do not express CD8β, and they are most likely to be decidual NK cells. Similarly, CD8β⁺, granzyme B⁺, and CD8β⁺granzyme B⁺ double-positive cells were counted (Fig. 4D). Consistent with the flow cytometry results, a small but slightly larger fraction of CD8β⁺ T cells in decidua basalis and decidua parietalis express granzyme B, whereas the majority of granzyme B⁺ cells do not express CD8β.

Expression profile shows an increase of mRNA encoding for cytolytic proteins in decidual CD8⁺ EM T cells

The mRNA expression profile of peripheral and decidual CD8⁺ T cell subsets was determined using qPCR. To obtain sufficient cells for mRNA isolation and qPCR analysis, the cells were sorted in a CCR7⁺CD28⁺ and CCR7⁻CD28⁻ CD8⁺ T cell fraction (FACSsort scheme is depicted in Supplemental Fig. 4). CCR7⁺CD28⁺ CD8⁺ T cells include EM1 and EM4 cells that showed low levels of perforin and granzyme B in the flow cytometric analysis.

![Figure 3](http://www.jimmunol.org/issue/figure/3.png)
CCR7<sup>+</sup>CD28<sup>+</sup>CD8<sup>+</sup> T cells include effector, EM2, and EM3 cells that all showed high levels of perforin and granzyme B in the flow cytometric analysis. As a control, the CCR7<sup>+</sup>CD45RA<sup>+</sup> naive CD8<sup>+</sup> T cell fractions that do not express cytolytic molecules were included. To confirm purity of the isolated CD8<sup>+</sup> T cell subsets, qPCR for CCR7 and CD28 was performed, and results confirm the FACS sort (Fig. 5A, 5B). As expected, all naive cell fractions do not contain mRNA of any of the cytolytic molecules analyzed (Fig. 5C–I). In addition, decidual E + EM2 + EM3 cells express higher levels of granzyme B, perforin, granulysin, and FasL, in comparison with EM1 + EM4 cells (Fig. 5E, 5G, 5H). Granzyme A and IFN-γ are equally expressed in EM1 + EM4 cells and E + EM2 + EM3 cells (Fig. 5F, 5G), whereas granzyme K mRNA is increased in EM1 + EM4 cells compared with E + EM2 + EM3 cells (Fig. 5H). Most interestingly, the IFN-γ, granzyme A, granzyme B, and FasL mRNA content of decidual EM1 + EM4 CD8<sup>+</sup> T cells is significantly increased in comparison with peripheral blood EM1 + EM4 cells (Fig. 5C–I). In addition, the granzyme B, granzyme K, perforin, and FasL mRNA content in decidual E + EM2 + EM3 CD8<sup>+</sup> T cells is significantly increased in comparison with E + EM2 + EM3 CD8<sup>+</sup> T cells from peripheral blood (Fig. 5C–I).

**Discussion**

This study demonstrates that decidual CD8<sup>+</sup> T cells mainly consist of highly differentiated EM and effector T cells, whereas unprimed naive cells are almost absent. In comparison with peripheral blood CD8<sup>+</sup> EM and effector cells, decidual CD8<sup>+</sup> EM and effector cells contain reduced levels of the cytolytic proteins perforin and granzyme B. Interestingly, the perforin and granzyme B mRNA content of decidual EM and effector CD8<sup>+</sup> T cell subsets is increased in comparison with that in peripheral blood EM and effector CD8<sup>+</sup> T cells. These data may suggest that decidual CD8<sup>+</sup> T cells may pursue alternative means of EM cell differentiation that includes a blockade in perforin and granzyme B mRNA translation into functional perforin and granzyme B proteins. In this respect, microRNAs (miRNAs), which are small RNAs that modulate gene expression by translational repression and/or mRNA degradation, may play a crucial role. miRNAs have also been shown to bind mRNA targets and inhibit protein synthesis through an unknown mechanism that preserves the stability of the mRNA (20, 21). In addition, miRNAs have been shown to be differentially expressed in naïve, effector, and memory CD8<sup>+</sup> T cells (22). The expression of miRNAs in decidual CD8<sup>+</sup> T cells remains to be examined but may explain the presence of high levels of mRNA encoding for perforin and granzyme B while the perforin and granzyme B proteins are reduced. Furthermore, analysis of miRNAs in decidual CD8<sup>+</sup> T cells may identify new target specificities of miRNAs.

Overall, our data suggest that local regulation of CD8<sup>+</sup> T cell differentiation may play a crucial part in maintenance of maternal immune tolerance to the fetus during human pregnancy. The decidual immune cell compartment is altered in a gestational age-dependent manner. In early pregnancy, T cells comprise 5–15% of lymphocytes; this percentage increases up to 40–80% at term (23). Furthermore, CD<sup>4+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T reg cells and activated T cells are shown to be highly enriched in decidual tissue, in comparison with maternal peripheral blood (3, 19). The dynamics of decidual T cell differentiation are difficult to study in humans; however, interaction with the changing immune environment during gestation may alter T cell function and differentiation at different...
stages during pregnancy. Furthermore, similar to decidual NK cells, decidual T cells may secrete soluble factors to influence decidual environment, promote angiogenesis, or even prevent cytotoxicity in target cells (24, 25). However, mRNA for VEGF-A or VEGF-C, molecules secreted by decidual NK cells, could not be detected in any of the decidual T cell subsets (data not shown).

The features of decidual CD8\(^+\) T cells do not correspond to those of their matching phenotype in peripheral blood. Peripheral blood effector, EM2, and EM3 cells highly express the effector molecules perforin and granzyme B, whereas in decidual CD8\(^+\) T cells, perforin and granzyme B expression is significantly reduced. Uptake of granzyme B by target cells is essential for DNA fragmentation and apoptosis of the target cells. Although granzyme B can be taken up by target cells through endocytosis independently of perforin, apoptosis of target cells by internalized granzyme B may not be induced until perforin is added (26–28). Besides perforin, human CTLs may express a second membrane-disrupting protein known as granulysin. Granulysin causes membrane-lipid degradation, but whether high enough concentrations can be reached in the immunological synapse to facilitate granzyme entry or target cell death is unclear (29). In this study we show that mRNA encoding for granulysin is present in decidual effector and EM T cells in levels comparable to those in peripheral blood effector and EM T cell subsets. In addition, FACS analysis shows the presence of low levels of intracellular granulysin in decidual CD8\(^+\) T cells. Therefore, granulysin may facilitate alternative pathways of cytotoxicity. Besides this, CTLs can also induce target cell death by engaging with cell surface death receptors, such as FAS that interacts with FASL on the CTL. FACS data show that FASL is expressed on CD8\(^+\) T cells in decidua, giving the decidual CD8\(^+\) T cells an alternative cytotoxic potential. However, the cytolytic capacity of decidual CD8\(^+\) T cell subsets remains to be confirmed in experimental killing assays; thus far, the target specificity of decidual CD8\(^+\) T cells remains unclear. Whether or not decidual CD8\(^+\) T cells can upregulate the expression of the cytolytic molecules and increase their cytotoxic capacity upon encountering their appropriate Ag and/or stimulus remains to be determined.

The presence of highly differentiated CD8\(^+\) T cells implies that fetal alloantigens, which are present at the fetal–maternal interface, may induce a CD8\(^+\) T cell response. Fetal trophoblasts at the fetal–maternal interface do not express HLA-A, -B, -DR, -DQ, and -DP molecules. Therefore, HLA-C and possibly also minor histocompatibility Ags are the main targets to which a decidual CD8\(^+\) T cell's response may be directed. HLA typing was performed on all fetal and maternal samples but did not show a correlation between the percentage of CD8\(^+\)CD28\(^{-}\) T cells and the presence or absence of a fetal–maternal HLA-C mismatch. Previously, minor histocompatibility Ag-specific CTLs have been shown to be induced in maternal peripheral blood during pregnancy (5). Our preliminary data reveal the presence of HY-specific CD8\(^+\) T cells in term pregnancy decidual tissue (T. Tilburgs and G.M. Swings, unpublished observations). It is difficult to establish which factors attract CD8\(^+\) T cells to the decidual tissue and cause CD8\(^+\) T cell differentiation at the fetal–maternal interface. Whether the differentiated decidual CD8\(^+\) T cells are the result of a local expansion and maturation or whether they originate from the
periphery, with a potential role for activation in decidual lymph nodes, is unknown. It is clear, however, that decidua CD8+ T cells are highly differentiated and possibly alternatively activated cells.

Many factors can influence the CD8+ T cell differentiation process. Studies have shown that at optimal Ag strength CD8+ T cells differentiate to full effector cells. If the signal strength is too high or too weak, CD8+ T cells undergo Ag-induced cell death or death by neglect, or they differentiate but do not obtain effector functions (13). In addition, cytokines like TGF-β and IL-10 and hormones like progesterone are important in the regulation of T cell activation and may determine the outcome of a T cell differentiation process (30, 31). At the fetal–maternal interface, both these mechanisms may explain the lack of effector function in the differentiated CD8+ T cells. Other mechanisms like the expression of HLA-G (32), IDO (33), and the presence of a high proportion of CD4+CD25bright regulatory T cells (3, 19), alternatively activated macrophages (34, 35), and CD56bright NK cells (36) in decidual tissue can also influence the CD8+ T cell differentiation process at the fetal–maternal interface. Further functional studies with isolated CD8+ T cell subsets from decidua and peripheral blood may clarify alternative mechanisms of CD8+ cytotoxicity or CD8+ T cell regulation. In addition, defects in decidual CD8+ T cell regulation or differentiation may play a role in placental pathological conditions in which both placental growth and fetal growth are impaired.

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Disclosures

The authors have no financial conflicts of interest.

References

Supplementary figure S1. Granzyme K expression in CD8+ T cell subsets in decidua and PBL

A) Shows representative histograms of intracellular granzyme K expression in CD8 T cell subsets from control PBL (n=4), maternal PBL (n=5) and decidua basalis (n=4) and decidua parietalis (n=4). B) shows average MFI of granzyme K and st.dev within cPBL, mPBL decidua basalis and decidua parietalis CD8+ T cell subsets. Bars indicate average percentage and st.dev.
Supplementary Figure S2: Granulysin and FASL expression in decidual CD8+ T cells and NK cells

Representative dotplots of CD8 expression with a) Granulysin, b) IgG, d) FASL and e) IgG expression in decidual CD45+ lymphocytes. Histogram overlays of c) Granulysin and f) FASL expression in CD8+ T cells (red line), NK cells (green line) and IgG control (blue line). Samples are representatives of 5 decidua samples. No difference was observed between decidua basalis and decidua parietalis samples.
Supplementary Figure S3: CD8β expression in CD8+ T cells

Representative dotplots of CD8 and CD8beta expression in CD8+ T cells and CD56+ NK cells in maternal PBL (A,B) decidua basalis (C,D) and decidua parietalis (E,F).
Supplementary figure S4. FACS sort scheme

A) depicts FACS sort scheme of one representative decidua basalis isolate. In short CD45+CD14- lymphocytes are selected (P1) and depicted in a FSC-A/SSC-A plot, the lymphocyte gate is selected (P2). Using FSC-H/FSC-W (P3) and a SSC-H/SSC-W (P4) aggregates are avoided. All gates are combined and CD4-CD8high cells are selected. CD8high cells are depicted in a CCR7 CD45RA plot. CD8+CD45+CCR7+ (P7) are the CD8+ Naive T cells. CD8+CCR7- (P6) are depicted in a CD28 histogram where the CD8+CD45+CCR7+CD28+ (P8) (EM1 + EM4), and CD8+CCR7-CD28- (P9) (effector + EM2 + EM3) are selected for cell sorting. B) depicts FACS ARIA configuration and used monoclonal
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