Human decidua contains differentiated CD8+ Effector-Memory T cells

*Manuscript in Preparation*


*Je hebt kleine leugens, je hebt grote leugens en je hebt statistiek*
Chapter 5

ABSTRACT

During pregnancy maternal lymphocytes at the fetal-maternal interface play a key role in the immune acceptance of the allogeneic fetus. Decidual NK cells contain immune modulatory properties and facilitate trophoblast invasion into maternal tissue. More recently, CD4+CD25bright regulatory T cells have shown to be concentrated in decidual tissue where they are able to suppress fetus-specific and non-specific responses. However, decidual CD8+ T cells form the largest fraction of T cells at the fetal-maternal interface but limited data is present on the characteristics of these cells. Therefore we performed phenotypic analysis of the decidual and peripheral CD8+ T cell pool with CD45RA, CCR7, CD28 and CD27 expression using nine-colour flowcytometry. In addition, we examined expression of the cytolytic molecules perforin, granzyme B and granzyme K to determine the cytotoxic potential of the decidual CD8+ T cell subsets. Our data demonstrate that decidual CD8+ T cells mainly consist of differentiated Effector-Memory cells while unprimed naïve cells are almost absent. Unlike peripheral blood Effector-Memory CD8+ T cells, the decidual Effector-Memory CD8+ T cells do not express perforin and have a reduced expression of granzyme B. Apparently, the functional features of decidual CD8+ T cells do not correspond their matching phenotype in peripheral blood. These data show that decidual CD8+ T cells may pursue alternative means of effector cell differentiation and indicate that decidual CD8+ T cell differentiation and regulation may play a crucial role in maternal immune tolerance to the fetus.
INTRODUCTION

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 in to maternal tissue (1,2). More recently, CD4+CD25bright regulatory T cells have shown to be concentrated in decidual tissue and are able to suppress fetus-specific and non-specific responses (3). These cells are suggested to prevent a destructive immune response to the allogeneic fetus. But so far the mechanisms for 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 to form antibodies and to induce specific CTLs to fetal HLA and minor histocompatibility antigens (mHags) (4-6). Recent data by our group indicate that a fetal-maternal HLA-C mismatch correlates with an increased decidual CD4+ T cell activation and regulation (T.Tilburgs et.al submitted document). Decidual CD8+ T cells form the largest fraction of T cells at the fetal-maternal interface and are main candidates to recognize and respond to fetal HLA-C at the fetal-maternal interface. However limited data is available on the phenotype and function of decidual CD8+ T cells during human pregnancy.

In healthy individuals and during viral infections, the CD8 effector T cell differentiation process has been studied extensively (7-9). These studies have identified particular CD8+ T cell subsets capable to elicit a cytotoxic response and identified many phenotypic markers to categorize these cells. In this study we use the cell surface markers CD45RA and CCR7 to discriminate Naïve (RA+CCR7+), Effector (RA+/CCR7-), Effector-Memory (EM) (RA-CCR7-) and Central-Memory (CM) (RA-CCR7+) CD8+ T cells (10-13) in decidual tissue. In addition we analyzed the expression of the co-stimulatory molecules CD28 and CD27 to determine additional the heterogeneity in CD8+ Effector-Memory cells (EM-1 28+27+; EM-2 28-27+; EM-3 28-27- and EM-4 28+27-) (12). Furthermore, the expression of the cytolytic molecules perforin, granzyme B and granzyme K was studied to examine the cytolytic capacity of the decidual CD8+ T cell subsets. Perforin is a membrane perturbing protein that delivers granzymes in the target cell (14). Among all known granzymes, granzyme B is essential to induce DNA fragmentation and apoptosis in target cells (14) whereas granzyme K may provide alternative mechanisms to kill target cells (15,16). We examined the CD8+ T cell pool at 2 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 decidual tissue to the maternal peripheral blood (mPBL). As a control, we analyzed peripheral blood CD8+ T cells of age matched healthy volunteer donors.
MATERIALS AND METHODS

Blood and tissue samples

Samples of decidua basalis, decidua parietalis and heparinised maternal peripheral blood (mPBL) were obtained from healthy women after uncomplicated term pregnancy (gestational age range: 37 – 42 weeks). Tissue samples were obtained after delivery by elective caesarean section or uncomplicated spontaneous vaginal delivery. mPBL samples were obtained either directly before or directly after delivery. Control PBL (cPBL) samples were obtained from healthy non-pregnant volunteer female donors. Signed informed consent was obtained from all women, and the study received approval by the LUMC Ethics Committee (P02-200).

Lymphocyte isolation

Lymphocyte isolation from decidua was done as described previously (17). 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 (Gibco-BRL, Grand Island, NY) and 0.02% DNAse I (Gibco) in RPMI-1640 medium, gently shaking in a waterbath at 37°C for 60 min and thereafter washed once with RPMI. The resultant suspensions were filtered through a 70μm sieve (BD, Labware; NJ) and washed once in RPMI. The decidual isolates were layered on a Percoll gradient of (7.5ml 1.080g/ml; 12.5ml 1.053g/ml; 20ml 1.023g/ml) for density gradient centrifugation (30min/800g), lymphocytes were isolated from the 1.080g/ml – 1.053g/ml interface. PBL samples were directly layered on a Ficoll Hypaque gradient (LUMC pharmacy; Leiden, The Netherlands) for density gradient centrifugation (20min/800g). Mononuclear cells were collected, washed twice with PBS containing 1% FCS and all cells were fixed with 1% paraformaldehyde and stored at 4°C until cell staining and flow cytometric analysis.

Flow cytometry

A nine-colour FACS panel was analyzed on a LSR-II Flowcytometer (Becton Dickinson) using FACS DIVA software. The LSR-II configuration and MoAbs used are listed in Table 1. All MoAbs were titrated to determine optimal dilutions. All CD8+ T cells are analyzed within the lymphocyte gate and are selected within the CD45+CD3+CD14-CD8+ phenotype. The gating strategy to determine the proportion of CD8+ T cell subsets is shown in figure 1A-C and depicted as percentage within CD8+ T cell fraction (figure 1D). To determine intracellular expression of perforin, granzyme B, and granzyme K, the cells were 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% saponine, 5% FCS and 0.05% sodium-azide in PBS) for 10 min and stained with perforin, granzyme B, granzyme K and all matching isotype controls in separate tubes. Perforin, granzyme B and granzyme K expression is depicted as Mean Fluorescence Intensity (MFI) within the Naïve, Effector, CM, EM-1, EM-2, EM-3 and EM-4 CD8+ T cell subsets. All matching isotype controls were analyzed in parallel and did not show positive MFIs. Data acquisition of decidua and PBL samples was done using the same LSR-II settings and data analysis was done using the same FACS DIVA analysis template.
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Statistical analysis
To determine differences between more than 2 groups, a non-parametric Kruskal-Wallis one way ANOVA was performed. If $p<0.05$ a Dunn’s multiple comparison post test was performed to compare all pairs of columns. P-values $<0.05$ are considered to reflect significant differences.

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$^1$Caltag; $^2$ImmunoTools GMBH; $^3$BD; $^4$BD Pharmingen; $^5$DAKO; $^6$e-biosciences

**Table 1.** LSR-II configuration and MoAbs used
RESULTS

Decidual CD8+ T cells mainly consist of differentiated Effector-Memory cells

Human peripheral blood CD8+ T cells can be separated into four functionally distinct cell populations based on CD45RA and CCR7 expression: Naïve (RA+CCR7+), Effector (RA+CCR7-), Central-Memory (CM) (RA-CCR7+) and Effector-Memory (EM) (RA-CCR7-) cells (Figure 1A, B). Subsequent analysis of CD28 and CD27 identifies 4 subsets of EM cells: EM-1 (28+27+); EM-2 (28-27+); EM-3 (28-27-) and EM-4 (28+27-) and 3 subsets of Effector cells: pre-Effector-1 (pE-1) (28+27+); pE-2 (28-27+) and Effector cells (28-27-) (Figure 1A, B). Analysis of peripheral blood of non-pregnant controls (cPBL) and maternal peripheral blood (mPBL) shows that Naïve, Effector and EM-1 cells are the most abundant cell types in peripheral blood, whereas CM, EM-2, EM-3 and EM-4 cells are minor cell populations (Figure 1D). In contrast, analysis of lymphocyte isolates from decidua basalis and decidua parietalis shows that EM-1, EM-2 and EM-3 cells form the largest fractions of CD8+ T cells in the decidua (Figure 1C, D). Thereby, the EM-2 and EM-3 cell fractions in both decidua basalis and decidua parietalis are significantly increased compared to the PBL samples whereas the proportion of Naïve cells in both decidua basalis and decidua parietalis is significantly reduced in comparison to the PBL samples (Figure 1C, D).

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 (12), peripheral blood CD8+ Effector and EM-3 cells express high levels of perforin, whereas the expression of perforin in EM-2 cells is detectable but reduced in comparison with Effector and EM-3 cells. Naïve, CM and EM-1 CD8+ T cells in peripheral blood do not express perforin (Figure 2A, B). Analysis of decidual T cell subsets shows significantly reduced levels of perforin in the CD8+ Effector, EM-2 and EM-3 cell populations in comparison with the peripheral blood CD8+ T cell subsets with the matching phenotype (Figure 2A, B). Alike peripheral blood Naïve and CM CD8+ T cells, decidual Naïve and CM CD8+ T cells do not express perforin. As a positive control for the lymphocyte isolation procedure and flowcytometric 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 (Figure 2C).

Reduced expression of granzyme B in decidual CD8+ T cells

Confirming previous studies, our data show that peripheral blood CD8+ Effector and EM-3 T cells were found to express high levels of granzyme B whereas EM-2 cells contain intermediate levels of granzyme B (Figure 3A, B). Comparison of control PBL and maternal PBL shows a slight but not significantly increased MFI of granzyme B in Effector and EM-3 cell in maternal PBL compared to control PBL. In contrast, Effector, EM-2 and EM-3 CD8+ T cells from decidua basalis display significantly reduced levels of granzyme B compared to the peripheral blood CD8+ T cell subsets with the matching phenotype (Figure 3A, B). In decidua parietalis the EM-3 cell fraction contain significantly reduced levels of granzyme B, whereas the Effector and EM-2 cells contain similar levels of granzyme B compared to their matching phenotype from peripheral blood. As a positive control for the lymphocyte isolation procedure and flowcytometric analysis, we analyzed CD45+CD3-CD56+ NK cells from decidua and peripheral blood for expression of granzyme B. High proportions of CD45+CD3-CD56+ NK cells from decidua and peripheral blood express granzyme B (Figure 3C).
Decidual CD8+ Effector-Memory T cells

Figure 1. CD8+ T cell subsets in peripheral blood and decidua
CD8+ T cells from (A) peripheral blood and (B) decidua are separated into 4 subsets based on CD45RA and CCR7 expression. Each of these subsets are analysed for CD27 and CD28 expression. C) Nine subpopulations of CD8+ T cells can be distinguished Naïve (N), Effector (E), pre-effector-1 (pE-1) and pE-2, Central Memory (CM), Effector Memory-1 (EM-1), EM-2, EM-3 and EM-4 cells. D) The proportion of Naïve, Effector (E, pE-1 and pE-2), CM, EM-1, EM-2, EM-3 and EM-4 cells are determined in PBL from non-pregnant control PBL (n=22), maternal PBL (n=20), decidua basalis (n=15) and decidua parietalis (n=14) isolates. Bars indicate average percentage and st.dev. *p<0.05, **p<0.01, ***p<0.001
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Granzyme K is expressed in decidual and peripheral blood EM-1 and EM-4 CD8+ T cells

Granzyme K is one of the least studied granzymes in humans. Although granzyme B is essential to induce DNA fragmentation and apoptosis of target cells, expression of Granzyme K may provide alternative means to induce cell death. Examining all different CD8+ T cell subsets from control PBL, maternal PBL and decidua shows that EM-1 and EM-4 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 cells subsets is observed between peripheral blood and decidual samples (Figure 4).

Figure 2. Perforin expression in CD8+ T cell subsets in decidua and PBL
A) Representative histograms of intracellular perforin expression in CD8+ T cell subsets from PBL and decidua. B) Shows average MFI of perforin and st.dev within control PBL (n=9), maternal PBL (n=10) decidua basalis (n=6) and decidua parietalis (n=6) CD8+ T cell subsets. Bars indicate average percentage and st.dev. *p<0.05, **p<0.01, ***p<0.001. C) Representative histograms of perforin expression in CD45+CD3-CD56+ NK cells from control PBL and decidua parietalis.
Decidual lymphocyte isolation procedure has no effect on decidual CD8+ T cell phenotype and intracellular expression of perforin and granzyme B

As decidual lymphocyte isolation procedures may alter the lymphocyte phenotype and functional activity of the isolated cells we included several controls to confirm the flowcytometric analysis. Firstly as a positive control for the FACS analysis we analyzed CD45+CD3-CD56+ NK cells for perforin and granzyme B expression. Data show that a high proportion of decidual NK cells express perforin and granzyme B (figure 2C and figure 3C respectively). Furthermore we treated peripheral blood samples with enzymes and percoll gradient centrifugation similar to the decidual lymphocyte isolation procedure. Subsequently we analysed CCR7, CD45RA, CD28 and CD27 expression and included analysis of perforin and granzyme B expression. No differences were observed between the CD8+ T cells subsets between the treated and untreated samples. In addition no differences were observed in perforin and granzyme B expression within the CD8+ T cell subsets (data not shown).

Figure 3. Granzyme B expression in CD8+ T cell subsets in decidua and PBL
A) Shows representative histograms of intracellular granzyme B expression in CD8 T cell subsets from PBL and decidua. B) shows average MFI of granzyme B and st.dev within control PBL (n=11), maternal PBL (n=11), decidua basalis (n=8) and decidua parietalis (n=7) CD8+ T cell subsets. Bars indicate average percentage and st.dev. *p<0.05, **p<0.01, ***p<0.001 C) Representative histograms of granzyme B expression in CD45+CD3-CD56+ NK cells from control PBL and decidua parietalis.
This study demonstrates that decidual CD8+ T cells mainly consist of highly differentiated Effector-Memory (EM) and Effector T cells, whereas unprimed naïve cells are almost absent. In contrast to peripheral blood CD8+ EM and Effector cells, decidual CD8+ EM and Effector cells do not express the cytolytic molecule perforin. In addition, decidual CD8+ EM and Effector cells do express granzyme B and granzyme K, but the expression of granzyme B is reduced in comparison with the peripheral blood CD8+ T cells of the matching phenotype. These data suggest that local regulation of CD8+ T cell differentiation may play a crucial role in maintenance of maternal immune tolerance to the fetus during human pregnancy.

The functional features of decidual CD8+ T cells do not correspond with the functional features of their matching phenotype in peripheral blood. Peripheral blood Effector cells, EM-2 and EM-3 cells highly express the effector molecules perforin and granzyme B.

**Figure 4.** 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 d.basalis and d.parietalis CD8+ T cell subsets. Bars indicate average percentage and st.dev.
whereas in decidual CD8+ T cells perforin expression is absent. Furthermore, granzyme B expression is reduced in decidual Effector, EM-2 and EM-3 cells whereas granzyme K is normally expressed in EM-1, EM-4 and Effector cells. 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 (18-20). 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 (21). Although our flow cytometric analysis may be confirmed with experimental analysis of the isolated CD8+ T cell subsets, by not expressing perforin decidual CD8+ Effector and EM T cells may not be able to elicit a full cytotoxic response. Besides perforin and granzyme mediated cytotoxicity CTLs can also induce target cell death by engaging with cell surface death receptors, such as FAS that interacts with FASL on the CTL but FASL is not expressed on any of the CD8+ T cell subsets in decidua. From this we conclude that decidual CD8+ T cells are highly differentiated cells that do not express conventional cytolytic mediators to induce target cell death.

Fetal trophoblasts at the fetal-maternal interface do not express HLA-A, -B, -DR, -DQ and DP molecules. Therefore HLA-C and possibly indirectly presented minor histocompatibility antigens (mHags) are the main targets to which a decidual CD8+ T cells response may be directed. However no correlation was found between the percentage of CD8+CD28- T cells and the presence or absence of a fetal-maternal HLA-C mismatch. Previously mHag specific CTLs have been shown to be induced in maternal peripheral blood during pregnancy (5). Our preliminary data do show the presence of HY specific CD8+ T cells in term pregnancy decidua tissue (data not shown). It is difficult to establish which factors attract CD8+ T cells to the decidua tissue and cause CD8+ T cell differentiation at the fetal-maternal interface. Whether the differentiated decidual CD8+ T cells originate from an influx from the periphery or are the result of a local expansion and maturation is also unknown. However the presence of highly differentiated CD8+ T cells does imply that fetal alloantigens are present at the fetal-maternal interface and attract a CD8+ T cell response.

Many factors can influence the CD8+ T cell differentiation process. Studies have shown that at optimal antigen strength CD8+ T cells differentiate to full effector cells. If the signal strength is too high or too weak CD8+ T cells die by antigen induced cell death (AICD), death by neglect or differentiate but do not obtain effector functions (11). In addition, cytokines are highly important in the induction of T cell activation and may determine the outcome of a T cell differentiation process. 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 (22) and IDO (23) and the presence of a high proportion of CD4+CD25bright regulatory T cells (3,17), alternatively activated macrophages (24,25) and CD56bright NK cells (26) 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 developing conditions of placental pathology where placental and fetal growth are impaired.
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REFERENCES
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