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The possible role of virus-specific CD8⁺ memory T cells in decidual tissue

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ABSTRACT

The most abundant lymphocyte present in decidual tissue is the CD8⁺ T cell. It has been shown that most decidual CD8⁺ T cells have an effector-memory phenotype, but expressed reduced levels of perforin and granzyme B compared with the peripheral CD8⁺ effector-memory T cells. The specificity of these CD8⁺ memory T cells has yet to be determined. One hypothesis is that the decidual memory T cells are virus-specific T cells that should protect the fetus against incoming pathogens. As virus-specific CD8⁺ memory T cells can cross-react with human leukocyte alloantigens, an alternative, but not mutually exclusive, hypothesis is that these CD8⁺ T cells are fetus-specific. Using virus-specific tetramers, we found increased percentages of virus-specific CD8⁺ T cells in decidual tissue compared with peripheral blood after uncomplicated pregnancy. So far, no evidence has been obtained for a cross-reactive response of these virus-specific T cells to fetal human leukocyte antigens. These results suggest that the virus-specific memory T cells accumulate in the placenta to protect the fetus from a harmful infection.

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1. Introduction

The maternal immune system has been shown to be aware of the presence of the fetus (Aluvihare et al., 2004; Bonney and Matzinger, 1997; Kahn and Baltimore, 2010; Tafuri et al., 1995). Previous studies have shown that antibodies to fetal human leukocyte antigen (HLA) are produced during pregnancy (van Kampen et al., 2002; van Rood et al., 1958) and functional cytotoxic T lymphocytes specific to fetal HLA and minor histocompatibility antigens are present in the peripheral blood of pregnant women (Bouma et al., 1996; van Kampen et al., 2001 Verdijk et al., 2004). In addition, the proliferative responses to fetal antigens are not different during pregnancy compared with non-pregnant controls (Lashley et al., 2011).

Previous studies in our laboratory focused on the local immune response in the uterus during pregnancy. Activated T cells in decidual tissue were found in uncomplicated pregnancies (Sindram-Trujillo et al., 2003) and the percentage of CD4⁺CD25^{bright} T cells was found to be higher in the decidua than in the peripheral blood (Tilburgs et al., 2006). Fetus-specific regulatory T cells from the peripheral blood were shown to be recruited to the fetal–maternal interface (Tilburgs et al., 2008). A proportion of decidual T cells express HLA-C-specific killer immunoglobulin-like receptors (KIRs) (Tilburgs et al., 2009a). Functional regulatory T cells were only found in HLA-C mismatched pregnancies and not in HLA-C matched pregnancies (Tilburgs et al., 2009b).

At the fetal–maternal interface the maternal immune cells come into contact with trophoblast cells. Several studies have shown that the trophoblast cells do not express HLA-A and HLA-B (Redman et al., 1984), but trophoblast cells express HLA-C, HLA-G and HLA-E on the cell surface (King et al., 1996, 2000a,b; Proll et al., 1999; Tilburgs et al., 2015). HLA-C molecules on the surface of trophoblast cells were only detected in beta(2) m-associated complexes, suggesting a stable HLA-C complex with little degradation to free heavy chains at the cell surface (Apps et al., 2008). The alternative HLA expression on trophoblast cells induces CD4⁺ regulatory T cells and NK cell tolerance (LeMaoult et al., 2004; Szekeres-Bartho, 2008). However, HLA-C and HLA-E, expressed on haematopoietic stem cells and phytohaemagglutinin (PHA) blasts respectively, have been

Abbreviations: CMV, cytomegalovirus; EBV, Epstein-Barr virus; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HLA, human leukocyte antigen; IFN, interferon; IL, interleukin; IMDM, Iscove's Modified Dulbecco's Medium; LCL, lymphoblastoid cell lines; mPBL, heparinized peripheral blood; Nk cell, natural killer cell; PBS, phosphate-buffered saline; PE, phycoerythrin; PHA, phytohaemagglutinin; UCB, umbilical cord blood.

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shown to serve as targets for allogeneic effector T cells (Romagnani et al., 2002; Heemskerk et al., 2005).

The most abundant type of T cell present in the decidual tissue is the $CD8^+$ T cell. In contrast to the peripheral blood, the decidual tissue contains a lower percentage of $CD4^+$ T cells and a higher percentage of $CD8^+$ T cells (Tilburgs et al., 2009a). The decidual $CD8^+$ T cells were suggested to follow an alternative differentiation pathway influenced by regulating cell populations, such as $CD4^+CD25^{bright}$ T cells, shown to be present at the fetal–maternal interface, resulting in an altered phenotype and function (Tilburgs et al., 2010b). The percentage of $CD8^+CD28^-$ T cells was increased in the decidua compared with the peripheral blood (Tilburgs et al., 2006). In addition, the $CD8^+CD28^-$ T cells found in the decidua do not express the cytolytic molecule perforin (Tilburgs et al., 2009c). Further investigation showed that decidual $CD8^+$ T cells were mainly of the effector-memory phenotype and that these cells expressed reduced levels of perforin and granzyme B compared with the peripheral $CD8^+$ effector-memory T cells (Tilburgs et al., 2010a).

Tilburgs et al. (2010b) suggested that fetal alloantigens at the fetal–maternal interface might induce an antigen-specific T cell response. The decidual $CD8^+$ T cells may directly recognize fetal HLA-C, although a correlation between the presence of an HLA-C mismatch and the percentage of $CD8^+CD28^-$ T cells in decidual tissue was not found. Another possibility could be that the decidual memory T cells are virus-specific T cells that should protect the fetus from incoming pathogens. Chronically persisting viruses such as the Epstein-Barr virus (EBV) and cytomegalovirus (CMV) are known to induce high frequencies of memory T cells (D'Orsogna et al., 2010). These virus-specific $CD8^+$ memory T cells commonly cross-react with allogeneic HLA class I molecules (Amir et al., 2010), a phenomenon called heterologous immunity. We therefore hypothesize that the virus-specific decidual $CD8^+$ memory T cells might potentially be cross-reactive with the mismatched HLA of the fetus.

So far, the specificity of the memory T cells found in decidual tissue is unknown. The aim of this study was to investigate the specificity of the $CD8^+$ memory T cells in decidual tissue. The percentage of virus-specific $CD8^+$ T cells was determined for lymphocytes isolated from the decidua basalis, decidua parietalis, and peripheral blood after uncomplicated pregnancy. In addition, T cell clones and cell lines were generated to investigate the possible alloreactivity against the paternal HLA-C expressed on the trophoblast.

2. Materials and methods

2.1. Tissue samples and lymphocyte isolation

Paired samples of decidua basalis, decidua parietalis, and heparinized peripheral blood (mPBL) were collected from healthy women after an uncomplicated term pregnancy. In addition, umbilical cord blood (UCB) was collected. Tissue samples were obtained after delivery by elective caesarean section. Decidual lymphocytes were isolated according to our standard protocol, as described previously (Tilburgs et al., 2006). In short, the decidua basalis and decidua parietalis were macroscopically dissected from the placenta or membranes and washed in phosphate-buffered saline (PBS). The tissue was minced and washed again to reduce contamination with blood and trophoblast cells. Decidual tissue was then incubated for an hour with 0.2% collagenase I and 0.02% deoxyribonuclease I in RPMI 1640 (Gibco, Paisley, Scotland) in a water bath (37 °C, gently shaking). The decidual fragments were washed in RPMI, filtered through a 70-μm sieve (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and washed again. The decidual isolates were layered on a Percoll gradient (10 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) and lymphocytes were isolated from the 1.080 g/ml–1.053 g/ml interface. Cells were washed twice and counted. Blood (mPBL and UCB) was layered on a Ficoll-Hypaque (LUMC pharmacy, Leiden, The Netherlands) for density gradient centrifugation (20 min/800 × g). Mononuclear cells were collected, washed twice with PBS and counted. The cells were frozen and stored in liquid nitrogen until analysis. HLA typing of the mother was performed at the typing laboratory of the Leiden University Medical Center using sequence-specific oligonucleotides (SSO) PCR.

Table 1
Human leukocyte antigen (HLA) molecules and viral peptides used to create tetramers.

Virus	HLA	Peptide
CMV	A2	NLVPMVATV
	A3	TVYPPSSTAK
	B7	RPHERNGFTVL
	B7	TPRVTGGGAM
	B35	IPSINVHHY
EBV	A2	GLCTLVAML
	A3	RLRAEAQVK
	A3	KIRLRPGGK
	A3	RVRAYTYSK
	A3	KHSRVRAYTYSK
	B7	RPPIFIRRL
	B35	EPLPQGQLTAY
	B35	HPVGEADYFEY
	B35	MGSLEVMPM
	B35	YPLHEQHGM
	B35	AVLLHEESM

2.2. Flow cytometric analysis

Two-colour immunofluorescence staining was performed to investigate tetramer-positive $CD8^+$ T cells. An allophycocyanin-conjugated CD8 antibody (Becton Dickinson) was used in concentrations according to the manufacturer's instructions to stain the $CD8^+$ lymphocytes. The specificity of these cells was examined by staining the cells with a mixture of phycoerythrin (PE)-conjugated HLA class I/peptide complexes. The tetrameric complexes used in this study were constructed as previously described (Burrows et al., 2000). Table 1 shows a list of the tetrameric complexes used in this study. Cells were stained for 30 min at 4 °C and washed in PBS (LUMC pharmacy) with 5% fetal bovine serum (FBS; Greiner Bio-One B.V., Alphen a/d Rijn, the Netherlands). Flow cytometric analysis was performed using a Calibur flow cytometer with Cellquest Pro software (Becton Dickinson). The data were analysed using the gating strategy shown in Fig. 1a. First, the lymphocyte gate was set around the viable lymphocytes and the $CD8^+$ T cells were selected. The percentage of tetramer-positive cells was determined within the $CD8^+$ T cell population.

2.3. Generation of virus-specific $CD8^+$ T-cell clones and cell lines

The lymphocytes isolated from the decidua basalis, decidua parietalis, and the peripheral blood were prepared for sorting as described before (Heemskerk et al., 2004). In short, cells were stained with a mixture of tetrameric complexes and antibodies directed against various cell surface molecules for 30 min at 4 °C in Iscove's Modified Dulbecco's Medium (IMDM) without phenol (Lonza/Biowhittaker), supplemented with 2% FBS. The antibodies used were fluorescein isothiocyanate (FITC)-conjugated and directed against CD4, CD19, CD14, CD40, CD16, and CD56 (Becton Dickinson). The cells were washed and cell sorted at 4 °C using

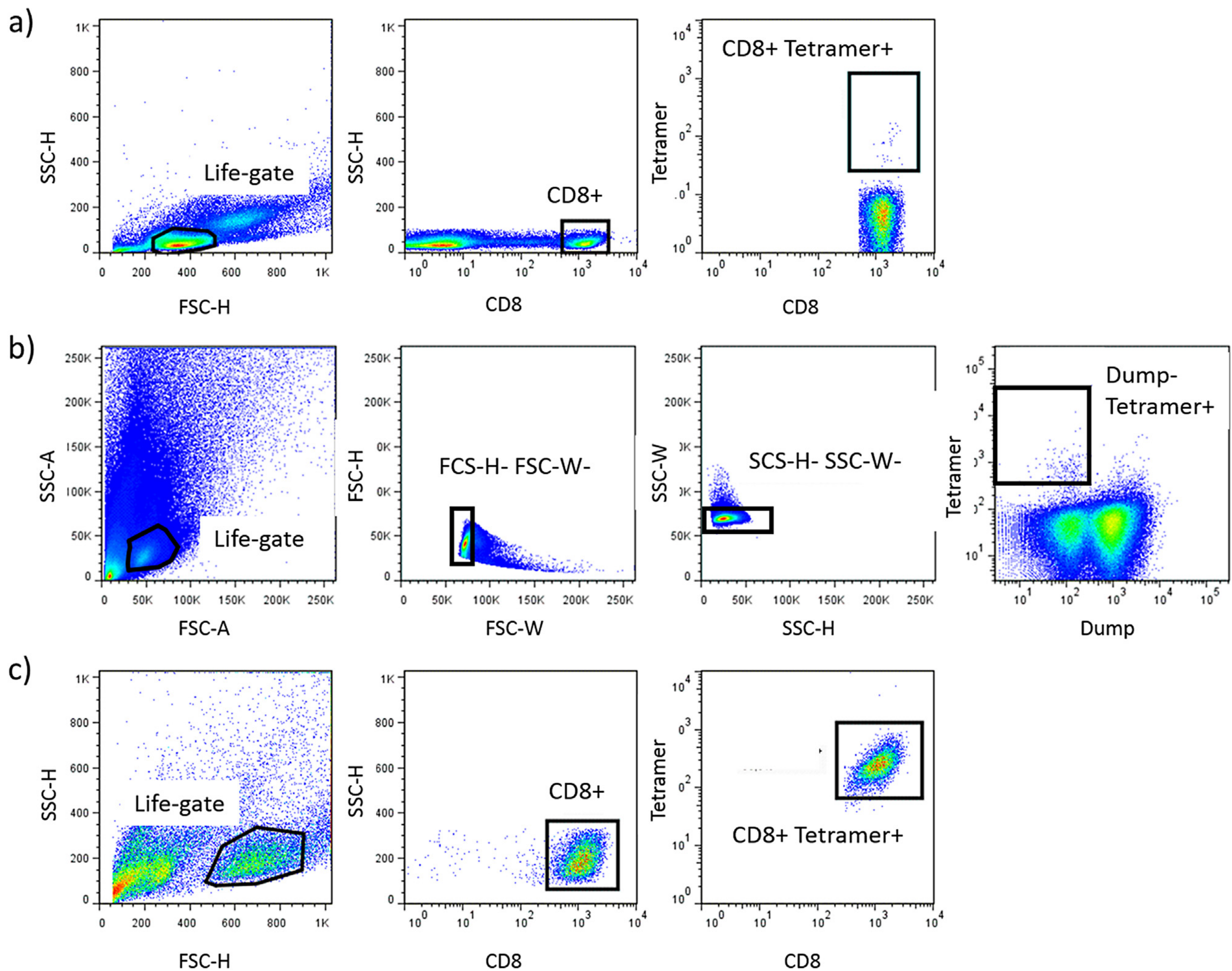

Fig. 1. Gating strategies. (a) Lymphocytes were stained for CD8 and a mixture of tetrameric complexes according to the maternal human leukocyte antigen (HLA) typing. The lymphocyte gate was set around the viable lymphocytes and the CD8+ T cells were selected. The percentage of tetramer-positive cells was determined within the CD8+ T cell population. (b) Lymphocytes were stained for CD4, CD19, CD14, CD40, CD16, CD56 and a mixture of tetrameric complexes according to the maternal HLA typing. The lymphocyte gate was set around viable lymphocytes and cell doublets were excluded. The cells positive for the mixture of tetrameric complexes, but negative for the aforementioned surface markers (dump-FITC), were sorted into 96-well plates. (c) This figure shows a representative example of the flow cytometric analysis of clones/cell lines generated after sorting using a mixture of tetrameric complexes. The tetramer positivity of the generated clones and cell lines was investigated by staining with the tetramer complexes from the mixture separately.

the fluorescence-activated cell sorter Aria Sorp (Becton Dickinson). Fig. 1b shows the gating strategy used to sort tetramer-positive cells. Cells that were negative for the cell surface molecules and positive for the mixture of tetramers were sorted and cultured at 1 cell/well (clones) or 10 cells/well (cell lines). The cells were sorted into a 96-well plate containing a feeder cell mixture with irradiated allogeneic PBMCs (4000 rad), 800 ng/ml PHA, 10 IU/ml IL-2 in IMDM supplemented with glutamine (Gibco), human serum (5%) and FBS (5%). Thereafter, sorted cells were non-specifically stimulated every two weeks with freshly prepared feeder cell mixtures. CD8 and tetramer positivity was confirmed by flow cytometric analysis, as described above, when clones and/or cell lines started to expand (Fig. 1c). In addition, the mono- or polyclonality of the generated T cell clones and cell lines was confirmed by determining the TCR Vβ usage with the TCR Vβ repertoire kit (Beckman Coulter).

2.4. Cytokine assays

A panel of EBV-transformed B cells (EBV LCLs) selected to cover the most frequently occurring HLA class I and II molecules were used to screen for allo-HLA cross-reactivity. In addition, mononuclear cells isolated from the umbilical cord blood of the women's own child were used to investigate a possible fetus-specific allo-response. Supernatants were collected 18 h after co-cultures with 10,000 virus-specific T cells and 50,000 irradiated stimulator cells (EBV LCLs or umbilical cord blood cells) in a final volume of 150 μl IMDM culture medium supplemented with 10% FBS and 100 IU/ml IL-2. The concentration of IFN-γ was determined in these supernatants using a Th1/Th2 kit (Bio-Rad).

2.5. Cytotoxicity assays

Cell-mediated lympholysis was used to analyse the cytotoxic capacity of the generated T cell clones (effector) towards the UCB of the women's own child (target). Maternal peripheral blood leukocytes, third party UCB with expression of HLA-A*29 and an EBV LCL with expression of HLA-A*02 (with or without the addition of the A2-EBV-GLC peptide) were used as target cell controls. The cytotoxic capacity of the effector cells was analysed in triplicate using four effector:target ratios (1:1, 5:1, 10:1 and 20:1). The target cells were labelled with ^{51}Cr, added to the effector cells in round-bottomed 96-well plates and incubated at 37 °C in a humid-

Table 2
Pregnancy characteristics ($n = 6$).

Pregnancy	
Gestational age (days)	274 (269–278)
Indication caesarean section	
- Breach presentation	1 (17%)
- Caesarean previous pregnancy	3 (50%)
- Obstetric history	2 (33%)
Anaesthesia (caesarean section)	
- Combined spinal epidural	6 (100%)
Mother	
Age (years)	32 (29–36)
Highest diastolic pressure (mmHg)	80 (65–95)
Gravidity	2 (1–3)
Parity	1 (0–2)
CMV seropositive	2 (33%)
EBV seropositive	5 (83%)
Child	
Birth weight (g)	3932 (3500–4510)
Gender (male)	3 (50%)

Table 3
Maternal HLA-A and -B typing.

Pregnancy	A-locus		B-Locus	
1	**A*02**		**B*07**	B*27
2	**A*02**	A*33	B*14	B*44
3	**A*02**	A*11	**B*07**	B*15
4	**A*03**	A*11	**B*35**	B*55
5	**A*02**	A*68	**B*35**	B*40
6	**A*02**	A*66	B*41	B*44

The HLA restriction of the tetrameric complexes used in the mixture are shown in bold.

ified atmosphere containing 5% CO_2. After incubating for 4 h, the ^{51}Cr that was released from the target cells was measured in the supernatant of the culture using a gamma counter (PerkinElmer, Waltham, MA, USA).

The percentage of specific lysis was calculated using spontaneous and maximal lysis, by incubating the target cells for 4 h in medium or 1% Triton X-100 respectively. The measured lysis was divided by the maximal lysis after correcting for the spontaneous lysis.

2.6. Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego, CA, USA). The Friedman test was used as a non-parametric one-way analysis of variance for matched observations to compare the percentage of CD8$^+$ T cells and the percentage of tetramer-positive CD8$^+$ T cells within decidua basalis, decidua parietalis and mPBL. Post-hoc analysis was performed using the Wilcoxon matched pairs test when a statistically significant difference was found among the three groups. p values < 0.05 were considered to denote significant differences.

3. Results

Lymphocytes from the decidua basalis, decidua parietalis and peripheral blood were collected from six healthy women after uncomplicated term pregnancy. Table 2 shows the characteristics of the pregnancies included.

3.1. Validating staining with a mixture of tetrameric complexes

The lymphocytes isolated from the decidua basalis, decidua parietalis and peripheral blood were stained with a mixture of tetrameric complexes rather than individual tetramers because of the low cell numbers of lymphocytes that can be isolated from decidual tissue. To rule out the possible introduction of false-positive CD8$^+$ T cells by staining with a mixture of tetrameric complexes instead of single tetrameric complexes, we validated the use of these mixtures with lymphocytes isolated from the peripheral blood of non-pregnant individuals. The peripheral blood of the selected individuals had previously been screened for the presence of virus-specific CD8$^+$ T cells with single tetrameric complexes and was found to harbour CD8$^+$ T cell populations with several virus specificities. In this study, the observed percentages of virus-specific CD8$^+$ T cells when using the mixture of tetrameric complexes were compared with the expected percentages calculated by adding up the percentages of virus-specific CD8$^+$ T cells stained with single tetrameric complexes (Fig. 2). The observed percentages determined after staining with mixtures containing three to eleven tetrameric complexes were similar to the expected percentages in four non-pregnant individuals.

3.2. Increased percentage of virus-specific CD8$^+$ T cells in decidual tissue

The lymphocytes isolated from the decidua basalis, decidua parietalis and peripheral blood were stained for CD8 and a mixture of tetrameric complexes according to maternal HLA typing. Table 3 shows the maternal HLA-A and -B typing for each pregnancy included in this study.

Paired analysis of the decidua basalis, decidua parietalis and peripheral blood was used to investigate the percentage of CD8$^+$ T cells and the virus specificity of these cells. Fig. 3a shows that the CD8$^+$ T cells were found to be more abundant in the decidua basalis and decidua parietalis than in the peripheral blood (Friedman test, $p = 0.029$). In addition, the percentage of tetramer-positive CD8$^+$ T cells was significantly different comparing the decidua basalis, decidua parietalis and peripheral blood (Fig. 3b; Friedman test, $p = 0.002$). A significantly higher percentage of virus-specific CD8$^+$ T cells was observed in both the decidua basalis and the decidua parietalis compared with mPBL.

3.3. Virus-specific CD8$^+$ T cell clones and cell lines

To generate virus-specific CD8$^+$ T cell clones and cell lines, isolated lymphocytes that stained negative for the cell surface molecules CD4, CD19, CD14, CD40, CD16 and CD56 and positive for the mixture of tetramers were sorted. In only 20% (1/5 pregnancies) expansion of the sorted cells was possible using an approach that had previously been validated in non-pregnant individuals. The mixture of tetrameric complexes used in this case contained two HLA-A2 restricted tetramers with either a CMV- or EBV-derived peptide. Although the percentage of tetramer-positive CD8$^+$ T cells was significantly higher in the decidua basalis and decidua parietalis compared with peripheral blood, the generation of clones and/or cell lines from the decidual tissue was more difficult than from peripheral blood. Not only the success rate of generating tetramer-positive T cell clones (20% versus 100%), but also the number of T cell clones generated from the decidua basalis, decidua parietalis and peripheral blood collected after uncomplicated pregnancy (median number 0.0; $n = 14$) was significantly lower compared with the number of T cell clones generated from peripheral blood collected from non-pregnant individuals (median number 4.2; $n = 8$; $p < 0.0001$).

The expanding clones and cell lines generated from the decidua basalis, decidua parietalis and peripheral blood were examined for CD8 and tetramer positivity. Fig. 1c shows an example of cells checked for CD8 and a single tetramer. All clones and cell lines generated from the decidua basalis, decidua parietalis and periph-

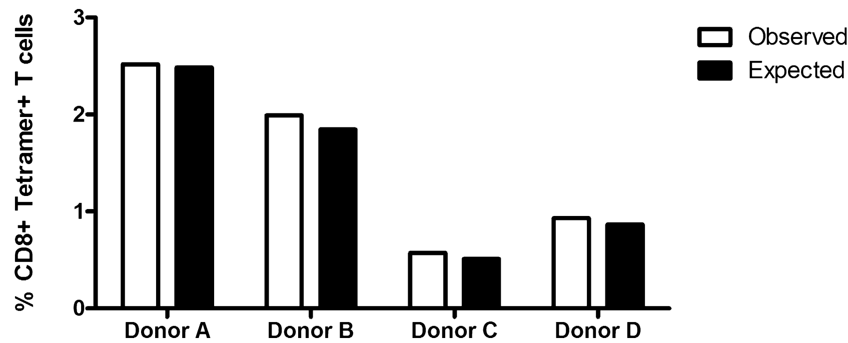

Fig. 2. Validation of staining with a mixture of tetrameric complexes. The percentage of virus-specific CD8+ T cells was determined in the peripheral blood of four non-pregnant individuals (donors A–D). The observed percentages represent the values measured after staining with a mixture of tetrameric complexes. The expected percentages were calculated by adding up percentages after staining with single tetrameric complexes. The mixtures contained three to eleven tetrameric complexes, based on the HLA typing of the donor.

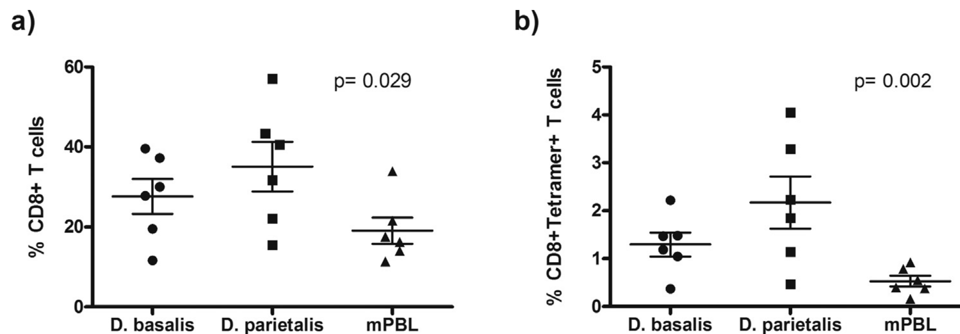

Fig. 3. Increased percentage of CD8+ and CD8+Tetramer+ T cells in decidual tissue compared with peripheral blood. (a) The lymphocytes isolated from decidual tissue and peripheral blood were stained for CD8. The percentage of CD8+ T cells was determined within the lymphocyte gate (Fig. 1a). (b) Lymphocytes were stained for CD8 and a mixture of tetrameric complexes according to the maternal HLA typing. The percentage of tetramer-positive cells was determined within the CD8+ T cell population. Percentages are shown as mean values ± SEM. p values < 0.05 were considered to denote significant differences.

Table 4
Vβ usage of the generated T cell clones and cell lines. # = not determined, mPBL = heparinized peripheral blood.

Tissue	Code	Clone/cell line	Vβ
Decidua basalis	A6	Clone Clone	Vβ2
	G2	Clone	Vβ2
	C1	Cell line	Vβ1
	C7	Cell line	Vβ1
	C8	Cell line	Vβ2+Vβ9
Decidua parietalis	A11	Clone	Vβ4
mPBL	A3	Clone	Vβ16
	B12	Cell line	#
	B1	Cell line	Vβ13.2
	C2	Cell line	Vβ3+Vβ5.1

eral blood were positive for the A2-EBV-GLC tetramer only. The phenotype of the T cell clones generated was shown to be of the effector-memory phenotype (CD8+CD45RA−CCR7−; Fig. 4a and b). Tetramer positivity within these CD8+ T cell subsets was not different when comparing the decidua basalis and decidua parietalis with peripheral blood (data not shown). The Vβ usage was determined for the virus-specific T cell clones and cell lines (Table 4). The Vβ used by T cell clones and cell lines generated from the decidua basalis and decidua parietalis were different from those used by T cell clones and cell lines generated from peripheral blood.

3.4. Cytotoxicity of generated T cell clones

The CD8+ T cell clones were analysed for their cytotoxic capacity towards UCB. None of the T cell clones tested showed cytotoxicity towards UCB in the cell-mediated lympholysis assay nor did these respond to UCB by producing IFN-γ (Fig. 4c). In contrast, significant cytotoxic responses were found towards maternal peripheral blood leukocytes and EBV-LCLs when these target cells were loaded with the HLA-A2-restricted EBV-GLC peptide before the incubation period (Fig. 4d and e). Fig. 4 also shows the expression of the cytolytic molecules perforin (Fig. 4 g) and granzyme B (Fig. 4 h) for the CD8+ T cell clones generated from the decidua parietalis.

3.5. Allo-HLA cross-reactivity by A2-EBV-GLC-specific T cell clones

Cross-reactivity to alloantigens was tested on the basis of IFN-γ production by stimulating the T cell clones with a panel of EBV LCLs, selected to cover the most frequently occurring HLA molecules (Fig. 4c). The T cell clone generated from the decidua parietalis produced IFNɣ upon recognition of HLA-A*02 expressing EBV LCLs loaded with exogenous GLC peptide (Pos ctrl) and some of the EBV LCLs expressing HLA-A*02 without the addition of exogenous GLC peptide, but did not produce IFN-γ when cultured in IL-2 containing medium alone (Neg ctrl). This A2-EBV-GLC-specific T cell clone also produced IFN-γ when stimulated with the HL-A*02-negative EBV LCLs 3 and 9. These EBV LCLs shared HLA-A*29, which was not expressed on any other EBV LCL. In addition, this T cell clone also showed a cytotoxic response towards a third party UCB expressing HLA-A*29 (Fig. 4f). Although we found that the CD8+ T cells did express FasL (Fig. 4i), blocking the Fas–FasL interaction in the cytotoxicity assay did not result in a decreased cytotoxic response towards the HLA-A*29-expressing third party UCB or target cells presenting the A2-EBV-GLC peptide (data not shown).

In contrast, the A2-EBV-GLC-specific T cell clones generated from the decidua basalis did not show a clear pattern of allo-

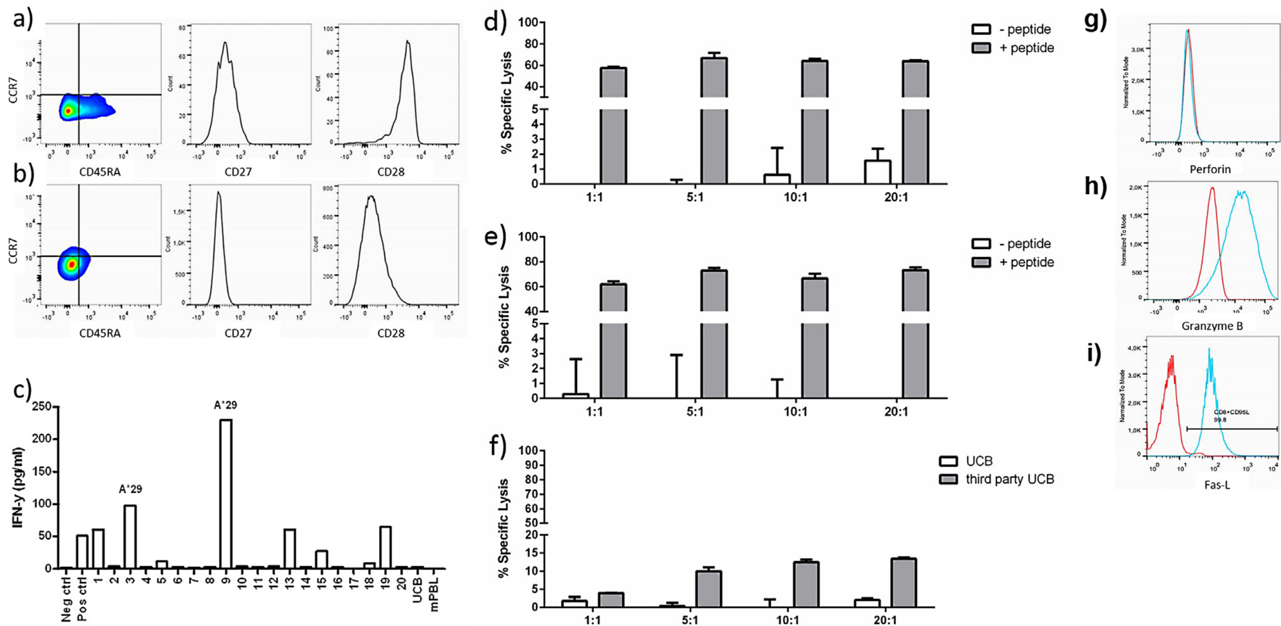

Fig. 4. Allo-HLA-A*29 cross-reactivity by the A2-EBV-GLC-specific memory T cell clone generated from the decidua parietalis. The phenotype of the CD8+ T cells was analysed using antibodies directed against CD45RA, CCR7, CD27 and CD28. The CD8+ T cell population analysed for (a) CD8+ T cells isolated from the decidua parietalis and (b) CD8+ T cell clones generated from the decidua parietalis are shown in this figure. Although the CD8+ T cells isolated from the decidua parietalis showed diversity in CD8 subsets, the CD8+ T cell clone generated from the decidua parietalis was mainly of the effector-memory phenotype (CD8+CD45RA−CCR7−). (c) The T cell clone was stimulated with a panel of EBV LCLs, selected to cover the most common HLA molecules. After 24 h the IFN-γ production was measured in the culture supernatant. The T cell clone produced IFN-γ in response to EBV LCL 3 and 9, which shared expression of the HLA-A*29 molecule, not expressed by other EBV LCLs. The T cell clones were also co-cultured with either maternal peripheral blood leukocytes (d) or an HLA-A*02 expressing EBV-LCL (e) with (+ peptide) or without (- peptide) loading the cells with the A2-EBV-GLC peptide before incubation. (f) In addition, we found a cytotoxic response towards third-party umbilical cord blood cells presenting HLA-A*29, but not towards the umbilical cord blood cells of the woman's own child. The specific lysis is shown as mean values ± SEM. The expression of the cytolytic molecules perforin (g) and granzyme B (h) are shown for the CD8+ T cell clones generated from the decidua parietalis. (i) The expression of Fas-L (CD95L) of the CD8+ T cell clones generated from the decidua parietalis is also shown.

HLA cross-reactivity, whereas the A2-EBV-GLC-specific T cell clones generated from maternal peripheral blood did not produce IFN-γ in response to any of the EBV LCLs.

4. Discussion

To our knowledge, this is the first human study investigating the specificity of CD8+ T cells in the decidua basalis and decidua parietalis. We found a significantly higher percentage of virus-specific CD8+ T cells in the decidua basalis (1.3%) and decidua parietalis (2.2%) compared with peripheral blood (0.5%). During murine pregnancy normal T cell responses were found following primary infection, including a significant infiltration of CD8+ T cells to the fetal–maternal interface (Constantin et al., 2007). In addition, in human pregnancy a dynamic maternal T cell response was detected with a peak in expanding T cell clones during the second trimester and normal levels before parturition (Hoger et al., 1996). Although it is not clear whether these virus-specific CD8+ T cells migrate from the peripheral blood to the decidual tissue or are locally activated, our results suggest that these cells are present in the decidual tissue to protect the fetus from harmful infections.

In previous studies, pregnancy was shown to affect CMV- and EBV-specific immune responses in different ways. The antigen-specific immune response towards EBV was found to be suppressed during pregnancy (Nakamura et al., 1993), whereas no effect of pregnancy on the immune response to CMV was observed (Revello et al., 2006). Actually, Lissauer et al. (2011) found an accumulation of CMV-specific memory cells during pregnancy. In the present study we found a higher number of virus-specific T cells in the decidua, but the generation of clones and/or cell lines from these tetramer-positive cells was very difficult. In only 20% of the pregnancies from which the tetramer-positive CD8+ T cells were sorted from the decidua basalis, decidua parietalis and peripheral blood could the cells be expanded. This success rate is significantly different from those of previous studies (Amir et al., 2010; D'Orsogna et al., 2009, 2010), which showed that in all cases clones and/or cell lines can be generated when tetramer-positive CD8+ T cells are found in the peripheral blood of non-pregnant healthy subjects. These results suggest that during pregnancy the CD8+ T cells might have different characteristics compared with CD8+ T cells in non-pregnant individuals, confirming previous studies describing the alternative phenotype of the decidual CD8+ cytotoxic T cells.

In the current study, lymphocytes isolated from the peripheral blood and decidual tissues were stained with a mixture of tetrameric complexes. In contrast to a previous study in which tetrameric complexes were labelled with different fluorescent dyes to detect and enrich several T cell specificities in a single sample (Newell et al., 2009), all available tetrameric complexes in our study were PE-conjugated. To rule out false-positive CD8+ T cells, we compared single tetramer stainings with the mixture of tetrameric complexes. The percentages of tetramer-positive CD8+ T cells of the single tetramer stainings were added up to determine the expected percentage of tetramer-positive CD8+ T cells. The expected percentage was not different from that found for the mixture of tetrameric complexes.

The distribution of CD8+ T cell subsets in decidual tissues is different from that of peripheral blood (Tilburgs et al., 2010a). In line with these observations, the CD8+ T cells in the decidual tissue collected in the present study were mainly of the effector-memory phenotype (CD8+CD45RA−CCR7−; Fig. 4a and b), whereas the peripheral blood mainly contained naive CD8+ T cells. Tetramer positivity within these CD8+ T cell subsets was not different

when comparing the decidua basalis and decidua parietalis with peripheral blood. These observations suggest that the increased percentage of virus-specific CD8+ T cells in the decidua basalis and decidua parietalis compared with peripheral blood might be due to the different composition of CD8+ T cell subsets in these tissues. The effector-memory cells found in the decidua basalis and decidua parietalis hardly express the effector molecule perforin and only low levels of granzyme B compared with peripheral blood (Fig. 4 g and h). In addition, the virus-specific T cell clones generated from the decidual tissues were considered EM-3CD8+ T cells based on the lack of CD45RA, CCR7, CD27 and CD28 expression (Fig. 4a and b). In line with previous findings (Tilburgs et al., 2010a), these EM-3CD8+ T cells did not express perforin and reduced levels of granzyme B, again confirming the alternative phenotype of decidual CD8+ T cells.

The HLA alloreactivity by virus-specific memory T cells was shown to be common (Amir et al., 2010). The EBV-specific T cell clone, which we could generate from the decidua basalis and decidua parietalis, showed allo-HLA cross-reactivity against HLA-A*29 and was able to lyse HLA-A29-positive allogeneic cells. A possible mechanism for the cytotoxic response, despite the low expression of perforin and granzyme B, could be FasL-Fas interaction. FasL-Fas interaction results in the elimination of pathogens by inducing apoptosis in the target cell, thereby limiting tissue damage and preserving the placenta. However, we did not find a decreased cytotoxic response toward the HLA-A*29-expressing third party UCB or target cells presenting the A2-EBV-GLC peptide when the FasL–Fas interaction was blocked. An alternative explanation might be that specific activation of the clone leads to the induction of granzyme and granzyme-mediated lysis. As the fetal HLA did not include HLA-A*29, the current data do not support the hypothesis that these tetramer-positive T cells might be fetus-specific, but suggest that these memory T cells are more likely to be present in the decidua basalis and decidua parietalis to protect the fetus from a virus infection. The virus-specific CD8+ memory T cells investigated in this study only kill virus-infected cells expressing HLA-A and/or HLA-B and fetal trophoblast cells do not express these HLA molecules.

Therefore, immune surveillance will mainly be focused on maternal cells in the placenta, indirectly protecting the fetus. However, if these cells pass the placenta barrier, these chimeric cells may also react to the maternal HLA on the fetal cells. So far, we were not able to investigate virus-specific CD8+ memory T cells that could potentially kill virus-infected cells that express HLA-C, as HLA-C tetramers were not available. Future studies, using functional assays, may determine whether HLA-C-restricted virus-specific CD8+ memory T cells are present in the decidual tissues.

Taken together, our data suggest that in uncomplicated pregnancy the presence of virus-specific CD8+ T cells in the decidua might protect the fetus from a harmful infection. Whether these cells are already present in early pregnancy remains to be determined. Furthermore, it cannot be excluded that these CD8+ T cells have different characteristics in pregnancies complicated by pre-eclampsia. Pre-eclampsia is predominated by Th1 immunity, with an increase in the production of Th1 cytokines, which induce inflammation (Saito et al., 2007). In pregnancies complicated by pre-eclampsia the virus-specific CD8+ T cells may be more prone to eliciting fetus-specific alloresponses because of the skewing towards Th1 immunity. Although the proportion of peripheral blood CD8+ T cells was comparable in women with pre-eclampsia and healthy controls (Moore et al., 1983; Toldi et al., 2008), the percentage of decidual CD8+ T cells was shown to be increased in placentas of pre-eclamptic women compared with healthy controls (Quinn et al., 2011; Stallmach et al., 1999). In addition, the cytotoxic T cell response to paternal antigens was higher in women with pre-eclampsia than in women with uncomplicated pregnancies (de Groot et al., 2010). The expression of HLA-G was reduced in pre-eclampsia compared with normal pregnancy, resulting in an increased generation of CD8+ T cell alloresponses (Kapasi et al., 2000). However, whether these virus-specific CD8+ T cells contribute to this increased alloresponse remains to be established.

5. Conclusion

The percentage of virus-specific T cells was found to be increased in the decidua basalis and decidua parietalis, suggesting an accumulation of memory T cells in the placenta to protect the fetus from a harmful infection. The list of tetrameric complexes used in this study does not cover the total number of possible specificities of the memory T cells found in either the peripheral blood or the decidual tissue. More extensive studies including more virus-specific T cells are necessary to establish their role in uncomplicated pregnancy and in pregnancy complications.

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