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Fetal-Specific CD8+ Cytotoxic T Cell Responses Develop during Normal Human Pregnancy and Exhibit Broad Functional Capacity

David Lissauer,* Karen Piper,† Oliver Goodyear,† Mark D. Kilby,*,1 and Paul A. H. Moss†,1

Tolerance of the semiallogeneic fetus presents a significant challenge to the maternal immune system during human pregnancy. T cells with specificity for fetal epitopes have been detected in women with a history of previous pregnancy, but it has been thought that such fetal-specific cells were generally deleted during pregnancy as a mechanism to maintain maternal tolerance of the fetus. We used MHC-peptide dextramer multimers containing an immunodominant peptide derived from HY to identify fetal-specific T cells in women who were pregnant with a male fetus. Fetal-specific CD8+ T lymphocytes were observed in half of all pregnancies and often became detectable from the first trimester. The fetal-specific immune response increased during pregnancy and persisted in the postnatal period. Fetal-specific cells demonstrated an effector memory phenotype and were broadly functional. They retained their ability to proliferate, secrete IFN-γ, and lyse target cells following recognition of naturally processed peptide on male cells. These data show that the development of a fetal-specific adaptive cellular immune response is a normal consequence of human pregnancy and that unlike reports from some murine models, fetal-specific T cells are not deleted during human pregnancy. This has broad implications for study of the natural physiology of pregnancy and for the understanding of pregnancy-related complications. *The Journal of Immunology*, 2012, 189: 1072–1080.

V iviparous pregnancy, in which the fetus develops within the mother's body, represents a significant challenge for the maternal immune system, as it requires a state of functional immunological tolerance of the semiallogeneic fetus. This immunological tolerance is not, as originally suggested, solely dependent on a strict anatomical separation of the maternal and fetal blood supply. Indeed, human hemochorial placentation leads to a close juxtaposition between placental villi and the uteroplacental circulation with intimate cellular contact between maternal and fetal tissue (1). However, the unique microenvironment at the decidual interface modulates potential fetal/maternal interactions through restricted MHC expression by human trophoblast (2), tryptophan depletion (3), regulation of complement (4), Fas ligand expression by trophoblast, corticotrophin releasing hormone (5), regulatory T cells (6), or LIF (7).

Moreover, it is now recognized that fetal cells and noncellular material, including placental microparticles (8, 9), are transferred into the maternal circulation early in gestation (10) and that fetal cells may then persist lifelong in the maternal circulation (11) and tissues (12), a phenomenon termed "fetal microchimerism." This transplacental exchange therefore permits immunological interactions between fetal and maternal tissue to occur during pregnancy at a peripheral interface and raises the important question as to how these are initiated and regulated (13). The physiology of murine placentation is markedly different to that of the humans, but murine models have been used to study the development of fetus-specific cellular immune responses during pregnancy. Fetal Ags have been shown to accumulate in peripheral and central lymph nodes that drain the uterus (14), and paternal Ags can be cross-presented by maternal APCs (15). Several murine models have demonstrated the presence of fetal-specific CD8+ T cells during pregnancy (16). An initial proliferation of fetal-specific T cells that are hyporesponsive to maternal tissue has been seen (17–19). Although some models demonstrate partial deletion of this fetal-specific response (17–19) through a process that may be Fas/Fas ligand-dependent (20), other reports have not documented deletion of fetal-specific T cells (21, 22) or any evidence of loss of cellular function (22). Thus, whereas murine models have been valuable for developing insights into fetal-specific immunity during pregnancy, they are inconclusive and sometimes conflicting.

The physiology of placentation and immunological recognition by cytotoxic cells have both been subject to intense evolutionary selection, and there are marked anatomical and immunological differences between murine and human pregnancy (23). Furthermore, the variability of the murine data and supraphysiological magnitude of fetal-specific T cells that often develop in transgenic models mean that insights into the natural physiology of human alloreactive immunity require studies in pregnant women (24).

A maternal cytotoxic T lymphocyte response specific for fetal minor histocompatibility Ags has been demonstrated in many women after human pregnancy (25–28). This is clinically important,

and one example is observed in the outcome of bone marrow transplantation using stem cells harvested from female donors, which leads to an increase in graft-versus-host disease (29–31) and an enhanced graft-versus-tumor effect after transplantation (32–34). It may also be potentially important in the pathogenesis of fetal loss with epidemiological evidence that women with recurrent miscarriage and a male first child may, due to priming against HY Ags, have a reduced chance of future successful pregnancy (35, 36). Additionally, when women with recurrent miscarriage have an HLA class II type known to be able to present immunodominant HY epitopes, this is associated with a reduction in subsequent live births (37).

Although maternal priming against fetal minor histocompatibility responses after pregnancy is now recognized, no studies have examined the development of a maternal CD8^{+} T lymphocyte response to fetal minor histocompatibility Ags during human pregnancy. We have performed an analysis of fetal-specific CD8^{+} T cells throughout human pregnancy through use of MHC-peptide dextramers (Immudex, Copenhagen, Denmark). This reagent consists of a dextran backbone to which multiple MHC-peptide complexes are bound. The MHC-peptide complexes contain an immunodominant epitope derived from an HY-encoded protein (38). In this study, we describe the magnitude and temporal dynamics of this response during uncomplicated human pregnancy and contrast this with the response noted after pregnancy. Additionally, fetal-specific T cells during pregnancy have been characterized, and the properties of fetal-specific T cell clones generated from women during pregnancy have been examined.

Materials and Methods
Study participants

Healthy pregnant women were recruited from antenatal clinics at Birmingham Women's National Health Service Foundation Trust, United Kingdom, between December 2008 and July 2010. Healthy parous non-pregnant controls were obtained from staff members at the hospital. A complete medical and obstetric history was obtained from all participants. Women with medical or obstetric problems, previous miscarriage, previous blood transfusion, or a twin sibling have been excluded from this analysis. Venous blood samples (25 ml) were obtained from HLA*0201 women with a male fetus (n = 42) between 8 wk gestation and delivery alongside an HLA*0201 nonpregnant control cohort with previous sons (n = 42, 8 mo–18 y after delivery). There was no significant difference in age, parity, number of previous sons, ethnicity, or CMV serostatus between the pregnant and nonpregnant cohorts. A further group of control patients was recruited who were currently pregnant with a female fetus, miscarriages, twin siblings, or blood transfusions and were at a range of gestational ages (8–39 wk) (n = 8). Eight additional HLA*0201-negative control donors were also assessed (data not shown).

A separate cohort of HLA*0201-positive women with a male fetus, confirmed on ultrasound (n = 20), were also followed longitudinally throughout pregnancy and into the postnatal period. Venous samples (25 ml) were collected prospectively in each trimester and within 6 wk after delivery. The fetus was confirmed as male postnatally.

The study was approved by the South Birmingham Research Ethics Committee (08/H1207/94).

Flow cytometric identification of HY-specific CD8^{+} T cells with HLA-peptide dextramer

PBMCs or T cell lines were stained with HY-specific dextramer (HLA*0201, FIDSYICQV; Immudex, Denmark) at 22°C for 15 min and washed twice in MACS buffer (PBS/0.5% BSA/2 mM EDTA; Sigma-Aldrich, Dorset, U.K.). Dextramer concentration was optimized by titration against previously obtained HLA*0201 FIDSYICQV CD8^{+} T cell clones and negative controls. An HLA*0201-negative control dextramer (Immudex) was also used. Following dextramer staining cells were washed twice in MACS buffer and then surface stained with the Ab panels used for either HY-specific T cell detection or full memory phenotyping as per Supplemental Table I. Analysis was carried out with an LSR II flow cytometer

(BD Biosciences, San Jose, CA) using FACSDiva software (version 6.1.3). The cytometer was calibrated daily with cytometer setup and tracking beads (BD Biosciences, USA). The flow cytometric gating strategy is shown in supplementary Fig. 1. Single color compensation control beads (BD Biosciences) were included for each experiment and offline automated compensation (FACSDiva version 6.1.3) was used. A positive response was reported when the frequency of HY-specific T cells was >10-fold background determined by the use of the negative dextramer and enriched with magnetic selection.

Expansion of HY-specific CD8^{+} T cells with peptide lines

Short-term cultures to expand HY-specific CD8^{+} T cells were carried out as previously described (19). Briefly, PBMCs were incubated with 15 μg/ml HY peptide (FIDSYICQV; Alta Biosciences, Birmingham, U.K.) in serum-free RPMI 1640 media for 1 h. Cells were then resuspended in RPMI 1640 complete media (containing penicillin/streptomycin (50 μg/ml), L-glutamine (2 mM; Invitrogen, Paisley, U.K.), 10% human serum (TCS Biosciences, Buckingham, U.K.) with the addition of IL-7 (25 ng/ml), and IL-15 (2 ng/ml) (PeproTech, London, U.K.). These cells were cultured for 10 d. From days 3 to 5 cultures were supplemented with IL-2 (100 U/ml; Chiron, Warwickshire, U.K.), and media were changed after 3–4 d with IL-2–containing complete media.

Generation of T cell clones

Following dextramer staining (as above) of cells obtained after expansion in a peptide line, cells were incubated with anti-PE magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 min at 4°C, washed twice, and enriched using "posseld" program on the autoMACS Pro (Miltenyi Biotec). The enriched HY-specific T cells were then cloned by limiting dilution over irradiated (40 Gy), PHA-stimulated PBMCs and EBV lymphoblastoid cell line (LCL), in complete media containing IL-2 (100 U/ml; Chiron), IL-4 (5 ng/ml), and IL-7 (5 ng/ml; PeproTech).

Functional assessment of T cell clones

Clones were initially screened by dextramer. Those clones that were confirmed to stain dextramer were further studied. Cytotoxicity was tested by standard 4 h chromium release assays and cytokine release measured by ELISA examination (BD Pharmingen, Oxford, U.K.) of supernatant following 16 h incubation with targets (24). Targets used were a female A2-positive LCL loaded with HY peptide, male LCLs (HY-positive), a female A2-negative LCL loaded with CMV-specific (irrelevant) peptide, an A2-negative LCL loaded with HY peptide, T cells alone, and LCLs alone. All assays were conducted in triplicate.

Imaging flow cytometry of fetal-specific T cell clones

Cells were stained with HY dextramer (HLA*0201 FIDSYICQV, PE; Immudex) or negative control dextramer (HLA*0201; Immudex) and CD8 (PE-Cy5; Abcam). Cells were kept on ice and run on the ImageStream multispectral imaging flow cytometer (Amnis, Seattle, WA). Images were analyzed using IDEAS version 4.0 image analysis software (Amnis). One thousand cells were collected in each sample, and single-stained compensation controls were used to compensate fluorescence between channel images on a pixel-by-pixel basis using IDEAS software offline compensation.

Gating identified single cells on the basis of aspect ratio versus area on the brightfield channel, and focused cells were selected by gating on a gradient root mean square histogram (rate of change of the image intensity profile) on the brightfield channel. Gating thresholds were set by visual examination of individual cells. Compensation and analysis settings were kept constant between positive and negative control samples.

Real-time PCR for maternal plasma fetal (male) DNA

A TaqMan real-time PCR assay for the DYS14 gene was used to detect and quantify male DNA in maternal plasma, relative to total DNA (39). DNA was extracted from maternal plasma for the assessment of free fetal DNA with the QIAamp DNA Blood Mini Kit (Qiagen, West Sussex, U.K.) as per the manufacturer's directions. DNA was extracted from 400 μl plasma and eluted into 40 μl eluent. Amplification of DYS14 and β-globin was performed as single-plex reactions. The primer and probe sequences were as follows: DYS14, forward, 5′-GGG CCA ATG TTG TAT CCT TCT C-3′, reverse, 5′-GCC CAT CGG TCA CTT ACA CTT C-3′, probe, FAM-MGB, 5′-TCT AGT GGA GAG GTG CTC-3′, β-globin, forward, 5′-GTG CAC CTG ACT CCT GAG GAG A-3′, reverse, 5′-CCT TGA TAC CAA CCT GCC CAG-3′, probe, VIC-MGB, 5′-AAG GTG AAC GTG GAT GAA GTT GGT GG-3′ (primers were obtained from Alta Bioscience, Birmingham,

U.K.; probes were from Applied Biosystems). DYS14 primers (300 nM each), DYS14 probe (100 nM), B2M primers (300 nM each), and B2M probe (100 nM) were used.

DNA amplification was carried out on the ABI Prism 7500 sequence detection system (Applied Biosystems). After an initial activation of uracil-N-glycosylase (2 min at 50°C) and 10 min at 95°C, amplification was carried out during 40 cycles (15 s at 95°C, 60 s at 60°C). A fluorescence threshold value was determined as 10 standard deviations above the mean of the background fluorescence emission for all wells between cycles 3 and 15. Fluorescence less than this threshold after 40 cycles was determined to be a negative result.

Standard curves for DYS14 and β-globin were produced using DNA extracted from male PBMCs and quantified spectrophotometrically (Nano-Drop; Thermo Scientific), and the DNA concentration was calculated based on each cell containing 6.6 pg DNA. Ten-fold dilutions were prepared ranging from 10^7 to 1 genomic equivalents per reaction. These standards were included in duplicate in every assay, and a standard curve was constructed from which the copy number for patient samples could be extrapolated. These standard curves demonstrated the assay was sensitive to 1 copy per reaction and linear to at least 10^7 copies. In each assay samples were prepared in duplicate with two male positive controls, two negative controls from women with no previous sons, and two water controls in each assay.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software). To determine differences between two independent groups, a Mann–Whitney U test was performed, and for paired data a Wilcoxon signed rank test was used. When analyzing parametric data with more than two groups, ANOVA with a Tukey post hoc test was used. The null hypothesis was rejected at a p value of <0.05.

Results

HY-Specific CD8^{+} T cells can be detected in 50% of women who are pregnant with a male fetus

HLA-peptide dextramers were used to identify CD8^{+} T cells specific for the HLA*0201-restricted HY minor histocompatibility Ag FIDSYICQV in the blood of women with a male pregnancy. The data were compared with a cohort of nonpregnant women who were matched for age and parity and had a history of at least one previous male pregnancy.

In initial experiments PBMCs were incubated with FIDS peptide and cultured for 10 d to increase the precursor frequency of Ag-
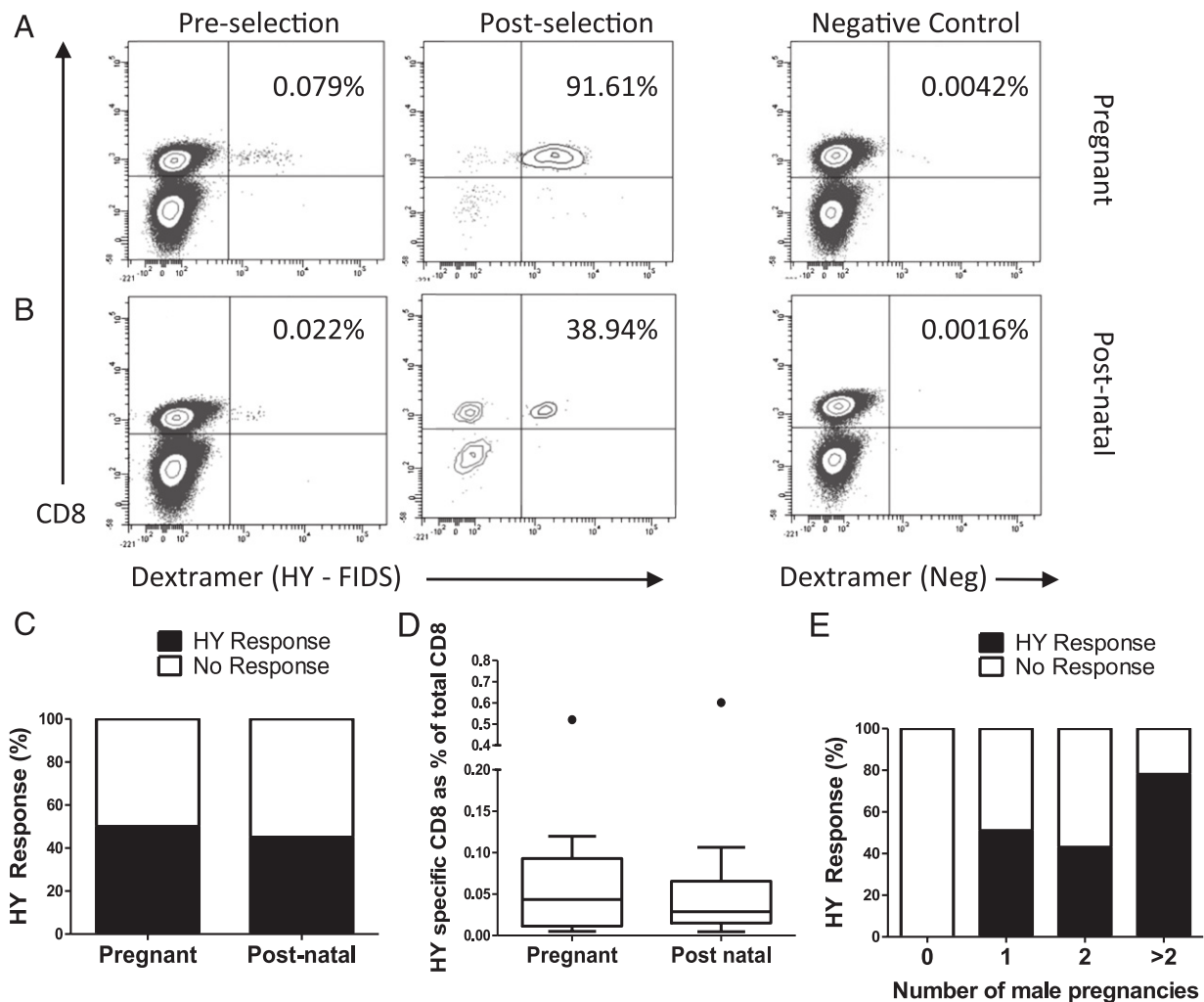

FIGURE 1. Detection of fetal-specific CD8^{+} T cells during and after human pregnancy. Flow cytometry contour plots illustrate the detection using MHC-peptide dextramers of fetal (HY minor histocompatibility Ag, FIDS)-specific CD8^{+} T cells in representative women with a male fetus, following 10 d peptide line expansion, during (**A**) and after (**B**) normal pregnancy. The *left column* shows the frequency of fetal-specific T cells prior to magnetic enrichment. The *middle column* demonstrates magnetic enrichment of fetal-specific T cells, and the *right column* shows a negative control using an HLA-matched dextramer with a negative control peptide. Percentages represent fetal specific T cells as a percentage of CD8^{+} T cells. The rate of detection of fetal-specific T cells is illustrated in women during (n = 42) and after (n = 42) pregnancy with a male fetus (**C**). In women in whom a fetal-specific T cell response was detected the frequency of fetal-specific T cells as a percentage of total CD8^{+} T cells is compared during (n = 21) and after (n = 19) pregnancy (**D**). The rate of detection of fetal-specific T cells with a woman's total number of male pregnancies (including current pregnancy) is shown (one male, n = 31; two males, n = 44; more than two males, n = 9). A negative control with no male pregnancies (n = 8) is included (**E**).
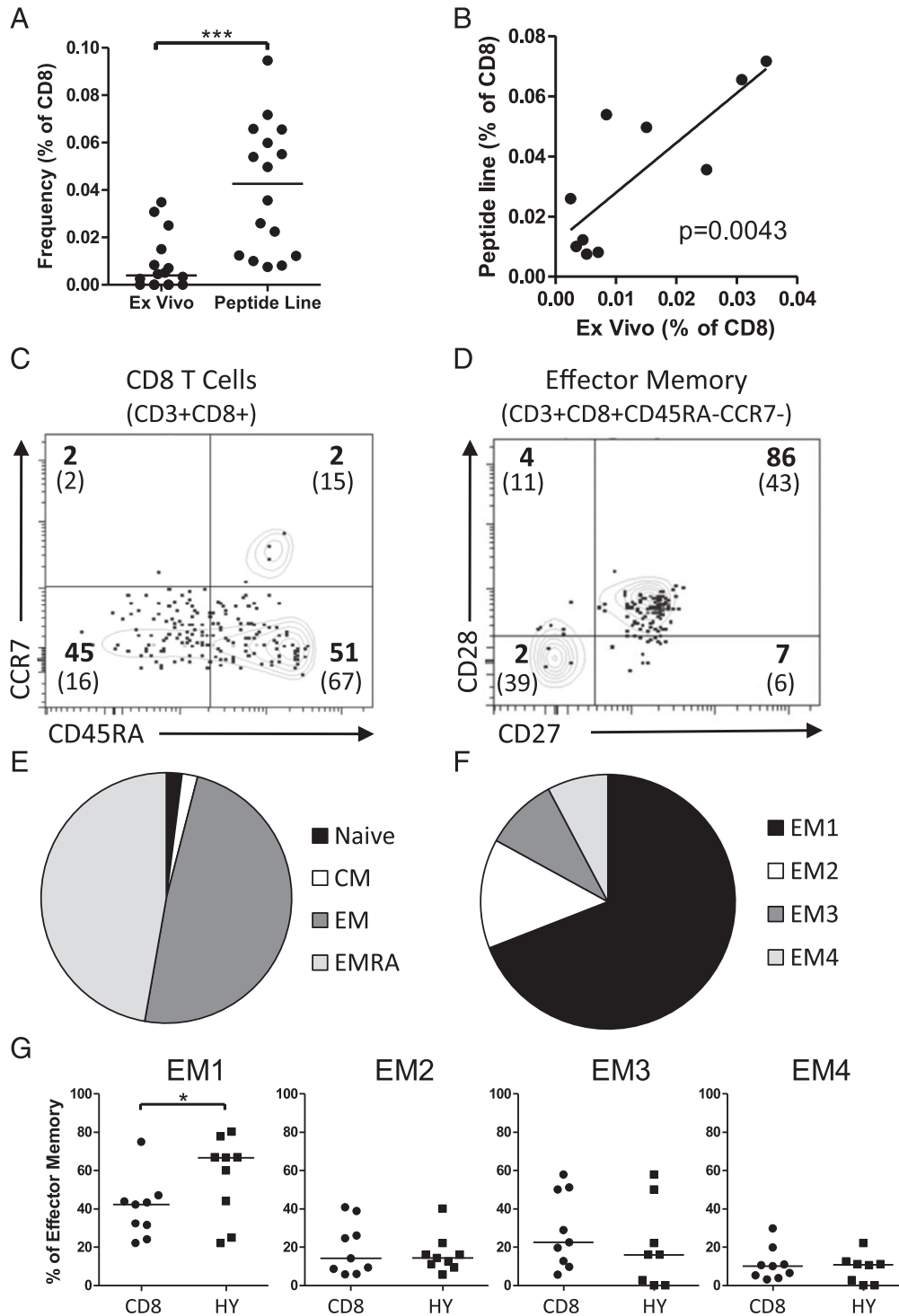
FIGURE 2. Ex vivo detection and memory phenotype of fetal-specific T cells during human pregnancy and their expansion in culture. The frequency of fetal-specific CD8^{+} T cells from women during pregnancy ex vivo and following peptide line expansion is compared in paired samples ($n = 16$) (**A**). The frequency of fetal-specific CD8^{+} T cells is correlated between peptide line and ex vivo conditions (**B**), in patients in whom a response was detected in both settings ($n = 10$). Representative examples of the memory phenotype of fetal-specific T cells illustrates fetal-specific T cells as a density plot in black, with percentages in each quadrant in bold, superimposed on a contour plot demonstrating the overall CD8^{+} T cells phenotype, with overall percentages in each quadrant in parentheses. The memory phenotype examined on the basis of CCR7 and CD45RA is illustrated (**C**) and EM (CD45RA⁻CCR7⁻) cells further examined on the basis of CD27 and CD28 expression (**D**). The memory phenotype (median of 10 ex vivo samples) is described as naive (CD45RA+CCR7+), central memory (CM; CD45RA⁻CCR7+), EM (CD45RA⁻CCR7⁻), and EMRA revertant (EMRA; CD45RA+CCR7⁻) (**E**). The median EM phenotype has been further subdivided into EM subsets on the basis of CD28 and CD27 expression into EM1 (CD27+CD28+), EM2 (CD27+CD28⁻), EM3 (CD27⁻CD28⁻), and EM4 (CD27⁻CD28+) (**F**). The EM phenotype of CD8^{+} T cells overall is contrasted with the HY-specific T cell subsets (*$p = 0.036$ by Wilcoxon signed rank test; bars indicate median) (**G**). ***$p \leq 0.001$ by Wilcoxon signed rank test; bars indicate median.
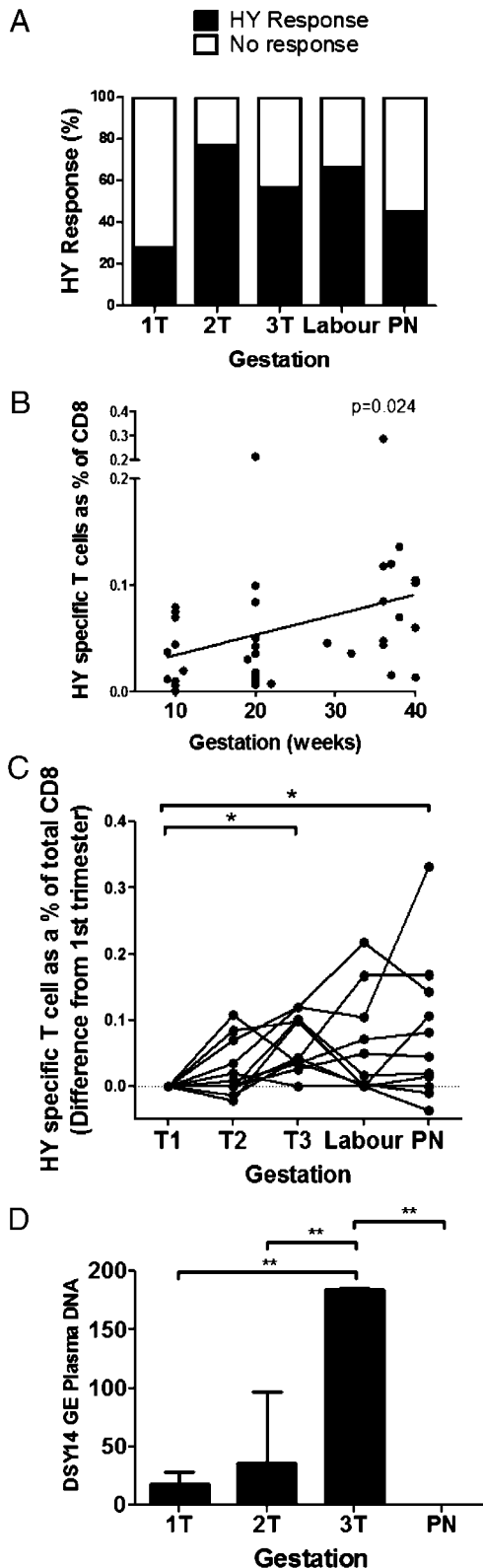
FIGURE 3. Fetal-specific CD8^{+} T cells increase in frequency during human pregnancy. Rate of detection by MHC-peptide dextramers of fetal (HY minor histocompatibility Ag, FIDS) Ag-specific T cells at different gestational ages is demonstrated in this cross-sectional analysis of women with a male pregnancy in the first trimester (1T) ($n = 11$), second trimester (2T) ($n = 12$), third trimester (3T) ($n = 11$), during labor ($n = 6$), or within 6 wk of delivery (PN) ($n = 17$) after peptide line expansion (**A**). In the same cohort, in women in whom a fetal-specific T cell response was detected ($n = 39$) the magnitude of response is correlated with gestational age in weeks during pregnancy (**B**). In a separate, prospectively recruited, longitudinal

specific cells and define the sensitivity of the detection method. Cells were then stained with PE-labeled dextramer, after which microbeads coated with PE-specific Ab were used for positive enrichment of the stained fraction. Representative examples show the detection of HY-specific CD8^{+} T cells in maternal blood during pregnancy (Fig. 1A) and in postpartum samples (Fig. 1B). These donors are selected to demonstrate examples of the frequency of staining in the quartile above (Fig. 1A) and below (Fig. 1B) the median. The specificity of the dextramers was illustrated by the lack of staining of CD8^{−} T cells and the low background staining using the negative control dextramer. HY-specific T cell responses were never identified in women without a previous gestation of a male fetus and who were currently pregnant with a female fetus ($n = 8$).

Fetal-specific T cells were detected in 50% of women during pregnancy, and this was comparable to the frequency of detection of 45% in the cohort with a previous male pregnancy history (50 versus 45%, $p = 0.57$) (Fig. 1C). The average magnitude of the HY-specific response after in vitro culture was 0.043% of the total CD8^{+} T cell pool during pregnancy, and this was also similar to the value measured from samples taken from women with a previous history of a male pregnancy (0.043 versus 0.029%, $p = 0.86$) (Fig. 1D). As previously reported (26), a history of multiple male pregnancies appeared to increase the proportion of women in whom HY-specific T cells could be detected, but this did not reach statistical significance (one male versus more than two males, $p = 0.15$) (Fig. 1E).

HY-specific T cells can be detected directly within peripheral blood of pregnant women with a median frequency of 0.008% of the CD8^{+} T cell pool

Having established that HY-specific T cells could be detected in many pregnant women following short-term expansion with peptide in vitro, we then went on to examine the precursor frequency and phenotype of these cells directly within peripheral blood. Blood samples were obtained from 16 HLA*0201 pregnant women with a male fetus in whom HY-specific T cells had been seen following in vitro expansion. HY-specific T cells were observed in 10 of these donors, with a median frequency of 0.008% of the CD8^{+} T cell pool. Interestingly, the cells expanded readily in vitro to a median value of 0.04% of CD8^{+} T cells, showing that fetal-specific CD8^{+} T cells retain proliferative potential and are not anergic to Ag ($p \leq 0.0001$) (Fig. 2A). Importantly, the frequency of HY-specific T cells detected within peripheral blood correlated closely with that observed following short-term 10 d culture, which reveals a uniform proliferative potential between donors and demonstrates the validity of in vitro culture as a measure to examine the temporal kinetics of immunity during pregnancy ($p = 0.0043$) (Fig. 2B). There were six women (38%) in whom fetal-specific T cells could not be detected directly in peripheral blood despite their presence following short-term culture, including one individual from whom HY-specific T cell clones was obtained. This demonstrates the increased sensitivity of detection

cohort of women with a male fetus, the frequency of fetal-specific T cells is examined in simultaneously assessed frozen specimens collected throughout pregnancy and the puerperium ($n = 11$) following peptide line expansion. The percentage of fetal-specific T cells relative to the response in the first trimester is plotted for each individual (*$p < 0.05$ by ANOVA with Tukey post hoc test) (**C**). Male free fetal DNA (DYS14 gene) in maternal plasma was measured by quantitative real-time PCR in each trimester and the postnatal (PN) period ($n = 12$, error bars indicate interquartile range; **$p \leq 0.01$ by ANOVA with Tukey post hoc test) (**D**).

that follows short-term culture, a finding that has been documented in many previous studies of immune function.

Fetal-specific maternal T cells in the peripheral blood of pregnant women have an effector memory phenotype

Dextramer staining was then combined with multiparameter flow cytometry to determine the phenotype of the HY-specific CD8$^+$ T cells within the peripheral blood of pregnant donors ex vivo. CD45RA and CCR7 were used to divide CD8$^+$ T cells into naive (CD45RA$^+$CCR7$^+$), central memory (CD45RA$^-$CCR7$^+$), effector memory (EM; CD45RA$^-$CCR7$^-$), and highly differentiated "revertant" CD45RA$^+$ EM subsets (CD45RA$^+$CCR7$^-$) (Fig. 2C) (40). The phenotype of HY-specific cells from 10 donors during pregnancy revealed a predominant effector memory phenotype with approximately equivalent proportions of cells with CD45RA$^-$ or CD45RA$^+$ expression ($p = 0.0039$) (Fig. 2E). Interestingly, very few HY-specific T cells displayed a central memory phenotype, which reveals a limited capacity for fetal-specific cells to migrate into secondary lymphoid tissue.

Further subdivision of CD8$^+$ memory subsets on the basis of expression of the costimulatory molecules CD27 and CD28 was also performed. In particular, within the CCR7$^-$CD45RA$^-$ EM subset four further subsets are defined on the basis of CD27 and CD28 expression and are termed EM1 (CD27$^+$CD28$^+$), EM2 (CD27$^+$CD28$^-$), EM3 (CD27$^-$CD28$^-$), and EM4 (CD27$^-$CD28$^+$) (Fig. 2D) (41). Analysis of EM subsets from 10 donors revealed that HY-specific T cells are less highly differentiated compared with the global CD8$^+$ pool, with 67% falling into the CD27$^+$CD28$^+$ EM1 pool compared with 42% of total CD8$^+$ T cells ($p = 0.039$) (Fig. 2F, 2G). Although the distribution of memory and EM subsets was relatively variable between donors, it was not possible to correlate this with pregnancy history or gestation in view of the relatively small size of the group (Supplemental Fig. 2).

As previously observed in respect to proliferation, the HY-specific cells demonstrated a functional response to presentation of HY peptide in vitro and underwent further phenotypic differentiation with induction of CD45RO expression and loss of CD27 and CD28 (data not shown).

HY-specific T cells become detectable during the first trimester and expand during pregnancy

The dynamics of the development of the fetal-specific CD8$^+$ T cell response during pregnancy was then examined in further detail. HY-specific T cell responses could be detected in 28% of women from as early as the first trimester of pregnancy (Fig. 3A). The proportion of women in whom a HY-specific response was detected increased during pregnancy, although this did not reach statistical significance ($p = 0.1057$). However, when the magnitude of the HY-specific CD8$^+$ T cell response was examined, a correlation was seen between the frequency of HY-specific T cells and gestational age ($p = 0.03$). The median frequency of the HY-specific T cell response after short-term culture was 0.035% of the CD8$^+$ population in women at 10 wk pregnancy and increased by 140% to a value of 0.085% at term (Fig. 3B).

To investigate this observation in more detail we performed a prospective study in an additional cohort of 20 HLA*0201-positive women who were pregnant with a male fetus. Blood samples were taken in each trimester, during labor, and in the postnatal period. HY-specific T cells were observed in 11 of these subjects, and longitudinal analysis confirmed that the frequency of HY-specific T cells increased between the first and the third trimester, with an overall frequency that was comparable to that documented in the cross-sectional study (Fig. 3C). The magnitude of the response was unchanged during labor but increased again in the postpartum period.

To relate the changes in the frequency of HY-specific T cells to Ag load, we went on to examine the presence of free fetal DNA in maternal plasma by real-time PCR quantification of the *DYS14* gene from the Y chromosome. Free fetal DNA is thought to be derived predominantly from fetal trophoblast that has been shed into the maternal circulation (42) and was used as a surrogate for fetal Ag. From the first to third trimester there was a >10-fold increase in free fetal DNA in the maternal circulation. (Fig. 3D)
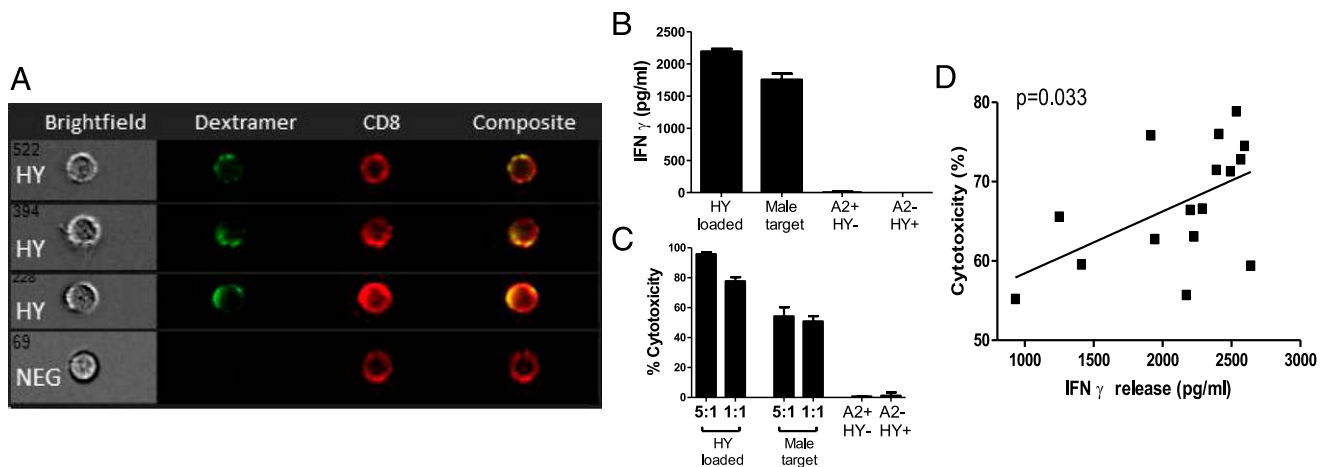

FIGURE 4. Specificity and function of fetal-specific CD8$^+$ T cell clones. HY-specific T cell clones were isolated from women during and after pregnancy. Following expansion by peptide stimulation and enrichment by magnetic selection, CD8$^+$ T cell clones were generated by limiting dilution analysis. Initial screening of clones was carried out by dextramer staining and flow cytometry. The dextramer staining of fetal-specific T cells is visualized by imaging flow cytometry. This demonstrates brightfield images, the dextramer staining (green), CD8 staining (red), and a composite image (colocalized staining yellow). The *first three rows* illustrate the HY HLA*0201 FIDS dextramer and the *bottom row* shows negative control cells (original magnification ×40) (**A**). Further characterization of clones was via assessment of IFN-γ release (**B**) and cytotoxicity by chromium release assay (**C**). Specificity was determined in both systems by assessment of function against female HLA*0201 cells loaded with HY peptide (HY loaded), male HLA*0201 targets (male target), HLA*0201 cells loaded with CMV peptide (A2$^+$HY$^-$), and HLA*0201-negative cells loaded with HY peptide (A2$^-$HY$^+$). Target cells and T cells alone were also assessed (data not shown). Error bars show range of triplicate experiments. Cytotoxicity assays were carried out at a 5:1 and 1:1 ratio. The cytotoxicity and IFN-γ release by clones was correlated (**D**).

This was rapidly cleared after delivery and became undetectable in the postpartum period, although after delivery the maternal immune response was maintained (Supplemental Fig. 3). Because the free fetal DNA was rapidly cleared after delivery, male free fetal DNA was not detected in women with a current female fetus, even when they had a history of previous male pregnancies.

HY-specific T cell clones generated from women during pregnancy are cytotoxic and secrete IFN-γ following recognition of male cells

In view of our frequent detection of HY-specific T cells in women during pregnancy, we went on to examine the functional capacity of the T cells in vitro. Initially we used imaging flow cytometry to examine the pattern of distribution of TCR and CD8 at the cell membrane to determine their colocalization in lipid rafts (43), which has been revealed as an important determinant of function and staining by HLA-peptide multimers (44). Dextramer-staining of the HY-specific T cell clones demonstrated a punctate pattern of membrane staining that colocalized with CD8 in all cases (Fig. 4A).

To determine the functional capacity of fetal-specific cells from pregnant women, HY-specific CD8⁺ clones were incubated with FIDSYICQV-pulsed target cells or primary male cells and the pattern of cytokine release (*n* = 17) and cytotoxicity of target cells (*n* = 18) were then measured (Fig. 4B, 4C). All of the clones demonstrated potent cytotoxic potential with the ability to lyse peptide-pulsed target cells at low E:T ratios. Moreover, clones isolated either during or after pregnancy were both able to recognize natural male target cells, which reveals their capacity to recognize endogenously processed Ag. Interestingly, the cytotoxic capacity and IFN-γ cytokine release of individual HY-specific T cell clones was highly correlated (Fig. 4D) and no differences were seen in the functional capacity of clones generated either during or after pregnancy (Fig. 5A, 5B).

HY-specific CD8⁺ T cell clones exhibit a wide variation of avidity for HLA-peptide multimers

Thirty-three HY-specific CD8⁺ T cell clones were isolated from women during pregnancy and a further 77 from blood taken during the postnatal period. The clones were screened for HY specificity by dextramer staining and, interestingly, the intensity of staining of individual clones showed marked variation. A consistent observation was the development of a population of low-intensity dextramer staining clones that was isolated from women during pregnancy (Fig 5C). Importantly, the intensity of staining remained consistent for individual clones during cell culture, indicating that differential avidity for dextramer was an intrinsic cellular property. At a functional level, the low-intensity dextramer staining clones remained capable of IFN-γ secretion and were able to lyse male target cells. Indeed, no correlation was observed between the intensity of HY dextramer staining and the pattern of cytotoxicity or cytokine release by individual clones (Fig. 5D).

Discussion

Cellular immune responses against fetal alloantigen have been previously reported in women with a history of pregnancy (25–28). To our knowledge, in this cohort study we have investigated for the first time whether fetal-specific CD8⁺ T cell immune responses can develop during pregnancy or are primed only after parturition. Our results demonstrate that many women develop T cell immune responses against fetal Ag from the first trimester of pregnancy and that these cells have potent functional capacity.

We used MHC-peptide multimers to identify and characterize alloreactive T cells present within maternal blood during human
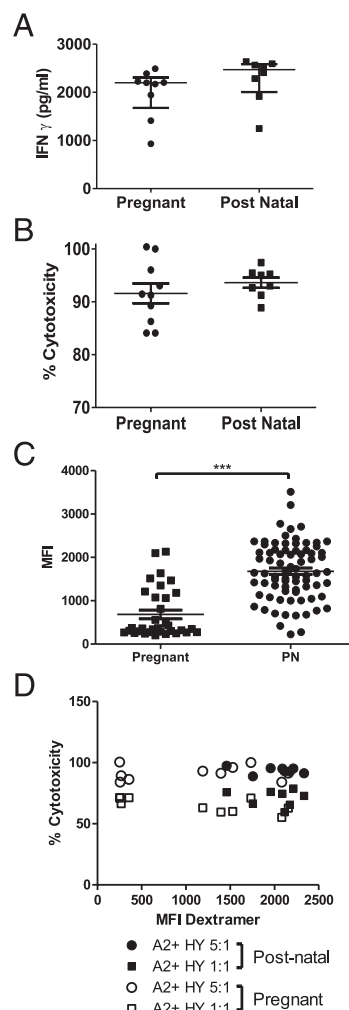

FIGURE 5. Comparison of fetal-specific T cell clone function, with clones derived during and after pregnancy. Production of IFN-γ (*n* = 17) (**A**) and cytotoxicity (**B**) (shown at a 5:1 ratio) (*n* = 18) are compared between T cell clones isolated during or after pregnancy. The intensity of MHC-peptide dextramer staining is compared between T cell clones isolated during (*n* = 33) or after (*n* = 77) pregnancy, with a large number of low-intensity T cell clones isolated during pregnancy (**C**). The lack of correlation between intensity of dextramer staining of clones isolated during (open symbols) and after pregnancy (filled symbols) and cytotoxicity at an E:T ratio of 5:1 (circles) and 1:1 ratio (squares) is illustrated (**D**). ***$p \leq 0.001$ by Mann–Whitney U test.

pregnancy. To maximize the sensitivity of detection of HY-specific T cells we elected to use short-term in vitro culture with peptide to expand the cells. This technique has been employed widely to study many Ag-specific responses, including those associated with alloreactivity (26, 27), viral infection (45), and cancer (46). We adopted a conservative approach and limited our assay to only a single episode of Ag exposure with a short 10-d culture period. This technique is unable to generate primary immune responses (47), and no responses were found in women without a history of male pregnancy. The magnitude of the HY-specific CD8⁺ T cell response detected following in vitro expansion correlated closely with the direct ex vivo frequency within peripheral blood, although, as anticipated, expansion in culture allowed greater sensitivity of detection.

One finding of our study was that fetal Ag-specific CD8⁺ T cells were detected in half of all women during pregnancy and were seen as early as 8 wk gestation. We found no evidence for an overall deletion of the fetus-specific response during gestation but

instead the alloreactive population increased in magnitude as pregnancy progressed. The results contrast with many murine models (16), which have often demonstrated maternal cellular "awareness" of fetal Ag during pregnancy but generally reveal deletion and anergy of the fetal-specific CD8^{+} T cell response (17–20).

HY-specific CD8^{+} T cells studied directly within maternal blood had an EM phenotype, although the relative proportion of central memory, EM, and revertant CD45RA^{+} memory cells was variable between different women with no clear correlation between memory subset distribution and clinical characteristics. One notable feature was that HY-specific EM cells retained high levels of expression of CD27 and CD28. CD27^{+}CD28^{+} EM1 cells share features with central memory cells and have longer telomeres than EM cells that have lost CD27 and CD28 (41). This relatively early differentiated phenotype is likely to reflect limited Ag exposure in vivo and may indicate that, after priming, there is limited exposure of HY-specific cells to fetal Ag. Alternatively, FOXP3^{+} regulatory T cells, which are suggested to be important in fetomaternal tolerance during pregnancy, may potentially operate to limit T cell differentiation (48).

We isolated HY-specific CD8^{+} T cell clones from women both during and after pregnancy. An interesting observation was that individual clones stained with different levels of intensity following incubation with the HY-HLA-A2 dextramer. This feature was an intrinsic property of each T cell clone and was retained throughout the culture period. The intensity of HLA-peptide multimer staining has been shown to be a reliable indicator of avidity for Ag, and the findings reveal a striking heterogeneity of T cell avidity in recognition of fetal tissue that we have not previously observed in many studies of virus-specific immune responses. At this stage we have not detected significant differences between functional properties of dim- and bright-staining T cell clones. Low-intensity multimer staining has also been observed on alloreactive T cells that exhibit regulatory properties, and this deserves further investigation in the setting of pregnancy (49, 50). HY-specific T cells studied in murine TCR-transgenic models can also show TCR downregulation, and it has been speculated that this is a mechanism to reduce functional capacity during exposure to male tissues (50). Whether modulation of the expression level of TCR is a particular feature of the fetal-specific immune responses that develop during human pregnancy will need further study.

There was no difference in the proportion of women in whom fetal-specific T cells could be detected during pregnancy or in the postpartum period. This is of interest as it shows that pregnancy is sufficient for priming of the alloreactive response, and there is no evidence of further induction of immunity after parturition. The magnitude of the fetal-specific response was also comparable with no evidence of potential boosting secondary to any fetomaternal hemorrhage during labor.

The finding of a fetal-specific response developing from early in gestation raises the question of whether a response would be initiated and maintained even if the pregnancy ended in miscarriage. The investigation of fetal-specific T cell responses in women with a history of miscarriage, who were excluded from the present study as the gender of the miscarried fetus would not have been known, will be an important area for future study.

These data are also intriguing in relation to the potential requirement for Ag during maintenance of the HY-specific immune response. Levels of fetal Ag in the maternal circulation are known to fall markedly following delivery (51), but this does not appear to compromise the magnitude or function of the HY-specific T cell population. Persistence of low levels of fetal Ag as fetal microchimerism following pregnancy (52) may be important in this regard. T cell clones isolated from women many years after pregnancy were

certainly fully functional with high levels of cytotoxicity and cytokine production.

The circulation of significant numbers of functional fetal-specific CD8^{+} T cells in pregnant women may seem surprising. However, these data are in agreement with some studies in HY transgenic mice that demonstrated that the presence of such cells did not affect litter size even when the CD8^{+} T cells were found at the maternal/fetal interface (22). This suggests that mechanisms other than deletion are critical factors enabling the fetus to escape from recognition by fetus-specific T cells. Downregulation of HLA expression on trophoblast and syncytiotrophoblast is one possible factor in this regard, although expression of HY Ag on these tissues did not affect pregnancy viability in a murine model (22). The functional potential of fetal-specific T cells was clear following in vitro expansion, but it is possible that in vivo, during healthy pregnancy, they remain in some way functionally attenuated or anergic. Further possibilities include suppression through regulatory T cells (6) or immunomodulatory hormones such as progesterone. HLA class I-restricted regulatory cells have been reported in pregnancy (50), but study of CD4^{+} regulatory cells is limited by poor definition of HLA class II HY epitopes and technical challenges associated with class II peptide-MHC multimers.

Finally, one may speculate on the potential physiological and pathological role of the cellular alloreactive T cell immune response that develops during pregnancy. Alloreactive responses in this context are not necessarily detrimental, and uterine NK cells certainly have an important role in promoting uterine vascular remodeling. Indeed, allogeneic pregnancy has been suggested to offer a survival advantage compared with syngeneic pregnancy (53), and a relative lack of CD8^{+} T cell infiltration into decidua has been associated with pregnancy complications such as preeclampsia (54).

However, there is also the potential that this phenomenon may have detrimental consequences. In "villitis of unknown etiology," excessive infiltration of maternal CD8^{+} T cells into the villous tree of the placenta is associated with fetal growth restriction, pregnancy loss, and stillbirth (55). Furthermore, the potential impact on fetal physiology was recently illustrated by the demonstration that maternal T cells trafficking into the fetal circulation are the main barrier to engraftment following in utero hematopoietic cell transplantation (56). Our observations indicate that the study of adaptive T cell immune responses against fetal tissue should be an important area for future obstetric investigation.

Disclosures

The authors have no financial conflicts of interest.

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