Cytomegalovirus sero positivity dramatically alters the maternal CD8+ T cell repertoire and leads to the accumulation of highly differentiated memory cells during human pregnancy

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BACKGROUND: Human pregnancy offers an immunological challenge for the immunocompetent women accommodating an allogenic fetus, while continuing to combat potentially infectious disease. Cytomegalovirus (CMV) infects the majority of the human population and establishes lifelong persistence, which can lead to the oligoclonal expansion of differentiated T cells. Primary CMV infection and, less commonly, secondary infection during pregnancy can cause fetal disease and morbidity. The balance between maternal immune competence and viral pathogenicity is thus delicately poised. Our objective was to investigate the influence of CMV serostatus on maternal CD8+ T-cell phenotype and cytokine profile in an apparently healthy cohort of pregnant women. Furthermore, we assessed if CMV serostatus modulated changes in CD8 T cells during gestation.

METHODS: CD8+ T-cell phenotype was investigated in 87 pregnant women with samples obtained both during pregnancy [CMV immunoglobulin G (IgG) + n = 39, CMV IgG− n = 21] and in the early post-natal period (IgG+ n = 16, IgG− n = 11). Multiparameter flow cytometry was used to study T-cell phenotype and HLA-peptide tetramers identified CD8 T cells specific for CMV. Levels of 26 plasma cytokines, chemokines and chemokine receptors were assessed in a separate cohort of 20 women (IgG+ n = 10, IgG− n = 10) followed longitudinally during and after pregnancy.

RESULTS: CMV seropositivity profoundly influenced the T cell repertoire and its dynamics during pregnancy. Naive CD8+ T-cells (CCR7+CD45RA+) were reduced by 50% in CMV-seropositive women. The proportion of CD45RA effector cells was not increased in CMV-seropositive donors, although this population was more highly differentiated with reduced CD27 and CD28. However, there was a doubling in the proportion of CD45RA+ revertant memory cells (CCR7−CD45RA+) in seropositive donors. Moreover, seropositive women during late pregnancy demonstrated an accumulation of highly differentiated CMV-specific T-cells. T-cell activation independent of CMV was also seen in late pregnancy. No CMV-related changes in plasma cytokines, chemokines or their receptors were observed.

CONCLUSIONS: Thus, CMV serostatus is a crucial consideration in studies of T cell memory and differentiation during pregnancy. The reduction in maternal naive T cells in CMV-seropositive donors could have implications for the maternal response to infections during pregnancy. These findings shed light on the delicate balance between host, fetus and chronic infection during healthy pregnancy and will inform studies in relation to the importance of CMV on maternal and fetal health.

Key words: cytomegalovirus / immunology / T cell / pregnancy
**Background**

During pregnancy, the maternal immune system must adapt to allow survival of the allogenic fetus while also controlling invasion of the uterine decidua by extralobular trophoblast (Scalf et al., 2006) and maintaining the ability to respond to infections (Constantin et al., 2007). It is now recognized that there is a complex immunological interaction between mother and fetus, resulting in maternal immunological awareness of the fetus. (Tafuri et al., 1995; Verdijk et al., 2004; Piper et al., 2007; Moldenhauer et al., 2009). CD8+ T cells are common at the decidual interface between the mother and fetus (Williams et al., 2009) and may have an important role in normal pregnancy and disorders such as recurrent miscarriage (Piccinni, 2006).

CD8+ cytotoxic T lymphocytes (CTL) demonstrate considerable heterogeneity with marked differences in the expression of molecules such as cytokines, chemokines and co-stimulatory molecules observed (Lanzavecchia and Sallusto, 2005; Appay et al., 2008). CTL may be separated into four major subsets on the basis of two surface cell markers: CD45RA, which is the high molecular weight isoform of the CD45 common lymphocyte marker, and the chemokine receptor CCR7 (Sallusto et al., 1999). These subsets are termed the naïve (N; CD45RA+CCR7+), central memory (CM; CD45RA−CCR7+), effector memory (EM; CD45RA−CCR7−) and highly differentiated ‘CD45RA revertant’ EM populations (EMRA; CD45RA+CCR7−). naïve and CM cells express CCR7 which is crucial for their ability to circulate through secondary lymphoid tissue. In contrast, loss of CCR7 and expression of alternative chemokine receptors allows EM and EMRA subsets to migrate to peripheral tissues. The CD45RA isoform of CD45 is expressed on naïve T cells but is spliced to CD45RO following T cell activation. CD45RA is re-expressed in subpopulations of memory cells and this EMRA pool is considered as a more highly differentiated subset with strong ex vivo cytolytic capacity but reduced replicative potential (Appay et al., 2008). The additional surface marker CD57 is also increased on this population and its expression is inversely proportional to telomere length (Brenchley et al., 2003).

Further subdivision of CD8 memory subsets on the basis of expression of the co-stimulatory and pro-survival molecules CD27 and CD28 can also yield extra information. In particular, within the EM subset (CCR7−CD45RA−) four further subsets can be defined based on their CD27 and CD28 expression. These functionally distinct subsets are termed EM1 (27+28+), EM2 (27+28−), EM3 (27−28−) and EM4 (27−28+). This classification is useful as assessment of CTL function shows that EM1 cells share features with CM cells while EM3 cells are functionally more similar to EMRA cells (Romero et al., 2007).

There has been no comprehensive assessment of the memory phenotype of peripheral CD8+ T cells during the course of human pregnancy. In contrast, the phenotype of cytotoxic T cells in the decidua has been studied in more detail and they appear mainly of the EM or EMRA subtypes (Tilburgs et al., 2010).

Recent studies have shown that cytomegalovirus (CMV) serostatus can have a profound influence on the profile of the human CD8+ T cell repertoire. CMV is a ubiquitous beta herpes virus, which establishes lifelong infection (Sinclair and Sissens, 2006). The prevalence of infection increases with age, geographic location and socio-economic group, and in women of reproductive age seropositivity is over 90% in developing countries, but <50% in many western countries (Kenneson and Cannon, 2007; Kaye et al., 2008). Infection gives rise to the development of a large population of CMV-specific T cells which are characterized by a highly differentiated phenotype with a predominant EM or EMRA phenotype, low expression of CD27 and CD28 and high levels of CD57 (Moss and Khan, 2004).

Primary maternal CMV infection is the leading cause of neonatal infectious morbidity (Pass et al., 2009). However, infection can also be transmitted to the fetus from mothers with CMV reinfecion or reactivation (Kenneson and Cannon, 2007) and the relative balance of host-virus immunity during pregnancy is therefore of considerable interest. The role of chronic CMV infection on the T cell repertoire in women during pregnancy, and the interaction of this infection with the unique immunological environment of pregnancy, have not been systematically evaluated.

We hypothesized that CMV seropositivity may alter the peripheral CD8+ T cell repertoire in human pregnancy through the expansion of highly differentiated CMV-specific T cells and cause immunological changes during healthy gestation. We therefore studied the global CD8+ T cell repertoire, frequency of CMV-specific CD8 T cells and plasma cytokine levels during apparently healthy human pregnancy, and related these observations to CMV serostatus.

**Materials and Methods**

**Study participants**

Between February 2009 and April 2010, 87 healthy pregnant women were recruited from antenatal clinics at Birmingham Women’s NHS Foundation Trust, UK. Women with medical or obstetric problems, recurrent miscarriage or prior blood transfusion were not recruited. Venous samples were obtained during pregnancy (CMV immunoglobulin G (IgG) + n = 39, CMV IgG− n = 21) and the early post-natal period (IgG+ n = 16, IgG− n = 11). In those pregnant women who were CMV IgG+ CD8 T cell responses to multiple CMV-specific epitopes were measured for each sample. The epitopes tested were determined by the maternal HLA type (n = 105).

Venous blood samples were obtained between 10 weeks gestation and delivery and post-natal samples were obtained within the first 6 weeks after delivery. Twenty millilitres of heparinized blood, and 5 ml of blood anticoagulated with EDTA were obtained at each time point.

The participants had a median age of 31 (range 18–46), with no significant difference in age, parity or miscarriages between CMV-seropositive and -seronegative women. There were significantly more women of white English ethnicity in the CMV-seronegative group (P ≤ 0.005), as would be expected (Supplementary data, Table S1). For the measurement of plasma cytokines, a separate cohort of women had plasma samples collected prospectively in each trimester and the early post-natal period (<1 week post-natal). Therefore, a total of four samples per participant were collected. (CMV IgG+ n = 10 participants, 40 samples, CMV IgG− n = 10 participants, 40 samples). The longitudinal assessment of the frequency of CMV-specific T cells was also carried out in a further cohort of five CMV-seropositive women who had blood samples prospectively taken in each trimester and the post-natal period. Peripheral blood mononuclear cells (PBMCs) were cryopreserved and then analysed simultaneously for each individual. The study was approved by the South Birmingham Research Ethics Committee (08/H1207/94).
Generation of HLA-peptide tetramers

Six major histocompatibility complex-peptide tetramers were utilized to identify CMV-specific T cells. HLA-A*0101 tetramers incorporating the pp65 residue YSEHPTFTSQY (YSE) and pp50 residue VTEHDLTLYL (VTE). HLA-A*0201 tetramers incorporating the pp65 residue NLVPQMIVAT (NLV). HLA-B*0702 tetramers incorporating the pp65 residues RPHRFNGFTVL (RPH) and TPRVTGGGAM (TPR) and HLA-B*8801 tetramers incorporating the IE-1 residue ELKRRKMIYM (ELK). These were all synthesized using standard methods (Altman et al., 1996).

Sample preparation

PBMCs were isolated from 20 ml heparinized venous blood by Ficoll density gradient centrifugation (Lymphoprep, Axis Shield, Oslo, Norway). Cells were cryopreserved and stored in liquid nitrogen prior to use for flow cytometry. A separate 2.7 ml sample anticoagulated with EDTA was used for the isolation of donor plasma and DNA. DNA was extracted from PBMC (QUIAGEN, Blood and Tissue Kit) and stored at −20°C prior to use. Plasma was stored at −80°C.

Donor selection

The CMV serostatus was determined by CMV IgG Enzyme Immunoassay test kit (BioCheck, Inc) as per the manufacturer's instructions. CMV IgG-seropositive donors were then HLA-typed using a PCR-based method (Bunce et al., 1995) to enable the appropriate tetramers to be used for each donor.

Flow cytometry

Thawed PBMCs were incubated with the appropriate tetramer at 37°C for 15 min. After washing with phosphate-buffered saline (containing 2% fetal calf serum), cells were incubated with a 10 colour antibody panel. Analysis was carried out on an LSR II flow cytometer (BD Biosciences) using FACS DIVA software (version 6.1.3). Instrument configuration and antibodies used are listed in Supplementary data, Table S2. The cytometer was calibrated daily with cytometer setup and tracking beads (BD Biosciences). A meticulous gating strategy was utilized to exclude aberrant binding events (Chattopadhyay et al., 2008), shown in Fig. 1. Briefly, single cells are first identified on an forward scatter-area (FSC-A) versus forward scatter-height (FSC-H) plot. Lymphocytes were gated on the basis of standard light scatter properties. A ‘dump’ channel was subsequently used to exclude cells which could bind tetramer and monoclonal antibodies non-specifically, including dead cells, monocytes and B-cells. T lymphocytes were then selected on the basis of CD3 expression. Finally, tetramer ‘positive’ cells were visualized against CD8 expression. Proportions of tetramer ‘positive’ cells are given as a percent of CD8-positive cells. ‘Fluorescence minus one’ controls were used to determine gating thresholds (Herzenberg et al., 2006). For each sample phenotypic gating was initially carried out by reference to the tetramer negative cell population, where populations could be clearly defined due to the larger cell populations and the same gating template was then applied to the tetramer positive population to determine the phenotype of the tetramer positive cells. Single colour compensation control beads (BD Biosciences) were included for each experiment and offline automated compensation (FACS DIVA version 6.1.3) was used.

Quantitative real-time PCR for CMV viral load

A Taqman real-time PCR assay was utilized to enable detection and quantification of CMV copy number from maternal peripheral blood. Amplification of the CMV UL132 gene (GenBank Accession AY446894) was performed using the Applied Biosciences Gene Expression Assay (Pa03453400_s1, Applied Biosciences) according to the manufacturer's instructions. DNA extracted from cultured CMV (strain AD169) was quantified spectrophotometrically (Nanodrop, Thermo Scientific) and the DNA concentration calculated. Ten-fold dilutions were prepared ranging from 10⁷ to 10² copies per reaction. These standards were included in duplicate in every assay and a standard curve constructed from which the viral load for patient samples could be extrapolated. These standard curves demonstrated that the assay was sensitive to <10 copies per reaction and linear up to 10⁷ copies. In each assay, samples were prepared in duplicate with two positive controls from patients with known viramia, two negative controls from CMV IgG-negative patients and two water controls in each assay. DNA amplification was carried out on the PE-ABI 7500 sequence detection system. After activation of the uracil-N-glycosylase (2 min at 50°C) and AmpliTaq Gold (10 min at 95°C), amplification was carried out over 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.

Maternal plasma cytokine measurement

A multiplex fluorescent bead-based immunoassay (Panomics, UK) was used to analyse plasma cytokine, chemokine and chemokine receptor levels. Factors analysed were interferon-γ, tumour necrosis factor-α (TNF-α), TNF-β, interleukin-1 beta (IL-1β), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13 and IL-15, vascular endothelial growth factor, monocyte chemotactic peptide 1 (CCL2), MIP1α (macrophage inflammatory protein-1 alpha), CCL3), MIP1b (CCL4), RANTES (regulated on activation and normally T cell expressed and presumably secreted), CCL5, CTACK (cutaneous T-cell attracting chemokine, CCL27), MIG (monokine induced by gamma-interferon), CXCL9, ITAC (interferon-inducible T-cell alpha chemoattractant, CXCL11), SDF1 (stromal cell derived factor-1, CXCL12), Fractalkine (CX3CL1), matrix metalloproteinase-3 (MMP-3), MMP-9, fibroblast growth factor-basic. Samples were analysed using Starstation Luminex software (version 3.0).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software). To determine differences between two groups, a non-parametric Mann–Whitney test was performed, and when multiple tests were performed the Bonferroni correction was used. When analysing more than two groups a non-parametric Kruskal–Wallis test was used, with a Dunn post test to compare all groups. The null hypothesis was rejected at a P-value of <0.05.

Results

CMV IgG seropositivity is associated with a marked reduction in the proportion of naïve T cells and an expansion of CD45RA⁺ memory cells in pregnant women

Flow cytometric analysis was used to determine the memory phenotype of CD8⁺ T cells within the peripheral blood of healthy pregnant women on the basis of CD45RA and CCR7 expression (Fig. 2a). These data were then related to the presence of CMV-specific antibody in order to determine how ‘latent’ CMV infection modified the CD8⁺ T cell repertoire. Example contour plots from two CMV IgG seropositive and two CMV IgG-seronegative donors are illustrated in Fig. 2.
CMV seropositivity was associated with a dramatic reduction in the proportion of naive CD8+ T cells which represented a median of only 23% in the CMV-seropositive group compared with 46% in CMV-seronegative donors (P = 0.0024) (Fig. 2d). The proportion of memory CD8+ T cells is therefore increased from 54% in CMV-seropositive donors to 77% in CMV-seropositive donors during pregnancy. (Box represents interquartile range and whiskers represent maximum and minimum values, ** denotes P < 0.01, *** denotes P < 0.001).

CMV infection did not significantly alter the proportion of EM or CM CD8+ T cells (Fig. 2e and f). However, the proportion of CMV-seronegative women to 77% in CMV-seropositive donors and it was of interest to determine the pattern of distribution in individual CD8+ memory subsets.

**Figure 1** Gating strategy. Contour plot of gating strategy involving: exclusion of doublets and other irregularly shaped cells, gating of lymphocytes on the basis of forward and side-scatter properties, a dump channel to exclude dead cells, monocytes (CD14) and B cells (CD19), then selection of CD3+ lymphocytes. In this example, tetramer positive cells are then displayed against CD8. The black box gate highlights tetramer positive CD8+ T cells.

**Figure 2** CMV-seropositive pregnant women have a more differentiated CD8+ T-cell phenotype. CD8+ T cells can be separated into naïve, CM, EM and highly differentiated EM re-expressing CD45RA (EMRA) on the basis of CCR7 and CD45RA expression (a). Two representative contour plots from CMV IgG positive (b) and CMV IgG negative (c) donors show the CD8+ T-cell phenotype on the basis of CCR7 and CD45RA expression. Summary data comparing the CD8 T-cell phenotype in CMV IgG seropositive (n = 39) and seronegative (n = 21) donors during pregnancy are displayed illustrating naïve (CD45RA+CCR7+) (d) effector memory (CD45RA−CCR7−) (e) CM (CD45RA−CCR7+) (f) and highly differentiated EM (TEMRA, CD45RA+CCR7−) subsets (g). Percentage of CD3+CD8+ T-cell positive for CD27 (h), CD28 (i) and CD57 (j) were also compared between CMV IgG-seropositive and -seronegative individuals. (Box represents interquartile range and whiskers represent maximum and minimum values, ** denotes P < 0.01, *** denotes P < 0.001).
‘revertant’ CD45RA+ effector cells was markedly increased in the CMV-seropositive group to a median of 38% compared with only 17% in CMV-seronegative women (P = 0.0002) (Fig. 2g). The expression of the anti-apoptotic and co-stimulatory molecules CD27 and CD28 was also examined on the CD8 repertoire. In CMV-seropositive women, there was a significant reduction in expression of both CD27 (median 59 versus 87%, P ≤ 0.0001) (Fig. 2h) and CD28 (median 47 versus 82%, P ≤ 0.001) on CD8+ T cells (Fig. 2i). In contrast, expression of CD57 which is a marker of late T cell differentiation was greatly increased in seropositive pregnant women (median 44 versus 19%, P ≤ 0.001) (Fig. 2j).

**CMV seropositivity drives EM CD8+ T cells towards a more differentiated phenotype in pregnant women**

CMV infection is associated with an increase in memory cells in healthy donors and it was therefore of interest to observe that there was no increase in the proportion of CD45RA−CCR7− effector cells in CMV-seropositive pregnant women. In order to investigate this observation in more detail, we studied the expression of CD27, CD28 and CD57 on CD8+ T cells within this subset. CD27 and CD28 expression can be used to classify effector cells into four functionally distinct memory subsets (Romero et al., 2007) termed EM1 (CD27+CD28+), EM2 (CD27+CD28−), EM3 (CD27−CD28−) and EM4 (CD27−CD28+) (Fig. 3a). A representative contour plot of these populations, obtained after gating on viable CD3+CD8+CD45RA−CCR7− cells, is shown in Fig. 3a.

Expression of CD27 and CD28 is lost during CD8+ T cell expansion and differentiation, with EM1 being the least-differentiated subset and EM3 representing the most differentiated compartment. This is confirmed by our analysis of CD57 expression on the EM subsets of all donors, with highest expression of this marker being seen on EM3 cells (Fig. 3b).

When EM cells were classified into these four subsets and compared according to CMV serostatus, clear differences could be demonstrated between CMV-seropositive and -seronegative pregnant women. The CD45RA−CCR7− subset within CMV-seropositive women exhibited a markedly more differentiated pattern, as demonstrated by an increase in the EM3 subset from 3% in seronegative donors to 15% in the CMV-seropositive group (P ≤ 0.0001). In contrast the size of the EM1 subset, which shares functional similarities with CM cells but is resident within tissue rather than secondary lymphoid organs, was reduced in seropositive donors (median 52 versus 72%, P ≤ 0.0001). The EM4 subset was also increased in CMV-seropositive women (median 13 versus 20%, P = 0.0002), whereas the proportion of EM2 cells was similar in both groups (median 9 versus 12%, P = 0.75) (Fig. 3c).

**The more differentiated CD8+ T cell repertoire seen in CMV-seropositive pregnant women is due to expansion of CMV-specific T cells**

As CMV serostatus was found to have such a marked effect on the global CD8+ T cell repertoire of pregnant donors, it became of

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![Figure 3](image-url)
interest to see if the effects were due to expansion of CMV-specific CD8+ T cells or reflected a more general effect on all CD8+ cells. HLA-peptide tetramers containing immunodominant CMV epitopes were therefore used to identify and phenotype CMV-specific T cells within the study cohort. Six tetramers were available and example contour plots contrasting the CMV-specific T-cell phenotype for four epitopes in two donors in comparison with the global CD8+ T-cell repertoire are shown in Fig. 4a. As expected, CMV-specific cells did not exhibit a naive phenotype and CM cells were also rarely observed. Significant heterogeneity of phenotype was observed both between different individuals and in relation to individual epitopes. However, the overall phenotype of CMV-specific T cells correlated precisely with the changes observed in the global CD8+ cell repertoire in CMV-seropositive women. In particular, the CMV-specific cells were largely focused in the revertant effector CD45RA+ subset and expressed high levels of CD57 with frequent loss of CD27 and CD28 (Fig. 4b).

**CMV serostatus does not alter the concentration of cytokine, chemokine or growth factor levels within peripheral blood**

As profound changes had been observed within the distribution of CD8+ memory subsets in relation to CMV serostatus, we proceeded to investigate if this was associated with an alteration in the level of cytokines, chemokines or growth factors within maternal plasma. However, multiplex analysis of 26 factors did not show any significant differences in plasma concentration between CMV-seropositive and -seronegative women (Supplementary data, Table S3). Changes in these factors were also compared within the three trimesters of
pregnancy and the early postnatal period but again no significant differences were observed (data not shown).

Prospective analysis within individual donors reveals that the proportion of CMV-specific T cells peaks in the third trimester and returns to baseline in the postnatal period

The unique and dynamic immunological environment of pregnancy raised the question as to how the CMV-specific immune response may be modulated in relation to gestational stage. Samples were therefore obtained longitudinally from each trimester and the postnatal period in five donors and these were studied in a prospective analysis. The frequency of CMV-specific T cells peaked in the third trimester in four cases, and in three of these the CMV-specific T cell response doubled between the first and third trimesters (Fig. 4c). Interestingly, this increase returned to baseline by 6 week postnatally. The CMV-specific immune response is known to increase the following episodes of viral reactivation (Cwynarski et al., 2001) and we therefore used a sensitive quantitative real-time PCR assay to look for evidence of subclinical viraemia. However, no evidence of CMV reactivation was detected within the peripheral blood mononuclear cells of any women included in the study (data not shown).

When the CMV-specific immune response was measured by cross-sectional analysis of the whole patient cohort in each trimester, no significant differences were seen in relation to gestation (Fig. 4d).

Gestational changes in global and CMV-specific T-cell phenotype

Examination of how the phenotype of the global CD8+ T cell repertoire changed during gestation and the puerperium was performed. The differences between seropositive and seronegative individuals in naive and EMRA proportions during pregnancy previously described (Fig. 2) remain statistically significant when each trimester is compared. Therefore, we divided the cohort into CMV-seropositive and -seronegative groups and contrasted these two populations. Within CMV-seropositive donors, there was a marked increase in the percentage of ‘revertant’ CD45RA+ effector cells which rose to a peak value of 46% in the third trimester (Fig. 5a), with a corresponding reduction in the proportion of EM cells throughout gestation. In CMV-seronegative women, this effect was not observed and in these individuals there were no statistically significant changes in phenotype during gestation (Fig. 5b).

The distinct pattern of changes during gestation in CMV-seropositive women was reflected in significant changes in the phenotype of the CMV-specific T cell pool during pregnancy. The major observation was a marked shift in the proportion of CMV-specific memory cells from CD45RA-EM to a ‘revertant’ CD45RA+ memory profile. This pattern was observed primarily between the second and third trimesters with EM cells falling from a median of 58–32% (P < 0.05) and EMRA increasing from a median of 35–65% (P < 0.01) (Fig. 5c).

Expression levels of CD38, a marker of T cell activation, show a selective increase during pregnancy on the CD45RA+ CD8+ memory subset

CD38 is an exonuclease and is often used as a marker of T lymphocyte activation and proliferation. Previous reports have identified an increase in expression during pregnancy and this was also observed in our cohort, where it was independent of CMV serostatus (Fig. 6a). In particular, the increased levels of CD38 were observed during late gestation and the early postnatal period (P < 0.05) irrespective of CMV serostatus. The use of multi-parameter flow cytometry allowed us to further characterize CD38 expression on CD8+ T cell subsets. CD38 expression was specifically increased in the EMRA subset and there was no statistically significant increase in naïve, EM or CM subsets (Fig. 6c). The expression level of CD38 increased by 2.5-fold on TEMRA cells from the first to the third trimester (P < 0.01) and this effect was stable during the early postnatal period (first trimester versus early postnatal P < 0.001).

Discussion

These data demonstrate that CMV seropositivity has a dramatic effect on the CD8+ T cell repertoire during pregnancy. CMV IgG-seropositive women have a more differentiated peripheral CD8+ T-cell phenotype with a 50% reduction in the proportion of naïve T cells and a significant increase in EMRA cells. In addition, even though the proportion of EM cells was not increased in the CMV-seropositive group there was a marked shift from the less differentiated EM1 to the more differentiated EM3 subset, characterized by the loss of the co-stimulatory molecules CD27 and CD28. This work has confirmed that these effects were directly due to the accumulation of CMV-specific T cells through the use of six HLA-peptide tetramers containing immunodominant CMV epitopes which revealed that the global changes were reflected in the phenotype of the CMV-specific T cells. Furthermore, CMV seropositivity was shown to cause a difference in the immunological changes observed in the maternal immune system in response to pregnancy. In the seropositive group, there was a large increase in the proportion of CMV-specific EMRA cells within the peripheral blood during the third trimester of pregnancy. This effect altered the overall CD8+ T-cell phenotype in CMV-seropositive women but no change was seen in seronegative women. ‘Latent’ CMV infection in seropositive women therefore alters not only the overall CD8+ repertoire but also the dynamics of the CD8 T cell response during pregnancy. We also noted an increase in T cell activation during gestation, predominantly due to increases in CD38 expression within the EMRA subset and this was independent of CMV infection.

Our group and others (Hooper et al., 1999; Looney et al., 1999; Chidrawar et al., 2009) have demonstrated CMV infection results in a more highly differentiated T-cell phenotype within peripheral blood and this is most clearly observed in the elderly. However, the influence of CMV infection on the CD8 T cell repertoire has not been investigated in the context of pregnancy. We have demonstrated that this effect extends to the pregnant population and while we do not believe this effect is unique to pregnancy it has been shown to be an important consideration in this patient group. There is an extensive reproductive immunology literature regarding the role of CD8+ T cells within maternal blood and decidua during pregnancy, but this has not accounted for CMV serostatus as a potentially important confounding factor. Rates of CMV infection are highly variable within different cohorts and our data reveal that CMV infection status is an important consideration when comparing CD8 T cell memory subsets and differentiation between different pregnant cohorts.
The finding in CMV-seropositive pregnant women of a relative increase in late differentiated CD8+ T cells with a loss of CD27 and CD28 expression is a pattern that is considered a marker of immune senescence and part of the 'immune risk phenotype'. Indeed, CMV seropositivity and elevated antibody titre appear, at least in the elderly, to be associated with excess mortality (Derhovanessian et al., 2009; Pawelec et al., 2009; Strandberg et al., 2009; Roberts et al., 2010). It is not yet clear if these detrimental effects occur only at the extremes of age or if their presence in a pregnant cohort could also influence maternal health or pregnancy outcome.

The relative reduction in naive lymphocytes and accumulation of CMV-specific clonal expansions has been proposed to have a detrimental impact on the ability of the host to respond to infections (Akbar and Fletcher, 2005). In support of this it has been observed that in CMV-seropositive elderly individuals (Trzonkowski et al., 2003) and in those with accumulations of CD8+ CD28− negative cells there is an inverse correlation with responsiveness to vaccination (Goronyz et al., 2001; Saurwein-Teissl et al., 2002). As the same phenotypic changes in the T cell repertoire have been observed during pregnancy further investigation is warranted to identify whether CMV seropositivity is detrimental to responses to infection or vaccination in a pregnant cohort.

Recent analyses have identified that CMV seropositivity is associated with a 1.5-fold relative risk for the development of pre-eclampsia (Xie et al., 2010) though these findings require confirmation in larger cohorts. It is interesting to speculate if the cellular changes we observed could contribute to this increased risk. However, the current study was limited to the examination of CD8 T cells in peripheral blood. Future work determining if these peripheral changes influence the phenotype of CD8 T cells found within the decidua, and if this in turn has effects on pregnancy outcome will be necessary.

In seropositive women in the third trimester, there was a significant increase in effector CTL (CD45RA+CCR7−) compared with the second trimester, which was not observed in seronegative women and was due to CMV-specific CTL. This demonstrates that infection history can alter the maternal immunological response to pregnancy. It is appreciated that this experimental design cannot determine if the change was caused by increased mobilization of EMRA cells or due to a change in phenotype with a re-expression of CD45RA on circulating EM cells. It has been shown that the immunological response to

**Figure 5** During gestation, there are changes in the global T-cell phenotype in CMV-seropositive women due to CMV-specific T cells. The frequency of naive (CCR7+CD45RA+), CM (CCR7+CD45RA−), EM (CCR7−CD45RA−) and EMRA (CCR7−CD45RA+) cells as a percentage of total CD8+ T cells is illustrated in CMV seropositive (n = 55) (a) and seronegative (n = 32) (b) women during pregnancy and the post-natal period. Changes in the frequency of CMV-specific CD8+ T cells identified by HLA-peptide tetramers containing immunodominant CMV epitopes are shown in the CM, EM and highly differentiated EM cell subsets as a percentage of total CD8+ T cells in CMV-seropositive women (c). (T1, first trimester; T2, second trimester; T3, third trimester, PN, post-natal. *denotes P < 0.05; ** denotes P < 0.01, error bars indicate standard error).
exercise is similarly effected by the previous infection history and that exercise preferentially mobilizes CMV-specific EMRA cells into the peripheral blood and this is thought to be driven by catecholamines (Turner et al., 2010). A potential explanation for the effect we observed during pregnancy could similarly be through a hormonally driven mechanism that alters T cell mobilization or differentiation. An alternative explanation is that, although CMV was not detectable in peripheral blood of these women during pregnancy, local reactivation during pregnancy is common, with virus excretion into breastmilk found in most seropositive women (Hamprecht et al., 2001). The increase in the tissue-homing EMRA population in late pregnancy could therefore be a response to this local reactivation during late pregnancy.

Prospective longitudinal analysis also revealed that the proportion of CMV-specific T cells overall peaked within the third trimester of pregnancy in a majority of individuals. This was not observed with a cross-sectional analysis due to the high variability between individuals in the magnitude of their CMV-specific CD8+ T cell response. This effect was not due to systemic viral reactivation or reinfection as sensitive quantitative PCR demonstrated that this was not accompanied by CMV viraemia. Changes in CMV-specific T-cell mobilization or a response to localized viral reactivation could again be responsible.

CD38 is a marker of T cell activation and proliferation. During pregnancy, there was a significant increase in expression of CD38 on maternal CD8 T lymphocytes. This overall effect was predominantly due to increased expression in the EMRA subset. This effect was not determined by previous CMV infection. This finding confirms previous studies suggesting a gestational increase in T cell activation (Mikyas et al., 1997; Truong et al., 2010), but the T cell subsets involved or the relationship with previous CMV infection had not been described. Understanding the expression of CD38 during pregnancy is important as high levels of CD38 expression are observed on decidual T cells (Abadia-Molina et al., 1996; Ho et al., 1996) and is an important determinant of prognosis in HIV infection (Liu et al., 1997; Froebel et al., 2000). Our findings reveal that CMV seropositivity does not appear to be responsible for the pregnancy-related

**Figure 6** T cell activation increased during gestation, independent of CMV serostatus. CD38 expression on CD8+ T cells was compared between CMV IgG-seropositive (n = 39) and seronegative (n = 21) individuals during pregnancy. (a) Changes in the expression of CD38 on CD8+ T cells was investigated during gestation and the early post-natal period (n = 87) (b) CD38 expression on CD8+ T cells was further characterized by its expression on naïve (CD45RA+CCR7+), EM (CD45RA−CCR7−), CM (CD45RA−CCR7+) and EMRA (CD45RA+CCR7−) T cells (c). (T1, first trimester; T2, second trimester; T3, third trimester; PN, post-natal, *denotes P < 0.05, **denotes P < 0.01, ***denotes P < 0.001).
increase in CD38 on CD8 T cells and suggest that this may therefore either be a hormonal effect, such as that seen to regulate levels of CD38 on the myometrium during pregnancy (Dogan et al., 2006) or be related to peripheral T cell activation by alloimmune fetal antigen.

It is interesting that these profound changes in CD8 T-cell phenotype were seen without significant differences being observed in plasma cytokines, chemokines and chemokine receptors when seropositive and seronegative individuals were compared. The measurement of plasma factors was conducted in a relatively small cohort and a conservative statistical approach was adopted, hence it is possible that very subtle CMV-induced changes could have been missed by this analysis. However, changes such as those observed for the cellular phenotype were clearly not present. Thus, while the cellular phenotypic changes are important and demonstrate altered cellular differentiation, it may be that in the context of latent infection, without antigenemia due to viral reactivation, the differences in effector cell secretory functions in vivo are less pronounced. Further studies investigating directly the modulation of T cell cytotoxicity and secretory functions in response to CMV antigens during pregnancy would enable this to be better defined.

The impact of previous CMV infection on the CD8 T cell repertoire during pregnancy was dramatic and CMV status must therefore be considered whenever investigating CD8 T cell memory subsets and differentiation between different pregnant cohorts. How these profound immunological changes during pregnancy that are induced by previous CMV infection alter maternal health or pregnancy-related outcomes such as the incidence of pre-eclampsia or responses to new infections during pregnancy now requires further investigation.

Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors’ roles
D.L., M.C., A.P. and O.G. significantly contributed to the study design, execution and manuscript drafting. P.A.H.M. and M.D.K. played a key role in study design, interpretation and manuscript revision. All authors contributed to the writing of the final manuscript and approved it for publication.

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