Homeostasis and function of T cells in healthy individuals and renal transplant recipients
Havenith, S.H.C.

Citation for published version (APA):
Havenith, S. H. C. (2012). Homeostasis and function of T cells in healthy individuals and renal transplant recipients

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
HUMAN VIRUS-SPECIFIC EFFECTOR-TYPE T CELLS ACCUMULATE IN BLOOD BUT NOT IN LYMPH NODES

Simone H.C. Havenith1,2,*, Ester B.M. Remmerswaal1,2,*, Mirza M. Idu3, Ester M.M. van Leeuwen1, Karlijn A.M.I. van Donselaar2, Anja ten Brinke4, Nelly van der Bom-Baylon1, Fréderike J. Bemelman2, René A. W. van Lier1,4 and Ineke J. M. ten Berge2

1 Department of Experimental Immunology, Academic Medical Center, Amsterdam, the Netherlands
2 Renal Transplant Unit, Department of Internal Medicine and
3 Department of Surgery, Academic Medical Center, Amsterdam, the Netherlands,
4 Landsteiner Laboratory, Sanquin Research, Amsterdam, the Netherlands.
* both authors contributed equally to this manuscript

Blood (2012) 119: 1702-12
ABSTRACT

It is believed that the size of the CD8+ T cell pool is fixed and that with every new viral challenge the size of pre-existing memory cell population shrinks to make way for the new virus-specific cells. CMV-seropositive individuals have high numbers of CMV-specific resting-effector type CD8+ T cells in their peripheral blood (PB). This prompted us to analyze if CMV infection limits immunological space at sites where immune reactions are initiated, i.e. in lymph nodes (LN). LN and paired peripheral blood samples were analyzed for CMV-, EBV- and influenza-specific CD8+ T cells. In marked contrast to blood, LN contained significantly low numbers of CX3CR1-expressing effector-type CD8+ T cells whereas the CMV-specific cells which were found in LN resembled poly-functional memory-type cells. In contrast, EBV- and influenza-specific CD8+ T cells were highly similar between peripheral blood and LN both in number and function. Thus, CMV-specific CD8+ T cells in LN unlikely restrain immunological space of other virus-specific cells.
INTRODUCTION

The elderly have an increased susceptibility to infections and impaired responses to vaccination, which may be caused by aging of the immune system, also referred to as immunosenescence. This poses a major challenge to public health, which may become even greater as the percentages of older people increase in the Western population. Various factors such as an impaired innate immune system but also reduced T and B cell responses may contribute to immunosenescence. Longitudinal studies in the aged (>85 years) have suggested that poor immunological responses are associated with an “immune risk profile”. Characteristics of this risk profile are an inverted CD4/CD8 ratio and increased numbers of CD27−CD28−CD57+ effector-type CD8+ T cells. We and others have shown that the presence of large numbers of these CD27−CD45RA− and CD57−CD28− CD8+ T cells, which are largely overlapping populations, is associated with latent cytomegalovirus (CMV) infection. Therefore CMV infection may contribute to immunosenescence.

It is generally believed that the size of the CD8+ T cell pool is fixed and that with every new viral challenge the size of the pre-existing memory cell population shrinks to make way for new virus-specific cells, a process referred to as memory cell attrition. However, Vezys et al, have challenged this notion by demonstrating in an elegant murine model system of repetitive antigenic challenge, that the CD8+ T cell compartment is remarkably flexible and has the capacity to expand and accommodate vast numbers of antigen-specific CD8+ T cells. This enlargement predominantly occurs within the effector memory subset and consequently the major enlargements of the CD8+ T cell pool take place in spleen, blood and solid tissues but not in lymph nodes. Importantly, memory CD8+ T cells specific for previously encountered infections were found to be largely preserved. In line with these observations in experimental animal models, we have shown in humans that the entrance of CMV-specific CD8+ T cells expanded the Ag-primed CD8+ T cell compartment rather than competing for “immune-space” with pre-existing memory T cells specific for persistent or cleared viruses. In this respect it is interesting that circulating CMV-specific CD8+ T cells in latent virus carriers do not express CCR7 but have a high expression-level of CX3CR1, the chemokine receptor for fractalkine, a cell-bound chemokine expressed on stressed endothelial cells. Thus, expression of CX3CR1 provides CMV-specific CD8+ T cells with the ability to migrate to inflamed vascular endothelium, whereas the absence of CCR7 indicates that these cells are unlikely to home to LN.

To test this notion directly, we questioned whether CMV infection limits immunological space at sites where immune reactions are initiated, such as in LN. We had the unique opportunity to study LN derived human T cells. These cells were isolated from LN gathered from surgical waste material collected during living donor kidney transplantation. We investigated the presence, phenotype and polyfunctionality of CMV-, EBV-, influenza-specific and total CD8+ T cells in the LN and compared this to the PB compartment.
MATERIALS AND METHODS

Subjects
We studied absolute numbers of CD4⁺ and CD8⁺ T cells, B-cells and NK-cells in a large group of patients on the waiting list for renal transplantation (n=560). In a smaller group of transplant recipients (n=21) we studied paired heparinized peripheral blood mononuclear cells (PBMC) and lymph node mononuclear cells (LNMC), which were isolated prior to, respectively during kidney transplantation. All these patients were EBV-seropositive and the majority of patients were also CMV-seropositive (16 of 21 patients). All patients were treated with quadruple immunosuppression, consisting of CD25 mAb induction therapy, Prednisolone, calcineurin-inhibitor and mycophenolic acid. Except for CD25 mAb, immunosuppressive treatment was started after transplantation. At the time the lymph node was gathered the first dose of CD25 mAb was already administered. However, we have demonstrated that CD25 mAb (basiliximab, Novartis Pharma, Basel, Switzerland) was not detectable in LNMC and that ex vivo CD25 expression on LNMC could be blocked with CD25 mAb (supplemental figure 1). The medical ethics committee of the Academic Medical Center, Amsterdam, approved the study and all subjects gave written informed consent.

Isolation of mononuclear cells from peripheral blood and lymph nodes
PBMCs were isolated using standard density gradient centrifugation. Lymph nodes were collected from kidney transplant recipients during living donor kidney transplantation. Briefly, LNMCs were isolated from surgical residual material of the recipient, gathered during the implantation of the transplanted kidney. Before anastomosing the arteria and vena renalis, the iliac artery and vein are dissected free. The residual tissue removed in this procedure, to obtain adequate vascular exposure, often contains lymph nodes. Directly after extraction, the gathered lymph nodes were chopped in small pieces. A cell suspension was obtained by grinding the material through a flow-through chamber. PBMCs and LNMCs were subsequently cryopreserved until the day of analysis.

Determination of absolute numbers of CD4⁺ and CD8⁺ T cells, B cells and NK cells
Absolute numbers were determined in EDTA whole blood with Multitest six color reagents (BD Biosciences, Erembodegem, Belgium) according to manufacturer’s instructions. Analysis was performed on FACSCanto II (BD) using FACSCanto software for analysis.

Virological analysis
To determine CMV serostatus, anti-CMV immunoglobulin G (IgG) was measured in serum using the AxSYM microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, IL, USA) according to the manufacturer’s instructions. Measurements were calibrated relative to a standard serum. EBV serostatus was determined by qualitative measurement of specific immunoglobulin G (IgG) against the viral capsid antigen (VCA)
and against nuclear antigen of EBV using respectively the anti-EBV VCA IgG enzyme-linked immunosorbent assay and the anti-EBV nuclear antigen of EBV IgG enzyme-linked immunosorbent assay (Biotest, Dreieich, Germany). All tests were performed following the instructions of the manufacturers.

**Tetrameric complexes**

HLA-peptide tetramer complexes were obtained from Sanquin (Sanquin Reagents, Amsterdam, the Netherlands). For CMV we used 8 different tetramers loaded with pp65- and IE-derived peptides, for EBV 6 different tetramers loaded with BMLF1-, EBNA3A- and BZLF1-derived peptides and for influenza (FLU) 2 different tetramers loaded with NP an MP derived peptide (supplemental table 1).

**Immunofluorescence staining; flow cytometry**

PBMCs and LNMCs were washed in phosphate-buffered saline (PBS) containing 0.01% NaN₃ and 0.5% bovine serum albumin. Two million PBMCs and LNMCs were incubated with an appropriate concentration of tetrameric complexes for 30 minutes at 4°C, protected from light. Fluorescence-labeled monoclonal antibodies (mAbs) were added and incubated for 30 minutes at 4°C, protected from light, at concentrations according to the manufacturer’s instructions. The following surface antibodies were used: CD8 V450, CD3 V500, CCR7 PE-Cy7, CD8 PerCP-Cy5.5, CD3 PE-Cy7, (BD Biosciences, San Jose, CA, USA), CD45RA eFluor 605NC (eBioscience Inc, San Diego, CA, USA), CX3CR1 FITC (BioLegend, San Diego, CA, USA), CD27 APC-Alexa Fluor 750 and CD4 PE-Cy5.5 (Invitrogen Ltd, Paisley, UK), CXCR3 PE (R&D Systems, Abingdon, UK).

For intracellular staining, cells were fixed after surface staining with FACS Lysing Solution (BD). Following permeabilization (FACS Permeabilizing Solution 2 (BD)) cells were stained with anti-granzyme K FITC (Immunotools, Friesoythe, Germany) and -granzyme B PE (Sanquin). Live/Dead fixable red cell stain kit (Invitrogen Ltd) was used in every staining to exclude dead cells from the analysis. Cells were measured on a LSR-Fortessa flow cytometer (BD) and analyzed with FlowJo software (FlowJo, Ashland, OR, USA).

**Detection of polyfunctional T cells**

Cytokine release after peptide or PMA/Ionomycin stimulation was performed as described by Lamoreaux et al 38, PBMCs and LNMCs were thawed and rested overnight in suspension flasks (Greiner) in RPMI supplemented with 10% FCS, penicilline and streptomycine (culture medium). Two million cells were stimulated with PMA/ Ionomycin or with the viral peptides in culture medium in the presence of CD107a FITC (eBioscience), αCD28 (15E8) (2µg/ml), αCD29 (TS 2/16) (1 µg/ml), brefeldin A (Invitrogen Ltd) (10µg/ml) and Golgistop (BD) in a final volume of 200µl, for 4 hours (PMA (10ng/ml/Ionomycin(1µg/ml)) or 6 hours (peptide) at 37°C 5% CO2. Stimulations were performed in non-treated round bottom 96-well plates (Corning). Subsequently, cells were incubated with the appropriate tetramers followed by incubation with CD3 V500, CD8 V450, CD4 PE-Cy5.5 and Live/Dead fixable red cell stain for 30 min at 4°C.
Then, they were washed twice, fixed and permeabilized (Cytofix/Cytoperm reagent, BD) and subsequently incubated with the following intracellular mAbs: anti-IFN$\gamma$ APC-Alexa Fluor 750 (Invitrogen), -TNF$\alpha$ Alexa Fluor 700, -IL-2 PE and -Mip-1$\beta$ PE-Cy7 (BD) for 30 min at 4°C. Cells were washed twice and measured on a LSRFortessa flow cytometer and analyzed with FlowJo software.

Statistical analysis

Statistical analysis of paired samples was done by two tailed Wilcoxon signed rank test with a 95% confidence interval. Non-paired samples were analyzed with two tailed Mann-Whitney test with a 95% confidence interval.

Figure 1) Absolute number of CD8$^+$ T cells, CD4$^+$ T cells, NK cells and B cells in CMV-seropositive and -seronegative patients awaiting renal transplantation. Comparison of absolute cell numbers in PB of CMV-seropositive (♦, n=430) and CMV-seronegative (■, n=130) patients prior to transplantation; (A) CD8$^+$ T cells; (B) CD4$^+$ T cells; (C) NK cells; (D) B cells. (E) Percentages of CMV pp65 YSE, CMV IE ELR and CMV IE QIK specific CD8$^+$ T cells within total CD8$^+$ T cells in one healthy individual.
RESULTS

CMV leaves a fingerprint in the PB CD8+ T cell compartment.

Since CMV has been shown to have a large impact on the composition of the PB CD8+ T cell compartment, we first studied its influence on the total number of CD8+ T cells in PB. In a large cohort of 560 patients awaiting kidney transplantation, we found nearly twice as many circulating CD8+ T cell numbers in 430 CMV-seropositive individuals compared to 130 CMV-seronegative ones (0.432x10^9/l versus 0.277x10^9/l; p<0.0001, figure 1A). CD4+ T cells, NK cells and B cells were not affected (figure 1B-D respectively). Even in healthy individuals up to 40% of the total CD8+ T cell compartment can be directed against one single peptide-HLA complex (figure 1E). These findings establish that CMV infection induces a lasting and profound CD8+ T cell expansion in the PB compartment.

CMV-specific CD8+ T cells are much less dominant in LN than in PB, but percentages of EBV- and FLU-specific CD8+ T cells are similar.

To investigate whether the CMV-specific CD8+ T cells in LN were as abundant as in PB, we compared the percentage of CMV-specific cells in PB CD8+ T cells to those in LN CD8+ T cells (figure 2). As a control, EBV- and influenza-specific (FLU-specific) CD8+ T cells were enumerated. CMV-specific CD8+ T cells were always found at significantly lower percentages in the LN than in the PB compartment. The presence of EBV- and FLU-specific CD8+ T cells in PB however was similar to those found in LN (figure 2B). Moreover, where CMV-specific CD8+ T cells exceeded EBV specific CD8+ T cells in PB, in LN this was found to be quite the opposite (figure 2C). Thus, in the LN, CMV-specific CD8+ T cells are only a minority of the total CD8+ T cell population.

CMV-specific CD8+ T cells as well as total CD8+ T cells in LN are enriched for CCR7 and CXCR3 and almost depleted of CX3CR1 expressing cells.

To understand why LN CD8+ T cells contained dramatically less CMV-specific CD8+ T cells, we studied chemokine receptor expression. One of the chemokines expressed abundantly on PB CMV-specific CD8+ T cells is CX3CR1. CX3CR1 allows for homing to inflamed endothelium and tissue via its ligand fraktalkine. The amount of cells expressing CX3CR1 in total CD8+ T cells as well as in CMV- and EBV-specific CD8+ T cells in LN was strongly reduced when compared to PB (p=0.001, P<0.005, P<0.001 respectively figure 3B), suggesting exclusion from the LN. We hardly found any CX3CR1 expressing FLU-specific cells, neither in the PB nor in the LN. Since CCR7 is required for the homing to resting LN, we next investigated the expression of this receptor. As expected, more CCR7 expressing total CD8+ T cells were found in LN (P=0.0001, figure 3C). Also CMV-specific CD8+ T cells were highly enriched for CCR7 expressing cells (p<0.0005). A similar trend was observed for FLU-specific CD8+ T cells, but not for EBV-specific CD8+ T cells. In mice, CXCR3 has been shown to be involved in the recruitment of T cells towards draining lymph nodes. Both total CD8+ T cells as well as CMV- and EBV-specific CD8 T cells in LN were enriched for CXCR3.
expressing cells. Strikingly LN CMV-specific CD8\(^+\) T cells resembled LN EBV-specific CD8\(^+\) T cells in respect to all chemokine receptors studied. Equal high percentages of FLU-specific cells in PB and LN express CXCR3. Together, these data suggest that the lower percentages of CMV-specific CD8\(^+\) T cells found in the LN might be caused by the exclusion of the CX3CR1-positive CMV-specific CD8\(^+\) T cells, the major phenotype
Figure 3) Chemokine receptor expression on (virus-specific) CD8+ T cells in PB and LN. (A) Representative dot plots of CCR7- and CX3CR1-staining on total CD8+ T cells (n=15), CMV- (n=10, 5 donors were analyzed with 2 different tetramers), EBV- (n=7, 4 donors were analyzed with 2 tetramers and 2 with 3 tetramers) and FLU- specific (n=5) CD8+ T cells. Comparison of percentages of (B) CX3CR1-, (C) CCR7- and (D) CXCR3-expressing cells within total CD8+ T cells and CMV-, EBV- and FLU-specific CD8+ T cells between PB and LN. All panels represent from left to right: total CD8+ T cells, CMV-, EBV- and FLU-specific CD8+ T cells. Paired samples were analysed using the Wilcoxon signed rank test.
in PB, and the recruitment of CCR7 expressing CMV-specific CD8+ T cells into the LN, the phenotype that is hardly found in PB.

Total CD8+ T cells as well as CMV-specific CD8+ T cells in LN contain less CD45RA+CD27- effector-type cells.

The difference in expression of chemokine receptors between the PB and LN CMV-specific CD8+ T cells prompted us to further investigate other phenotypical differences. Since most CMV-specific cells in PB are CD45RA+CD27-, markers previously shown to depict an effector phenotype, we studied the LN CMV-specific CD8+ T cells for their expression of these markers (figure 4). Already when analyzing the total CD8+ T cell compartment the lack of effector type cells in the LN became apparent. The difference between the PB and LN CD8+ T cells was highly significant (p=0.0001, figure 4B). This finding was reflected in the lower percentage of effector-type cells in LN CMV- and EBV-specific CD8+ T cells (both p<0.005) compared to PB. Since hardly any PB FLU-specific CD8+ T cells with an effector phenotype could be detected, the difference between PB and LN was not significant. In short, a substantially greater part of the LN total CD8+ T cells as well as CMV- and EBV-specific CD8+ T cells displayed a memory phenotype when compared to PB.

Only few granzyme B+granzyme K- CMV- and EBV-specific CD8+ T cells as well as total CD8+ T cells are found in LN.

We next assessed whether LN CMV-specific CD8+ T cells, besides differences in abundance, homing potential en effector phenotype, also differed in function. Therefore the cytolytic capacity of the LN CMV-specific CD8+ T cells by analyzing granzymes was studied. In PB the majority of the CMV-specific CD8+ T cells contained vast amounts of granzyme B. PB EBV-specific CD8+ T cells however usually contained granzyme K instead (figure 5). In LN the percentage of granzyme B containing CD8+ T cells was significantly lower than the PB CD8+ T cells (P=0.001, figure 5B). This was also the case, albeit to a lower extent, for LN CMV-specific CD8+ T cells (P<0.01). LN EBV- and FLU-specific CD8+ T cells consisted of equal amounts of granzyme B containing cells compared to PB. For granzyme K no differences between CD8+ T cells from LN and PB were seen. The LN CMV- and EBV-specific CD8+ T cells however did comprise more granzyme K positive cells than the PB CMV- and EBV-specific CD8+ T cells (P<0.005 and p<0.001, figure 5C). FLU-specific CD8+ T cell populations in LN showed a tendency towards less granzyme B and K containing cells. Most striking however less granzyme B+ granzyme K- cells were found in all LN (virus specific) CD8+ populations studied (except for the FLU-specific cells, which do not contain detectable amounts of these cells even in PB) (CD8 P=0.001, CMV P<0.005, EBV P<0.0005).

In summary, also when regarding cytolytic function, LN derived CMV- and EBV-specific as well as total CD8+ T cells contain less effector-type cells.

More polyfunctional CMV-specific CD8+ T cells are found in LN.

To analyze cytokine secretion capacity, CMV-specific CD8+ T cells were stimulated with their cognate peptides. Strikingly, LN CMV-specific CD8+ T cells needed less peptide
to induce the production of IL-2 and a larger percentage of all LN CMV-specific CD8+ T cells were capable of producing IL-2 (figures 6A). The production of TNFα, IFNγ, MIP-1β and the expression of CD107a were similar in LN and PB CMV-specific CD8+ T cells. Remarkably, the percentage of polyfunctional cells (producing all 4 cytokines and expressing CD107a) was almost solely dependent on the percentage of IL-2-
Figure 5) Granzyme-expression on (virus-specific) CD8+ T cells in PB and LN. (A) Representative dot plots of granzyme K- and granzyme B-staining on total CD8+ T cells (n=11), CMV- (n=6, 2 donors were analyzed with 2 different tetramers), EBV- (n=10, 3 donors were analyzed with 2 tetramers and 1 donor with 3 tetramers) and FLU-specific (n=5) CD8+ T cells. B-D) Comparison of percentages of granzyme-expressing cells within, from left to right: total CD8+ T cells, CMV-, EBV- and FLU-specific CD8+ T cells between PB and LN. B) Granzyme B containing cells; C) Granzyme K containing cells; D) Granzyme B containing and granzyme K negative cells. Paired samples were analysed using the Wilcoxon signed rank test.
producing cells, since the other cytokines and CD107a each reached a plateau at 1-10 ng/ml of cognate peptide. Stimulation with ionomycin and PMA revealed a substantial increase in IL-2 producing total as well as CMV-specific CD8+ T cells in LN (both p<0.005, figure 6B). For EBV-specific CD8+ T cells no difference was observed. A lower percentage of IFNγ- (p<0.001, figure 6C) producing LN CD8+ T cells was observed, while IFNγ was produced by an equal percentage of LN EBV-, CMV- and FLU-specific CD8+ T cell when compared to their PB counterparts. The same pattern was also found for TNFα, Mip-1β and CD107a (supplemental figure 2). The lower percentage of IFNγ-, TNFα- and Mip-1β-producing and CD107a positive cells is likely due to the higher amount of naive cells within the total LN CD8+ T cells (figure 4C). As a result, total LN CD8+ T cells do not contain more polyfunctional cells. LN CMV-specific CD8+ T cells however contain more polyfunctional cells (p<0.05), figure 6D) and they have a higher responsiveness towards their cognate peptide, suggesting once again that the CMV-specific CD8+ T cells found in the LN resemble memory phenotype CD8+ T cells. To address the question whether LN derived CD8+ T cells are more activated and proliferate better, we did a comparative analysis of the proliferation and activation status of the LN and PB derived CD8+ T cells. We did not observe any differences in the expression of the activation markers PD-1, HLA-DR, CD38 or in the expression of the proliferation marker Ki-67 (data not shown).

CMV does not leave a fingerprint in the LN CD8+ T cell compartment.

Finally we studied the impact of CMV on the LN total CD8+ T cell compartment. When we compared the subsets that, in PB, are associated with CMV seropositivity (CD45RA+CD27--; CX3CR1+CCR7+ and granzyme B+ granzyme K), we observed no apparent differences when comparing LN from CMV seronegative individuals to LN from seropositive ones (figure 7). Thus as a result of the minute amounts of CMV-specific CD8+ T cells in LN and their resemblance, in both phenotype and function, to memory CD8+ T cells, CMV seropositivity does not leave a clear imprint in the total CD8+ T cell compartment of the LN.

DISCUSSION

Latent CMV infection is characterized by the presence of large numbers of circulating CMV-specific CD8+ T cells. We and others previously showed that expansion of the CD8+ T cell pool occurring during primary CMV infection does not affect absolute numbers of pre-existing memory CD8+ T cells directed against other persistent (e.g. Epstein Barr) or cleared (influenza) viruses. Together, this indicates that the expansion of CMV-specific CD8+ T cells found in the peripheral blood compartment do not displace memory CD8+ T cells with other viral specificities, but rather enlarges the total CD8+ T cell pool. These findings are in agreement with the earlier mentioned study in mice, demonstrating enlargement of the CD8+ T cell pool after repetitive antigen challenge.
VIRUS-SPECIFIC CD8⁺ T CELLS IN LYMPH NODES

Figure 6) Cytokine producing (virus-specific) CD8⁺ T cells in PB and LN. Percentage (A) IL-2-, TNFα-, IFNγ-, MIP-1β-producing, CD107a-expressing and polyfunctional cells (PF cells) of CMV-specific CD8⁺ T cells after stimulation with various concentrations of cognate peptide for 6 hours in the presence of brefeldin A and Golgistop. The filled circles (●) with bold line represent the mean ± SEM of PB of four patients, the open circles (○) with dotted line represent the mean ± SEM of LN cells of the same four patients. * = p<0.05 Percentage of (B) IL-2-, (C) IFNγ- and (D) polyfunctional cells (PF cells) within, from left to right; total CD8⁺ T cells and CMV-, EBV- and FLU- specific CD8⁺ T cells after stimulation with PMA and ionomycin for 4 hours in the presence of brefeldin A and Golgistop. Paired samples were analysed using the Wilcoxon signed rank test.
Figure 7) Summarizing phenotypic comparison of PB and LN derived CD8+ T cells. Representative dot plots of CMV-seronegative (n=4 CD27/CD45RA and CCR7/CX3CR1, n=2 GranzymeB/K, upper rows in each panel) and CMV-seropositive (n=10 CD27/CD45RA and CCR7/CX3CR1, n=9 GranzymeB/K, lower row in each panel) PB (upper panel) and LN (lower panel) CD8+ T cells for expression of CD27 and CD45RA, CCR7 and CX3CR1, and granzyme K and granzyme B.
Murine naïve T cells and also central memory T cells, expressing CCR7 and L-selectin are known to continuously re-circulate through LN, where they can encounter antigen and rapidly give rise to effector T cells. Human LN derived CD8+ T cells concordantly appear to contain more naïve and memory CD8+ T cells, expressing more CCR7 and CXCR3 and hardly any CX3CR1, as compared to their PB counterparts. We and others have previously shown that circulating CMV-specific CD8+ T cells in latent virus carriers hardly express CCR7 and are high in their expression of CX3CR1, which precludes localization to LN and favors migration to inflamed endothelium. We here extend these findings, showing that CMV-specific CD8+ T cells in LN are much less dominant than in PB, while the percentages of EBV- and FLU-specific CD8+ T cells in PB and LN are similar. To identify the different virus-specific CD8+ T cells, we used tetramers loaded with peptides of the most immunodominant epitopes. The shortcoming of this technique is that it is not possible to analyze the total virus-specific response. Still, because we analyze the same tetramer in PB and LN, for these epitopes this is a valid comparison. The few CMV-specific CD8+ T cells that are detected in LN express more CCR7 compared to their PB counterparts. However, a significant percentage of LN derived CMV-specific CD8+ T cells does not express CCR7. This can very well be due to the down-regulation of CCR7, through ligand-induced internalization once these cells have reached the LN. T cells can also make use of other chemokine receptors to home to LN. During infection and the concurrent inflammation, T cells can reach the draining LN without making use of CCR7. Instead inflammatory chemokines and their receptors, such as CXCL10 and CXCR3, play a more important role and indeed LN derived total, CMV- and EBV-specific CD8+ T cells express significantly more CXCR3 compared to their PB counterparts. The LN analyzed in our study are resting LN. However, humans are constantly exposed to a wide variety of pathogens. The para-iliacal LN studied here drain the pelvic area and parts of the intestine. Therefore, it is possible that low levels of CXCL10 are continuously produced in the LN, attracting CXCR3 expressing T cells from the blood.

LN derived CMV-specific and total CD8+ T cells differ substantially from their PB counterparts regarding their functional capacity. Concordant with the absence of effector-phenotype (CD27) CD8+ T cells in the lymph nodes, effector molecules are lacking in the LN derived CD8+ T cells. The functional profile of memory CD8+ T cells is very diverse, including cytolysis and the production of various chemokines and cytokines. Roederer et al have developed an assay to simultaneously study five CD8+ T cell functions, including degranulation (CD107a); cytokine (IFNγ, TNFα, IL-2) and chemokine (MIP-1β) production. They observed that the presence of polyfunctional HIV-specific CD8+ T cells inversely correlates with disease progression and therefore polyfunctional cells can be regarded as “the most fit” cells. We here demonstrate that both LN and PB derived CMV-specific CD8+ T cells equally produce IFNγ, TNFα and MIP-1β and express CD107a. However, LN derived CMV-specific CD8+ T cells contain more polyfunctional cells, the difference lying in the higher production of IL-2. The fact that LN derived CMV-specific CD8+ T cells contain more IL-2 producing cells
appears to be directly related to the high number of memory-type cells that are known to produce more IL-2 than effector type cells do\textsuperscript{24}. Furthermore, we observed that LN derived CMV-specific CD8\textsuperscript{+} T cells express less granzyme B and more granzyme K, which is a feature of memory phenotype (CD27\textsuperscript{+}CD45RA\textsuperscript{−}) cells in PB CD8\textsuperscript{+} T cells\textsuperscript{24,25}.

During latency, CMV is known to reside in endothelial cells\textsuperscript{26,27} and myeloid cells\textsuperscript{28}. Functionally, PB CX3CR1\textsuperscript{+}GranzymeB\textsuperscript{+} effector-type CMV-specific CD8\textsuperscript{+} T cells are equipped to migrate to inflamed endothelium and adequately kill infected cells, preventing further viral reactivation. We speculate that memory phenotype CMV-specific CD8\textsuperscript{+} T cells patrol LN and become more important when systemic reactivation occurs. Memory phenotype cells have a superior ability to proliferate\textsuperscript{29} and produce IL-2\textsuperscript{24} and are therefore less dependent on helper signals provided by IL-2 producing CD4\textsuperscript{+} T cells.

From our findings, we conclude that the strong immune response directed against CMV, which leaves a fingerprint in the PB CD8\textsuperscript{+} T cell compartment but not in LN, is unlikely to restrict immunological space for naïve CD8\textsuperscript{+} T cells and memory CD8\textsuperscript{+} T cells with other antigenic specificities. Thus, the observation that the elderly have an increased susceptibility to infections asks for other explanations than the occupation of space by CMV-specific CD8\textsuperscript{+} T cells. Possible causes are the reduction in absolute numbers of naïve T cells due to thymic involution\textsuperscript{30} or replicative senescence of primed memory T cells. However, these factors can not entirely explain the earlier mentioned concept of the “immune risk profile” in older people which has been associated specifically with CMV\textsuperscript{31}. Previously, we have shown that CMV induces a sustained chronic inflammation apparent from increased serum levels of IFN\textsubscript{γ} and CRP\textsuperscript{32}. In addition to being a very potent activator of the immune system, IFN\textsubscript{γ} has been reported to exert suppressive actions, such as inhibition of human T cell responses\textsuperscript{33}. In a CD70TG sustained co-stimulation mouse model, IFN\textsubscript{γ} has been shown to induce B cell depletion in bone marrow, spleen and lymph nodes. Over-expression of CD70 leads to T cell differentiation toward an effector-phenotype (CD27\textsuperscript{−})\textsuperscript{34}. Human CMV-seropositive individuals likewise have consistently more circulating CD27\textsuperscript{−} effector-type CD8\textsuperscript{+} T cells and higher serum levels of IFN\textsubscript{γ} compared to CMV-seronegative ones. However, we did not observe a lower absolute number of circulating B cells. Possibly, B cells are depleted from other lymphoid compartments as demonstrated in the above mentioned mouse study. Furthermore, CMV is famous for having a large amount of proteins that subvert immune surveillance in the host\textsuperscript{28}. One example is the viral IL-10 (hcmvIL-10) ortholog\textsuperscript{35}, which is able to bind to the human IL-10 receptor\textsuperscript{36} and can inhibit both innate and adaptive immune responses\textsuperscript{37}. Possibly, hcmvIL-10 can also more systemically affect innate and adaptive immune responses directed against other infections.

In summary, we found that large numbers of CMV-specific CD8\textsuperscript{+} T cells, generated during latent infection, increase the total CD8\textsuperscript{+} T cell pool without affecting other virus-specific CD8\textsuperscript{+} T cells in the PB. Furthermore, we demonstrate that CMV-specific CD8\textsuperscript{+} T cells are hardly present in the LN and thus do not limit immunological space.
at sites where immune reactions are initiated. Together, these data argue against the possibility of memory cell attrition due to space competition as a cause for low responses to vaccination in the elderly who have high percentages of effector CD8+ T cells in the PB compartment.

REFERENCE LIST


Supplemental Figure 1) Basiliximab is not found on LN derived T cells. (A) Percentages of CD25 expressing cells within total LN CD3⁺ T cells before (left) and after (right) in-vitro CD25mAb (basiliximab) incubation (1 ug/ml). (B) Percentages of ex-vivo basiliximab⁺ LN CD3⁺ T cells. Basiliximab (humanized IgG mAb) was detected with anti-human IgG PE before (left) and after (right) in-vitro CD25mAb (basiliximab) incubation (1 ug/ml)
Supplemental Figure 2) TNF-α, MIP-1β producing and CD107a-expressing (virus-specific) CD8⁺ T cells in PB and LN. Percentages of (A) TNF-α-producing, (B) CD107a-expressing and (C) MIP-1β-producing cells within, from left to right; total CD8⁺ T cells, CMV-, EBV- and FLU-specific CD8⁺ T cells after stimulation with PMA and ionomycin for 4 hours in the presence of brefeldin A and Golgistop.
### Supplemental table 1. MHC class I loaded with different peptides

<table>
<thead>
<tr>
<th>Name</th>
<th>HLA</th>
<th>Virus</th>
<th>Protein</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV IE ELR</td>
<td>B*0801</td>
<td>CMV</td>
<td>IE 1</td>
<td>ELRRKMMYM</td>
</tr>
<tr>
<td>CMV IE ELK</td>
<td>B*0801</td>
<td>CMV</td>
<td>IE 1</td>
<td>ELKRKMIYM</td>
</tr>
<tr>
<td>CMV IE QIK</td>
<td>B*0801</td>
<td>CMV</td>
<td>IE 1</td>
<td>QIKVRVKMV</td>
</tr>
<tr>
<td>CMV IE VLE</td>
<td>A*0201</td>
<td>CMV</td>
<td>IE 1</td>
<td>VLEETSVML</td>
</tr>
<tr>
<td>CMV pp65 YSE</td>
<td>A*0101</td>
<td>CMV</td>
<td>pp65</td>
<td>YSEHPTFSQY</td>
</tr>
<tr>
<td>CMV pp65 NLV</td>
<td>A*0201</td>
<td>CMV</td>
<td>pp65</td>
<td>NLVPMVATV</td>
</tr>
<tr>
<td>CMV pp65 IPS</td>
<td>B*3501</td>
<td>CMV</td>
<td>pp65</td>
<td>IPSINVHY</td>
</tr>
<tr>
<td>CMV pp65 TPR</td>
<td>B*0702</td>
<td>CMV</td>
<td>pp65</td>
<td>TPRVTGGGAM</td>
</tr>
<tr>
<td>EBV BMLF GLC</td>
<td>A*0201</td>
<td>EBV</td>
<td>BMLF-2</td>
<td>GLCTLVAML</td>
</tr>
<tr>
<td>EBV BZLF EPL</td>
<td>B*3501</td>
<td>EBV</td>
<td>BZLF-1</td>
<td>EPLPQGQLTAY</td>
</tr>
<tr>
<td>EBV BZLF RAK</td>
<td>B*0801</td>
<td>EBV</td>
<td>BZLF-1</td>
<td>RAKFKQLL</td>
</tr>
<tr>
<td>EBV EBNA HPV</td>
<td>B*3501</td>
<td>EBV</td>
<td>EBNA-1</td>
<td>HPVGEADYFEY</td>
</tr>
<tr>
<td>EBV EBNA RPP</td>
<td>B*0702</td>
<td>EBV</td>
<td>EBNA-3A</td>
<td>RPPIFIRRL</td>
</tr>
<tr>
<td>EBV EBNA FLR</td>
<td>B*0801</td>
<td>EBV</td>
<td>EBNA-3A</td>
<td>FLRGRAYGL</td>
</tr>
<tr>
<td>EBV EBNA IVT</td>
<td>A*1101</td>
<td>EBV</td>
<td>EBNA-4</td>
<td>IVTDFSVIK</td>
</tr>
<tr>
<td>FLU NP CTE</td>
<td>A*0101</td>
<td>Influenza A virus</td>
<td>Nuclear protein</td>
<td>CTELKLSDY</td>
</tr>
<tr>
<td></td>
<td>A*0201</td>
<td>Influenza A virus</td>
<td>Matrix protein 1</td>
<td>GILGFVFTL</td>
</tr>
</tbody>
</table>