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J Immunol 2013; 190:5363-5372; Prepublished online 1 May 2013;
doi: 10.4049/jimmunol.1203267
http://www.jimmunol.org/content/190/11/5363

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/05/01/jimmunol.1203267.DC1

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Age-Associated Increase of Low-Avidity Cytomegalovirus-Specific CD8+ T Cells That Re-Express CD45RA

Stephen J. Griffiths,*†† Natalie E. Riddell,*†† Joanne Masters,* Valentina Libri,* Sian M. Henson,* Anne Wertheimer,† Diana Wallace,* Stuart Sims,‡ Laura Rivino,§ Anis Larbi,§ David M. Kemeny,§ Janko Nikolich-Zugich,† Florian Kern,† Paul Klennerman,§ Vince C. Emery,§ and Arne N. Akbar*

The mechanisms regulating memory CD8+ T cell function and homeostasis during aging are unclear. CD8+ effector memory T cells that re-express CD45RA increase considerably in older humans and both aging and persistent CMV infection are independent factors in this process. We used MHC class I tetrameric complexes that were mutated in the CD8 binding domain to identify CMV-specific CD8+ T cells with high Ag-binding avidity. In individuals who were HLA-A*0201, CD8 T cells that expressed CD45RA and were specific for the pp65 protein (NLVPMATV epitope) had lower avidity than those that expressed CD45RO and demonstrated decreased cytokine secretion and cytolytic potential after specific activation. Furthermore, low avidity NLVPMATV-specific CD8+ T cells were significantly increased in older individuals. The stimulation of blood leukocytes with CMV lysate induced high levels of IFN-γ that in turn induced IL-15 production. Moreover, the addition of IL-15 to CD45RA−CD45RO+ CMV-specific CD8+ T cells induced CD45RA expression while Ag activated cells remained CD45RO+. This raises the possibility that non-specific cytokine–driven accumulation of CMV-specific CD8+CD45RA+ T cells with lower Ag-binding avidity may exacerbate the effects of viral reactivation on skewing the T cell repertoire in CMV-infected individuals during aging. The Journal of Immunology, 2013, 190: 5363–5372.

The reduction in thymic output during aging suggests that T cell memory has to be maintained by periodic proliferation of the pre-existing T cell pool in older individuals (1). However, the repeated episodes of T cell activation throughout life leads to phenotypic and functional differentiation toward an end-stage T cell that is associated with the loss of proliferative capacity (2). This process, known as replicative senescence, may arise from telomere erosion, oxidative damage to DNA as well as stress induced responses (1). However, despite their proliferative dysfunction, highly differentiated end-stage–like CD8+ T cells are increased in older individuals (3) possibly attributable to their relative resistance to apoptosis in vivo (4).

Multiple lines of evidence indicate that the presence of expanded populations of highly differentiated CD8+ T cells is detrimental to immunity. For example, mice that have large T cell expansions have greater disease severity after HSV challenge in vivo (5). Also, aged rhesus monkeys harbor large expanded populations of T cells that are associated with poor responses to vaccinia vaccination (6). In humans, infection with CMV and the concurrent accumulation of CMV-specific T cells is detrimental to immunity for coresident EBV infection (7). In addition, the accumulation of large numbers of effector memory CD8+ T cells in CMV positive older humans is predictive of earlier mortality (8). Therefore, clarification of how expanded populations of highly differentiated T cells are generated and maintained in older humans and whether they are functionally competent is essential.

Highly differentiated T cells in both CD4+ and CD8+ compartments in humans can be identified by loss of the surface chemokine receptor CCR7 and/or the costimulatory molecules CD27 and CD28, and reduction of their telomere length (3, 9, 10). In addition, a highly differentiated (CCR7−, CD27−, CD28−) subset of effector memory T cells that are considered to be close to an end stage (3) can re-express the CD45RA molecule (EMRA T cells) (11). This particular subset of T cells is considerably expanded during aging and has characteristics of senescent T cells (2, 3, 12). Previous studies and also data included in the current report show that the increase in EMRA CD4+ and CD8+ T cells may also result from persistent CMV infection independent of age (12–14). However, the reason why CMV induces substantially greater numbers of EMRA T cells compared with other persistent viruses, such as EBV and varicella zoster virus, is not clear, and the functional properties of this population in older humans are not known (15).

In this study, we show that the expanded CMV-specific CD8+ T cell population specific for a HLA-A*0201–restricted epitope (NLVPMATV [NLV]) of the immunodominant pp65CMV pro-
tein can show either high or low avidity, as identified by tetramers that have been mutated in their MHC binding domain for CD8 (16–18). This low-avidity population accumulates in older subjects, preferentially expresses CD45RA, and has reduced functional responses to Ag specific stimulation compared with their high-avidity CD45RO-expressing counterparts. Furthermore, we found that CD45RA re-expression could be induced in CMV-specific CD8⁺CD45RO⁺ T cells by IL-15, but not TCR activation, suggesting that cytokine-mediated homeostatic proliferation may be in part, a mechanism for the generation of EMRA T cells in vivo.

Materials and Methods

Blood sample collection and PBMC isolation

Written informed consent was obtained and whole blood was collected in standard heparinized tubes from healthy volunteers. Young donors were between 18 and 35 y and old donors were between 65 and 95 y of age. The study was approved by the Local Research Ethics Committee of the Royal Free and University College Medical School. Donors did not have any comorbidity, were not on any immunosuppressive drugs, and retained physical mobility and lifestyle independence. PBMCs were isolated using Ficoll–Hypaque (Amersham Biosciences) and either analyzed immediately or cryopreserved in 10% DMSO/FCS.

Determination of donor CMV status

The CMV status of donors was obtained by the overnight stimulation of fresh PBMCs with CMV viral lysate and identification of IFN-γ production by CD8⁺ T cells as described previously (9). There was total concordance between IFNγ⁺ responses and seropositivity obtained from IgG serology obtained from the diagnostic laboratory of University College London Hospital (9).

Flow cytometric analysis

PBMCs were analyzed by flow cytometry using a combination of the following Abs: CCR7 PE, CD3 allophycocyanin, CD3 FITC, CD8 FITC, CD8 PerCP, CD8 APC-Cy7, CD11a PE, CD14 2D Horizon V450, CD16 FITC, CD19 PerCP, CD27 FITC, CD27 PE, CD28 PE-Cy7, CD45RA allophycocyanin, CD45RO PE, CD56 ECD (Beckman Coulter), HLA-DR eFluro 450 (eBioscience), and IL-15 PE (all from BD Biosciences, Oxford, U.K., unless stated). Occasionally, cells were also stained with either Live/Dead Fixable Blue Dead Cell Stain (Invitrogen, Paisley, U.K.) or BD Via-Probe (BD Biosciences). Tetramer staining was conducted on HLA-matched donor PBMCs for 20 min at 37°C with either HLA-A*0201 (NLVPVMATV) or HLA-B*0701 (TPRTVGGGAM [TPR]) CMVpp65-specific conventional tetramers or “null” tetramers that have been mutated in the α3 domain in the conserved binding site for CD8 as previously described (18). Conventional tetramers identify all specific CD8⁺ T cells that are able to bind specific peptide, whereas the null tetramers identify CD8⁺ T cells that have high avidity for specific peptide without the need for CD8 binding. Therefore, using conventional and null tetramers to identify CMVpp65-specific T cells in the same individuals, we can determine the proportion of these cells that have high avidity (19). All samples were fixed with 2% paraformaldehyde then analyzed on either the BD FACSCalibur or BD LSR/LSRII using CellQuest software or BD FACSDiva software, respectively.

Functional activity

Multifunctional responses of CMVpp65-specific CD8⁺ T cells was performed by staining whole PBMCs initially with either conventional or high-avidity HLA-A*0201 (NLVPVMATV) CMVpp65-specific tetramers and CD107a FITC (BD Biosciences) for 2 h at 37°C. Titrated concentrations of CMVpp65 peptides (ProImmune, Oxford, U.K.) were added in the presence of monensin and brefeldin A for an additional 4 h at 37°C. Subsequently, PBMCs were stained intracellularly with IFN-γ PE-Cy7 or BD Horizon V450 and TNF-α APC (BD Biosciences) via Caltag Fix and Perm in accordance with the manufacturer’s guidelines.

Activation of CD8⁺CD45RA⁺ and CD8⁺CD45RO⁺ T cells

CD8 fraction of PBMCs was collected by magnetic bead separation (MACS; Miltenyi Biotec, Surrey, U.K.). The non-CD8 fraction was retained for use as APCs, which were pulsed with either medium containing NLVPVMATV (NLV) peptide (ProImmune) or with medium only for 1 h at 37°C prior to irradiation (40-Gy gamma radiation). The CD8⁺ population was stained with CD45RA allophycocyanin (Caltag Laboratories, Buckingham, U.K.) and CD45RO FITC (Dako, Stockport, U.K.) and sorted to isolate CD45RA and CD45RO populations of >95% purity using a BD FACSARia. A total of 200,000 cells of each population were then placed in sterile FACS tubes with different ratios of irradiated peptide pulsed APCs. After 2 h of incubation at 37°C, brefeldin A was added, and cells were further incubated for 4 h. Subsequently PBMCs were stained for intracellular expression of IFN-γ (BD Horizon V450; BD Biosciences) via Caltag Fix and Perm in accordance with the manufacturer’s guidelines.

Polyclonal activation of CD8⁺ T cells

Total PBMCs, or sorted CD45RA⁺ or CD45RO⁺ CD8⁺ T cells which were added to irradiated APCs (1:1 ratio), were activated with immobilized anti-CD3 (0.5 µg/ml) or PMA/ionomycin (both 0.5 µg/ml). After 2 h of incubation at 37°C, brefeldin A was added, and cells were further incubated for 16 h at room temperature. PBMCs were then stained for CD8, CD27, CD45RA, and intracellular expression of IFN-γ (BD Horizon V450; BD Biosciences) via Caltag Fix and Perm in accordance with the manufacturer’s guidelines.

Measurement of IL-15 mRNA production following CMV lysis or cytokine stimulation

The mRNA levels of IL-6, IL-15, TNF-α, and IFN-α were measured in total PBMCs directly ex vivo and after 4 and 24 h of culture in the presence of CMV lysis from infected cells (1:10 of stock solution of 0.64 mg/ml). In addition, IL-15 mRNA levels were measured in total PBMCs before and after 24 h of culture in the presence of either IFN-α (500 U/ml; α 2a, 11100-1 from PBL IFN source), IL-6 or TNF-α (both 50 ng/ml; R&D Systems). Cytokine mRNA was measured in different leukocyte subsets from PBMCs that were stimulated with CMV lysis (24 h). T cell populations were isolated by CD3 MAC separation and the negative fraction (non-CD3⁺ cells) were then incubated with a cytokine mixture (CD14 FITC, CD3 APC, CD56 ECD [Beckman Coulter], HLA-DR eFluo 450 [eBioscience], CD19 PE-Cy7 [eBioscience], CD16 PE and UV live dead [Invitrogen], all BD Biosciences unless stated) prior to sorting. Leukocyte populations were identified by: T cells (CD3⁺), NK cells (CD3⁺CD56⁻), B cells (HLA-DR⁺CD3⁺CD56⁺), monocytes (Mono) (HLA-DR⁺CD5⁵CD56⁻CD19⁺CD16₁CD14⁺) and dendritic cells (DCs) (HLA-DR⁺CD3⁺CD56⁻CD19⁺CD16⁻CD14⁻). Total RNA was purified with RNeasy columns (Qiagen). Reverse transcription was performed with random primers using the murine leukemia virus reverse transcriptase (Invitrogen). The mRNA level of genes was determined by real-time quantitative PCR (Applied Biosystems) with the TaqMan primers for IL-15 (Hs00999093_m1), IFNα2 (Hs02621172_s1), IL-6 (Hs00985639_m1), and TNF-α (Hs01113624_g1). The housekeeping gene for the same DNA reaction mixture was the TaqMan primers for Hs09298695_g1). Each sample was run in duplicate, and target mRNA level was expressed as a ratio to the level of 18S to control for differing levels of cDNA in each sample.

Induction of CD45RA re-expression by IL-15

A CD8⁺positive fraction was collected from PBMCs by MACS then stained with 1) anti-CD45RA allophycocyanin (Caltag Laboratories) or 2) anti-CD45RA APC and anti-CD27 FITC, and sorted using a BD FACSARia. Cells were individually cultured at 100,000 cells/well with a single dose of IL-15 (10 U; R&D Systems, Abingdon, U.K.) for up to 21 d. At different times, post-IL-15 addition, cells were harvested, counted, then stained with the following: 1) NLV/HLA-A*0201 tetramer followed by surface staining with CD45RA allophycocyanin and CD45RO FITC or 2) CD45RA allophycocyanin and CD27 FITC for 30 min at 4°C, respectively. We incubated CD45RA⁺CD45RO⁺ cells with IL-2 instead of IL-15 as a control.

Statistical analysis

Graph Pad Prism version 5 was used to construct all graphs, dot plots, and bar charts. The D’Agostino–Pearson omnibus K2 normality test was used to determine whether data fitted a Gaussian (normal) distribution. Statistical significance was evaluated using the Student t test if data followed a normal distribution, and if data were also paired, it was assessed using a Student paired t test if data followed a normal distribution, or a variant of the paired t test if data were not normally distributed. Pearson and Spearman rank.

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LOW-AVIDITY CMV⁺ T CELLS ACCUMULATE IN OLD HUMANS
Results

CMV infection and aging are associated with decreases in the naive CD8+ T cell compartment and concomitant increases in the highly differentiated T cell pool (14, 20). CMV infection is also associated with large oligoclonal expansions of CD8+ T cells in older individuals (7, 20). Using MHC class I tetrameric complexes (tetramers) that are directed to epitopes of the immunodominant CMVpp65 protein in HLA-A*0201 (NLV) or HLA-B*0701 (TPR)–positive individuals (representative results for NLV in one young and one old individual are shown in Fig. 1A) we observed an increase in CMVpp65-specific CD8+ T cells during aging (p < 0.01, R² = 0.16; Fig. 1B) confirming previous reports (7, 14). When separated further on the basis of HLA-A*0201 (NLV)– and HLA-B*0701 (TPR)–specific responses the increase in CMV-specific CD8+ T cells during aging was still observed (n = 36, p < 0.05, R² = 0.13, and n = 25, p < 0.05, R² = 0.23; respectively; Supplemental Fig. 1A).

We used the relative expression of the surface markers CD45RA and CD27 to define naive (CD45RA+CD27+), central memory (CD45RA-CD27+), effector memory (CD45RA+CD27-), and CD45RA re-expressing effector memory populations (EMRA; CD45RA+CD27-) (21). We confirmed that there was an increase in CMVpp65-specific CD8+ EMRA T cells (NLV/HLA-A*0201 and TPR/HLA-B*0701) during aging (representative staining and cumulative data shown in Fig. 1C, 1D), regardless of whether these specific T cells were defined on the basis of CD27–CD45RA+ or CD45RA–CD45RO+, CD45RA–CD45RO–, or CD45RA+CD11a+ (Supplemental Fig. 1B). In addition, we found that within the NLV/HLA-A*0201–specific CD8+ T cell population, < 5% of EMRA cells expressed CD28 (data not shown), confirming that these cells exhibit a late differentiated phenotype (8, 22, 23).

FIGURE 1. CMV-specific CD8+ T EMRA cells accumulate during aging. Representative dot plots showing the size of the CMVpp65–specific population as a percentage of total CD8+ T cells for one young and one old individual (A). Pooled data (n = 61) showing the size of the CMVpp65–specific population as a percentage of total CD8+ T cells as stratified by age (B). Line of best fit was generated by linear regression and the correlation assessed by Pearson and Spearman rank (GraphPad Prism). Representative dot plots (one young and one old donor) of CMVpp65–specific CD8+ T cell CD45RA–CD27 subset distribution (C). Cumulative data comparing the percentage of CMVpp65–specific CD8+ T EMRA cells (CD45RA–CD27-) within the total CMV-specific compartment in different age groups (D). One dot represents one donor. Results from two-tailed paired t test are shown.

CMV-specific CD8+ T cells with low Ag binding avidity accumulate in old HLA-A*0201+ individuals

Two tetramers directed toward the pp65 peptides NLV (HLA-A*0201) and TPR (HLA-B*0701) were generated using standard recombinant MHC molecules. In addition, NLV and TPR tetramers were generated using HLA-A*0201 or HLA-B*0701, respectively, with an altered α3 domain in the conserved binding site for CD8 (17–19). These mutations have been extensively investigated across a range of human and mouse MHC class I molecules and consistently impact on CD8 binding but not TCR interactions (24–27). The alteration weakens CD8 coreceptor binding that normally stabilizes the interaction between peptide–MHC and the TCR. Binding to this mutated tetramer is therefore more reliant upon the ability of the TCR to bind peptide and hence requires high avidity of interaction (CD8 independence). Using the two types of tetramers (conventional and null), the size of the conventional (total) and high-avidity CMVpp65–specific populations were compared in the same donor (Fig. 2A).

In the young subjects, the size of the conventional and high-avidity NLV/HLA-A*0201 or TPR/HLA-B*0701–specific T cell populations were similar, suggesting that most of the tetramer-defined cells were of high avidity in this age group (Fig. 2B, 2C). However, in older humans, there were significantly fewer high avidity (null tetramer positive) compared with conventional avidity CMV-specific CD8+ T cells in NLV/HLA-A*0201 in the same individuals, but this was not observed in TPR/HLA-B*0701 subjects (Fig. 2B, 2C). This suggests that the NLV-specific cells that accumulate in older HLA-A*0201–positive individuals have reduced TCR binding avidity. In contrast, all TPR-specific cells that are identified by the conventional tetramer in HLA-B*0701–positive individuals appear to have high TCR binding avidity. However, in some individuals using just the conventional tetramer, we found TPR/HLA-B*0701 binding cells that had a high or low tetramer staining pattern (Supplemental Fig. 2A, top three panels). Previous work on tumor Ags showed “low”–avidity cells to be identified by lower tetramer staining intensity indicating that TPR/HLA-B*0701–specific CD8+ T cells with weak tetramer binding may be a population with low avidity (28). Therefore, cells with low avidity can also be identified in some HLA-B*0701–positive individuals. Collectively, our results indicate that CMVpp65–specific CD8+ T cells with low avidity accumulate in some individuals during aging and that this may be associated with HLA type. High-avidity NLV/HLA-A*0201–specific CD8+ T cells have superior functional responses to challenge with specific Ag

The functional response profiles of the conventional and high-avidity NLV/HLA-A*0201–specific CD8+ T cell populations were compared by measuring IFN-γ, CD107a expression and TNF-α production in the tetramer-defined population after activation of total PBMCs with increasing doses of a CMVpp65 peptide pool (Fig. 3, Supplemental Fig. 3). As shown in Fig. 2B and 2C, some individuals showed equal binding of the conventional and null tetramers, indicating that all the cells were of high avidity, whereas others showed lower null compared with conventional tetramer binding, indicating that cells of low avidity were found in these subjects. When we investigated individuals that showed lower null versus conventional tetramer binding, we found that the high avidity (null tetramer binding) population expressed higher levels of IFN-γ, CD107a, and TNF-α following activation compared with the conventional tetramer binding population that contains a mixture of high- and low-avidity cells (representative staining, Fig. 3A; dose response to peptide, Fig. 3B). This was confirmed in a second experiment (Supplemental Fig. 3A). In contrast, when we investigated participants that had equal...
proportions of null and conventional tetramer binding, the expression of IFN-γ, CD107a, and TNF-α following peptide activation was similar in both null and conventional tetramer positive cells (Fig. 3C). This was confirmed in two further subjects who showed equal null and conventional tetramer binding (Supplemental Fig. 3B). These results collectively indicate that the presence of lower avidity NLV-specific CD8+ T cells is associated with decreased functionality of this population.

The phenotypic characteristics of high-avidity CMVpp65-specific CD8+ T cells in HLA-A*0201 (NLV) individuals

We next investigated whether the high-avidity CMV (NLV)-specific CD8+ T cells expressed CD45RA or CD45RO (Fig. 4). In a representative example, using the conventional tetramer, the characteristic CD45RA/CD45RO spread is observed in the CMV (NLV)-specific population and the specific cells predominantly express CD45RA (Fig. 4A, top and bottom left panels). However the high-avidity CMV (NLV)-specific cells that were identified with the null tetramer in the same individual predominately expressed CD45RO (Fig. 4A, top and bottom right panels).

![Image](http://www.jimmunol.org/)

We confirmed the observation that CD45RA expression was associated with lower avidity by staining PBMCs from the same individuals with either the conventional tetramer (to quantify CD45RA expression by both high- and low-avidity cells) or the null tetramer (to determine the percentage of high-avidity cells within the NLV-specific population). We found that greater percentages of CD45RA+ tetramer+ T cells in the total tetramer pool was associated with a lower proportion of high-avidity tetramer+ cells within the same pool (p < 0.0001; Fig. 4B, left panel). Conversely, increased expression of CD45RO by the NLV-specific CD8+ T cells was associated with increased proportion of high-avidity cells (Fig. 4B, right panel).

When we investigated pp65-specific CD8+ T cells in HLA-B*0701 (TPR) individuals using the conventional and null tetramer, the correlation between avidity and CD45RA/RO phenotype was not observed (both p > 0.3, R² < 0.09; Supplemental Fig. 2B). As described above, some HLA-B*0701–positive individuals had both high and low conventional tetramer binding cells, and we found that that high tetramer binding cells in these individuals were mainly CD45RO+, whereas the cells with low tetramer binding were CD45RA+ (Supplemental Fig. 2A, bottom three panels). Therefore, the association between CD45RA expression and lower avidity also applies to CMVpp65-specific (TPR) CD8+ T cells in some subjects who are HLA-B*0701.

NLV/HLA-A*0201–specific CD8+ EMRA T cells have low functional responses to Ag

We investigated whether the lower avidity CD45RA+ NLV/HLA-A*0201–specific T cells had altered functional activity by comparing their response to stimulation with NLV peptide to their CD45RO counterparts in the same individuals (n = 4). IFN-γ production was used to determine the percentage of reacting cells, because this has been shown to be a better single marker of activation to CMV than TNF-α or IL-2 (29). CD8+ T cells that had a mixed CD45RA and CD45RO phenotype were sorted into CD45RA and CD45RO populations (Fig. 5A, top row). This individual also has CMV-specific (NLV) CD8+ T cells that were found before sorting and after sorting in the CD45RA or CD45RO populations (Fig. 5A, bottom row).

Stimulation with increasing numbers of APCs pulsed with NLV peptide induced a dose-dependent increase of IFN-γ–positive cells within the CD45RO population; however, the response in the NLV-stimulated CD45RA population was considerably lower (Fig. 5B, Supplemental Fig. 3C). Therefore CMV (NLV) T cells that express CD45RA have lower TCR avidity and also lower functional responsiveness to specific TCR stimulation by peptide compared with those that express CD45RO.

To determine whether the EMRA CD8+ T cell population was functionally deficient in general or only defective when responding to the CMV-specific peptide, we activated PBMCs with a polyclonal stimulus (anti-CD3) and examined the capacity of CD8+ T cell subsets, identified by relative CD45RA and CD45RO expression, to secrete IFN-γ (Fig. 5C). We found that the EMRA population was very functional. We also ruled out the possibility that reduced avidity of CD45RA expressing CMV-specific T cells was due to lower expression of CD3 or CD8 by showing that the CD45RA+ NLV/pp65–specific CD8+ T cell population expressed similar levels of CD8 and greater level of CD3 (p < 0.05) compared with the CD45RO+ NLV/pp65–specific populations in the same individuals (Fig. 5D, 5E).

We also investigated whether NLV-specific CD45RA expressing T cells that have poor responses to specific peptide can respond to polyclonal stimulation. In one individual, we stimulated CD8+ T cell populations that were sorted into CD45RA and CD45RO subsets with NLV peptide or either anti-CD3 or PMA/ionomycin and assessed IFN-γ production by the NLV-specific cells (Supplemental Fig. 3D). We found that although the NLV-specific CD45RA+ T cells did not respond to NLV peptide
compared with the CD45RO population (Supplemental Fig. 3C, left), the same cells responded to a similar extent to anti-CD3 or PMA/ionomycin stimulation as their CD45RO counterparts (Supplemental Fig. 3D), indicating that the CD45RA re-expressing T cells are not inherently functionally defective. Therefore the reduced response of the CMV-specific CD45RA expressing CD8+ T cells to peptide stimulation (NLV) is likely to be related to their reduced avidity for Ag.

CMV can induce IL-15 expression in monocytes indirectly via induction of IFN-α secretion by DCs

Previous studies have shown that CMV induces the secretion of high levels of IFN-α (9, 30) that, in turn, induces IL-15 (31) that leads to considerable bystander activation during certain viral infections (32). This together with the observation that the addition of IL-15 to cultured primed/memory CD45RO+ T cells without any antigenic stimulus can induce their re-expression of CD45RA (33–36) prompted us to test the hypothesis that IL-15–driven homeostatic proliferation could be a mechanism for the CMV infection induced increase in EMRA CD8+ T cells.

We confirmed that CMV lysate–stimulated PBMCs produced a high level of mRNA to IFN-α that was also found previously at the protein level (Fig. 6A) (9). In addition, we also showed that IL-15 was induced in these cultures after 24 h (Fig. 6A). We next showed that the addition of IFN-α but not IL-6 or TNFα could induce the potent (>6- to 10-fold increase) production of IL-15 by PBMCs (Fig. 6B). In addition, when we isolated leukocyte subsets, we found that although IFN-α production after CMV lysate stimulation was mainly produced by the DC population, IL-15 was mainly produced by monocytes (Fig. 6C). Finally, we showed a link between the secretion of IFN-α and IL-15 by PBMCs after CMV lysate stimulation because the addition of an anti–type 1 IFNR Ab to CMV-stimulated PBMCs partially blocked the increase in IL-15 mRNA expression by these cells (Fig. 6D).
Two isoforms of IL-15 exist; a short signal peptide isoform that remains within the cytoplasm and has unknown function, and a long signal peptide isoform that can induce NK and T cell proliferation when transpresented on the cell surface to neighboring cells, or if secreted (37, 38). The membrane bound form of IL-15 identifies the bioactive cytokine that is mainly involved in CD8 expansion (39). We therefore investigated whether the IL-15 that is induced at the transcriptional level in monocytes after CMV lysate stimulation is also detected at the protein level, on the surface of these cells by flow cytometry (Fig. 6E). We stimulated PBMCs with CMV lysate and investigated the expression of IL-15 in different leukocyte subsets gated as shown (Fig. 6E). We found that monocytes but not T, B, NK cells, or DCs showed membrane staining with this cytokine (representative experiment shown in Fig. 6F). In addition in six separate individuals, we found that monocytes but not T cells expressed surface IL-15 after CMV lysate stimulation (Fig. 6G). Because T cells express IL-15R yet do not show membrane staining for this cytokine, it is unlikely that the IL-15 observed on the surface of monocytes is due to the binding of secreted IL-15. This indicates that the CMV lysate stimulation can induce the expression of IL-15 at both the transcriptional and translational level.

FIGURE 4. Low-avidity NLV/HLA-A*0201–specific CD8+ T cells were mainly CD45RA+. Representative example of one donor stained with either the conventional or high-avidity NLV/HLA-A*0201 tetramer together with CD45RA and CD45RO Abs (A). Collective data (n = 29) showing correlation between per cent binding high-avidity tetramer within the total NLV/HLA-A*0201–specific populations, with the percentage of cells expressing CD45RA (B, left panel) or CD45RO (B, right panel). Lines of best fit were generated by linear regression and the correlation assessed by Pearson and Spearman rank (GraphPad Prism).

FIGURE 5. NLV/HLA-A*0201–specific CD8+ CD45RA+ T cells showed poor response to Ag activation. Isolated CD8+ T cells were further sorted into CD45RA+ and CD45RO+ populations (A, top row). NLV/HLA-A*0201–specific cell were identified within the unfractionated or sorted CD8+ T cells using conventional tetramers (A, bottom row). Numbers represent percentages within the quadrants. The CD45RA+ and CD45RO+CD8+ T cell populations were stimulated separately with unpulsed APCs or increasing ratios of NLV peptide-pulsed APCs and the IFN-γ response recorded (B). U.S. represents the unstimulated control (containing no APCs). Three additional experiments showing similar results are shown in Supplemental Fig. 3C. PBMCs (n = 7) were stimulated with anti-CD3 and the IFN-γ responses of CD8+ T cell populations defined by relative expression of CD45RA v CD27 were measured (C). The levels of CD8 (D) and CD3 (E) coreceptor expression on the different NLV/CMVpp65-specific CD8+ T cell subsets was measured by flow cytometry (n = 9). Results from two-tailed paired t test are shown.
IL-15 induces CMV-specific CD8^+CD45RO^+ T cells to re-express CD45RA in vitro

Next, CD8^+ T cells were isolated from HLA-A^*0201–positive donors (Fig. 7A). The cells were stained for both CD45RA and CD45RO, and NLV-specific cells were identified by staining with the CMV (NLV) tetramer (Fig. 7A, bottom left panel). Subsequently, we sorted CD45RA^-CD45RO^+ cells from the CD8 population and <2% of the total CD8 population and <1% of the CMV (NLV)-specific cells within this remaining population expressed CD45RA. IL-15 induced the expansion of CD8^+ T cells but maintenance of the NLV-specific population by day 14 (Supplemental Fig. 4A) and induced CD45RA re-expression in both the total CD8^-CD45RO^+ and CMV (NLV)-specific CD8^-CD45RO^+ T cell subset (Fig. 7A). However, the CD45RA re-expressing cells also expressed CD45RO. In a previous study, we showed that compared with IL-2 and IL-7, IL-15 induces greater levels of CD45RA re-expression on Ag-specific cells (34). In contrast, when CMV (NLV)-specific CD8^-CD45RO^+ T cell populations were stimulated with CMV peptide-pulsed irradiated autologous APCs and IL-2, they proliferated and retained CD45RO expression but did not express CD45RA (Supplemental Fig. 4B, top row). CMV (NLV)-specific CD8^-CD45RA^+ T cells also expanded and switched expression from CD45RA to CD45RO following TCR stimulation (Supplemental Fig. 4B, bottom row).

IL-15 induces different populations of CD45RA re-expressing CD8^+ T cells

The four CD8^+ T cell population determined by CD45RA and CD27 expression (21) were cultured with IL-15 for 21 d (representative example of three experiments; Fig. 7B). This cytokine induced the expansion of all four subsets (~3-fold); however, the kinetics of expansion was slower in the EMRA subset (Supplemental Fig. 4C). Both the naive and the EMRA populations remained CD45RA^-CD27^+ and CD45RA^-CD27^+ phenotype, respectively, in the presence of IL-15; however, a large proportion of both populations now also expressed CD45RO (Fig. 7B). In the presence of IL-15, up to 69% of central memory CD8^+ T cells now expressed CD45RA, but these cells also expressed CD45RO (Fig. 7B). Similarly, ~61% of CD45RA^-CD27^- effector memory T cells re-expressed CD45RA and became CD45RA^+CD27^-, but these cells retained CD45RO expression. The level of CD45RA re-expression with IL-2 was between 2 and 25% of that induced by IL-15 (data not shown). Collectively, these data show that although IL-15 can induce the expression of CD45RA on CM and EM T cells, these cells retain CD45O expression and therefore are...
not identical to the CM and EM cells that are freshly isolated from peripheral blood in vivo.

**Discussion**

Previous studies have identified CMV infection as a risk factor to survival in older humans (8, 13) possibly through the induction of large expanded populations of highly differentiated CD8+ T cells (7, 14). However, it is not clear how these expanded populations of T cells exert their negative influence. The main aims of the current study were to determine how highly differentiated CMV-specific CD8+CD45RA+CD27+ (EMRA) T cell populations are generated and whether they are functionally competent in older humans. These cells accumulate during aging and are also CD28− and constitute part of the increased effector cell populations defined within the “immune risk phenotype” found in some older individuals in the OCTA/ NONA studies (8, 13).

Human CD45RA+ T cells that are activated by TCR ligation express CD45RO (40). This also applies to CD8+ EMRA T cells that are reactivated in vitro (4, 35, 41) raising the question of how CMV-specific CD8+CD45RA+CD27− (EMRA) T cell populations are generated and whether they are functionally competent in older humans. These cells accumulate during aging and are also CD28− and constitute part of the increased effector cell populations defined within the “immune risk phenotype” found in some older individuals in the OCTA/ NONA studies (8, 13).

Although CMV-specific CD8+ T cells with lower avidity accumulate in older subjects, we have not tested directly if they also had reduced functionality. However, because CD8+ T cells with lower avidity also have decreased functional capacity and these cells increase in older HLA-A*0201 subjects, it is very likely that these cells in older subjects have decreased functionality. In support of this, Ouyang and Pawelec (42) and Hadrup et al. (43) have already shown that CMVpp65-specific CD8+ T cells in older humans are dysfunctional in terms of IFNγ after specific stimulation compared with younger subjects.

**FIGURE 7.** IL-15 induces CD45RA expression. CD45RA−CD45RO+CD8+ T cells were isolated from a CMV-seropositive donor and incubated with IL-15 (A). The CD45RA/CD45RO phenotype was determined at different times in the total CD8 population (top panels) or in the NLV-specific CD8+ T cells (bottom panels). Naive (CD45RA+CD27+), central memory (CD45RA−CD27−), effector memory (CD45RA−CD27+), and EMRA (CD45RA+CD27−) CD8+ T cell subsets were isolated and their CD45RA/CD45RO phenotype was determined before (day 0) and after culture for different times with IL-15 (B). Numbers represent percentages in quadrants. Experiments shown are representative of three performed with very similar results.
An extensive study by Chidrawar and Moss (14) showed that the absolute number of CD8+ EMRA (CD45RA re-expressing memory) T cells increases during aging and that CMV infection accentuates this increase. In the current study we show an increase in the percentage of CMVpp65 (conventional) tetramer positive cells that are CD45RA+/CD27+ during aging. Because this population contains the lower avidity cells, the data collectively suggests that the absolute number of lower avidity pp65 specific T cells accumulate in older subjects. Still it is likely that there are sufficient numbers of highly avid and functional CMV-specific T cells available to control viral reactivation during aging because older CMV-positive individuals do not experience increased pathology that is attributable to the virus. However, it is possible that the association of CMV seropositivity and decreased health during aging (8) may be due to the increase in low-avidity cells that interfere with efficient responses to other pathogens.

A novel point in the current study is that we link IFN-α secretion induced by CMV stimulation to IL-15 secretion and CD45RA re-expression by CD8+ T cells. This suggests that lifelong CMV reactivation may have a direct effect on specific T cells, where excessive differentiation through repeated stimulation occurs (9) but also a nonspecific effect that may arise from excessive cytokine induced homeostatic proliferation. Bone marrow–derived granulocyte/monocyte progenitor cells are a major site for CMV latency (44) and compelling evidence suggests that the bone marrow may host virus-specific memory T cells (45). This is particularly evident in older subjects who have increased levels of IL-15 present in the bone marrow (46). Furthermore, T cells may be found in close proximity to IL-15–producing cells in the bone marrow (47). The CD8+ T cell expansion induced by IL-15 may be considerable, and it is interesting to note that in macaques the infusion of IL-15 led to a 100-fold expansion of effector memory CD8+ T cells (48) in vivo.

T cells recognizing the same Ag presented by MHC can vary in their sensitivity by several orders of magnitude (19), and it is the highly avid CD8+ T cells (49), with multifunctional potential (29), that are most efficient at controlling viruses. An important observation in this study was that CMVpp65–specific T cells that expressed CD45RA in individuals who were HLA-A*0201 had lower avidity and functionality than those that expressed CD45RO in the same individuals. This was less robust for individuals who were HLA-B*0701, suggesting that HLA type of the individual plays a role in determining the avidity and functionality of Ag-specific CD8+ T cells consistent with previous reports (50, 51). Other HLA-A*0201–restricted CD8+CD45RA+ T cells that are specific for different CMV viral proteins may also have lower avidity and/or functional capacity than their CD45RO counterparts and a recent study in a limited number of participants identified a large population of IE-1 CMV–specific HLA-A*0201–restricted CD45RA+CD8+ T cell population with low TCR binding avidity (52).

Previous studies have shown that highly differentiated EMRA T cells in both the CD4 and CD8 compartments have characteristics of senescence including loss of CD28 expression (1, 2, 12, 20, 23). This suggests the intriguing possibility that homeostatic cytokines may induce senescence signaling pathways in T cells and may also give rise to CD8+ T cells with lower avidity. Despite exhibiting senescence, the EMRA population is not functionally deficient because they can secrete high levels of cytokines and mediate cytotoxic activity very efficiently in response to TCR stimulation (34, 53, 54). However, the CD45RA+/CD8+ T cells that are specific for the pp65 (NLV) epitope in individuals who are HLA-A*0201 positive do not secrete high levels of cytokines in response to Ag-specific stimulation, probably reflecting their lower avidity for specific Ag rather than their inherent dysfunction. As high functional avidity is critically important for optimal pathogen clearance (19), the current data suggests that expansions of low-avidity NLV/HLA-A*0201–specific CD8+ T cells would provide suboptimal immunity to CMV reactivation in vivo. However, it is not clear whether immunity to this protein is protective. It is possible that the removal of low avidity CMV-specific CD8+ T cells may increase the efficiency of the immune system in older CMV-infected humans, as suggested by studies in rodents (5). In practice this may prove difficult and costly in humans. It is possible that targeting homeostatic cytokines to prevent nonspecific T cell accumulation may counteract the negative effects of large expansions of effector T cells that have been shown to be detrimental in older humans.

Acknowledgments

We thank Prof. Andy Sewell whose group developed the null tetramers that we used. We also thank the blood donors who participated in this study.

Disclosures

The authors have no financial conflicts of interest.

References


Low Avidity CMV⁺ T Cells accumulate in Old Humans

Supplementary Figure 1

A)

B)
Low Avidity CMV⁺ T Cells accumulate in Old Humans

**Supplementary Figure 2**

A)  

<table>
<thead>
<tr>
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B)  

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<td>% CD45RA⁺ vs % Binding High Avidity Tetramer</td>
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<tr>
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<tr>
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<tr>
<td>% CD45RO⁺  0    20  40  60  80  100</td>
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Supplementary Figure 3

A) Participants with mixed high and conventional avidity populations:

- IFNγ+ T cells
- CD497a+ T cells
- TNFα+ T cells

B) Participants with all high avidity binding populations:

- IFNγ+ T cells
- CD497a+ T cells
- TNFα+ T cells

C) T cell: APC

1:1 1:1 2:1 1:2

APC APC + NLV

D) CD45RO+ CD45RA+

% IFNγ+ T cells

1:1 APC PMA/Ionomycin
Low Avidity CMV⁺ T Cells accumulate in Old Humans

Supplementary Figure 4

A) CD8⁺ T Cells

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NLV/HLA A*0201

B) Day 0

CD45RA⁺

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CD45RA⁺

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C) Naive

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Control Memory

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Effector Memory

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EMRA

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Low Avidity CMV + T Cells accumulate in Old Humans

Supplementary Figure Legends

Supplementary Figure 1. CD45RA expressing CMVpp65-specific T cell populations accumulate within HLA-A*0201 and HLA-B*0701 individuals
Pooled data showing the size of the NLV/HLA-A*0201-specific (A, top left) and TPR/HLA-B*0701-specific (A, top right) population as a percentage of total CD8+ T cells as stratified by age (n= 36 and 25 respectively). Line of best fit was generated by linear regression and the correlation assessed by Pearson and Spearman rank (GraphPad Prism). Cumulative data comparing the percentage of CMVpp65-specific CD8+ T EMRA cells (CD45RA+CD45RO−, CD45RA−CCR7+, CD45RA−CD11a+) from the total CMV-specific compartment by age group (B, bottom panel, left, middle and right respectively).

Supplementary Figure 2. Phenotype of high avidity CMV pp65-specific CD8+ T cells in HLA-A*0701 (TPR) individuals.
Representative dotplots showing the presence of two populations of conventional TPR tetramer binding CD8+ T cells based on tetramer fluorescence intensity (A, top panel). The CD45RA/CD45RO expression profile of the high and low tetramer binding populations was assessed (A, bottom panel). Donor PBMCs were stained with either the conventional or high avidity TPR/HLA-A*0701 tetramer together with CD45RA and CD45RO antibodies (B). Collective data (n=16) showing no significant correlation between percentage of CD45RA+ expression (left) and CD45RO+ expression (right), with per cent binding high avidity tetramer within the total TPR/HLA-A*0701 specific populations). Lines of best fit were generated by linear regression and the correlation assessed by Pearson and Spearman rank (GraphPad Prism).
Low Avidity CMV⁺ T Cells accumulate in Old Humans

Supplementary Figure 3. Additional data demonstrating the superior functional response of high avidity CD45RO⁺ NLV-specific cells.

CMV pp65 peptide pool induced functional response (IFNγ, CD107a and TNFα) of null and conventional binding tetramer populations from one individual who demonstrated a mixed population of NLV-specific cells (0.48% conventional tetramer binding, 0.07% null binding) (A). Similar assessment of functionality in two individuals who had all high avidity cells(0.03%conventional vs 0.04% null, top panels and 0.01% conventional vs 0.01% null, bottom panels) (B). N.D. represents not done. Sorted CD45RA⁺ (closed symbols) and CD45RO⁺ (open symbols) CD8⁺ T cell populations were stimulated separately with unpulsed APCs or increasing ratios of NLV peptide-pulsed APCs and the IFNγ response recorded as described previously (C). U.S. represents the un-stimulated control (containing no APCs). The results from 3 additional different donors are shown. The IFNγ response of the NLV-specific cells within the sorted CD45RA⁺ and CD45RO⁺ population to polyclonal CD3 stimulation and PMA/Ionomycin activation (D) were also measured in the same donor whose NLV specific response is shown in C, left panel).

Supplementary Figure 4. IL-15 and CMV specific expansion of CD8⁺ T cell populations

The number of CD8⁺ T cell were counted and the percentage survival compared to the original number of cells seeded were calculated at each time point during the IL-15 incubation (CD45RA⁻CD45RO⁺CD8⁺ and NLV/C MVpp65⁺ T cells, A). CD45RA⁺CD45RO⁻ and CD45RA⁻CD45RO⁻ cells were sorted via FACS Aria (B). FACs plot analysis showing CD45RA/CD45RO expression on gated NLV-specific CD45RA⁺CD45RO⁺ cells (top) and CD45RA⁻CD45RO⁻ cells (bottom) at days 0 and 7 of incubation with CMV pp65 peptide pulsed irradiated APCs and IL-2. Numbers represent percentages in quadrants.
Low Avidity CMV^+ T Cells accumulate in Old Humans

CD27/CD45RA CD8^+ T cell subsets were isolated and incubated with IL-15 and the percentage survival compared to the original number of cells seeded was, determined at the time points indicated (C).