DIFFERENTIAL CO-STIMULATION THROUGH CD137 (4-1BB) RESTORES
PROLIFERATION OF HUMAN VIRUS-SPECIFIC “EFFECtor MEMORY” (CD28-
CD45RA^hi) CD8^ T CELLS

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Running Title: CD137 restores proliferation of CD8 T cells

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Abstract

In healthy carriers of human cytomegalovirus (HCMV), the virus-specific memory CD8+ T cell population is often dominated by CD28- CD45RA\textsuperscript{hi} cells which exhibit direct ex vivo cytotoxicity but whose capacity for proliferation and generation of further memory cells has been questioned. We show that when highly purified CD28- CD45RA\textsuperscript{hi} CD8+ T cells are stimulated with viral peptide presented by autologous monocytes, the virus-specific T cells show early upregulation of CD137 (4-1BB) and CD278 (ICOS), re-express CD28, and proliferate with similarly high cloning efficiency in limiting dilution analysis as CD28+ CD45RO\textsuperscript{hi} cells or CD28- CD45RO\textsuperscript{hi} cells. Using peptide-pulsed autologous fibroblasts transfected with individual costimulatory ligands as antigen presenting cells, we show that CD137L is a key costimulatory ligand for proliferation of CD28- CD45RA\textsuperscript{hi} CD8+ T cells and not CD80, CD86 or CD275 (ICOSL). Therefore CD28- CD45RA\textsuperscript{hi} CD8+ T cells are not terminally differentiated but require a specific co-stimulatory signal for proliferation.
Introduction

Understanding the heterogeneity of human antigen-experienced CD8+ T cells has advanced by studying combinations of different phenotypic cell surface molecules. Sallusto et al subdivided human antigen-experienced CD8+ T cells on the basis of chemokine receptor CCR7 expression into CCR7+ “central memory” cells capable of homing to secondary lymphoid organs, and CCR7- “effector memory” cells ¹. The low molecular weight isoform of the common leucocyte antigen CD45RO ² is expressed by antigen-experienced CD28+ CD45ROhi cells, most of which express CCR7, and by CD28-CD45ROhi cells most of which lack CCR7. The high molecular weight isoform CD45RA is expressed by naïve CD28+ CD45RAhi cells which express CCR7. CD28- CD45RAhi cells have been termed ‘effector memory RA’ (TEMRA ) or ‘revertant memory’ cells because of their re-expression of CD45RA ³⁻⁵. On the basis of expression of costimulatory molecules CD28 and CD27, human antigen-experienced CD8+ T cells have also been subdivided into early CD28+ CD27+ cells (most of which express CD45RO and CCR7), intermediate CD28- CD27+ and late CD28- CD27- cells (most of which lack CCR7). In association with different human persistent virus infections, the phenotypes of circulating virus-specific CD8+ T cells in long-term virus carriers vary according to the virus ⁶,⁷. It appears that the phenotype of virus-specific CD8+ T cells may be associated with the site(s) in which virus antigen is expressed. Thus, in carriers of Epstein-Barr virus (EBV), CD8+ T cells directed against the EBV latency-associated antigens expressed in infected B cells are predominantly CD28+ CD45ROhi cells, whereas CD8+ T cells directed against EBV lytic antigens expressed in pharyngeal epithelial cells are predominantly CD28- CD45RAhi cells ⁸.

The beta herpesvirus human cytomegalovirus (HCMV) establishes life-long infection with latency in cells of the myeloid lineage ⁹. The CD8+ T cell response to HCMV in immunocompetent subjects is
usually strong and can constitute up to 10% of an individual’s antigen-experienced compartment\(^{10}\). We have previously shown that during primary HCMV infection, the highly activated HCMV-specific CD8\(^+\) T cells in peripheral blood are predominantly CD28- CD45RO\(^{hi}\). During convalescence, circulating populations of HCMV-specific CD28+ CD45RO\(^{hi}\) cells and CD28- CD45RA\(^{hi}\) cells emerge and accumulate in the peripheral blood\(^{4}\). In long-term virus carriers, the CD8\(^+\) T cell response against a defined viral peptide is often dominated by a relatively small number of individual clones which have undergone massive expansion in vivo and which are maintained for years\(^{5}\). Cells of a single expanded HCMV-specific clone are usually distributed in both CD28+ CD45RO\(^{hi}\) cells and in CD28- CD45RA\(^{hi}\) cells\(^{5}\); in many virus carriers, the response against a viral peptide is dominated by CD28- CD45RA\(^{hi}\) cells\(^{4,5}\). These HCMV specific CD28- CD45RA\(^{hi}\) T cells are heterogeneous for the expression of CD27\(^4\) and as such can be viewed as a mixture of intermediate and late stage antigen experienced CD8\(^+\) T cells as defined by Appay et al\(^6\).

Antigen-experienced CD45RA\(^{hi}\) CD8\(^+\) T cells have some characteristics of effector cells, including direct ex vivo antigen-specific cytotoxicity, intracellular perforin expression and prompt secretion of IFN gamma following antigen recognition\(^{11-13}\). However, the capacity of antigen-experienced CD45RA\(^{hi}\) CD8\(^+\) T cells to proliferate in vitro has been questioned by some investigators. When stimulated in vitro with monoclonal anti-CD3 antibody plus anti-CD28 antibody or allogeneic dendritic cells, CCR7- CD45RA\(^{hi}\) CD8\(^+\) T cells showed reduced proliferation\(^{14,15}\). In contrast, when stimulated in vitro with specific viral peptide presented by autologous PBMC in the presence of exogenous IL-2, highly purified CCR7- CD28- CD45RA\(^{hi}\) CD8\(^+\) T cells or CCR7- CD27- CD45RA\(^{hi}\) CD8\(^+\) T cells proliferated strongly\(^{4,16}\). Because of their reduced proliferation under some but not all experimental conditions, our hypothesis was that CD28- CD45RA\(^{hi}\) CD8\(^+\) T cells might have a strict
requirement for specific co-stimulation following stimulation with antigen. The CD28 costimulation pathway was the first to be described and is considered to be the most important for activation of naïve T cells. A number of alternative co-stimulatory pathways belonging to the CD28/B7 family and TNF/TNFR family have subsequently been identified. Costimulation through CD137:CD137L has been demonstrated to promote proliferation of CD8+ CD28- memory T cells when administered with anti-CD3 stimulation but to date the co-stimulatory requirement(s) of antigen-specific CD28-CD45RA hi CD8+ T cells following antigen exposure remain unclear. This is an important question, bearing on whether the assumption of end stage differentiation, and the designation of this population as “effector memory” is fully justified. Therefore in highly purified populations of CD28-CD45RA hi CD8+ T cells, we analysed the antigen-stimulated cloning efficiency, kinetics of co-stimulatory molecule expression, and proliferative response to antigen-expressing autologous fibroblasts that expressed individual co-stimulatory ligands.

Materials and Methods

*Ethical approval was obtained from the Addenbrookes NHS Hospital Trust institutional review board (Cambridge Research Ethics Committe) for this study. All recipients provided informed consent according to the Declaration of Helsinki (LREC 97/092).*

Donors

Ten healthy HCMV-seropositive long-term virus carriers were studied. Serostatus was determined with an IgG ELISA (Trinity Biotech, Didcot, UK).

Peptides and MHC class I multimers
Peptides from the HCMV tegument protein pp65 (Proimmune, Oxford, UK) were used to stimulate virus-specific CD8+ T cells. NLVPMVATV presented by HLA-A2 and TPRVTGGGAM presented by HLA-B7 were diluted in unsupplemented RPMI-1640 medium (Sigma, Dorset, UK) and used at the dilutions indicated. MHC class I multimers, either tetramers or pentamers, of NLVPMVATV plus HLA-A2 or TPRVTGGGAM plus HLA-B7 (Proimmune, Oxford, UK or a kind gift of Professor Paul Klenerman, University of Oxford) were used to identify peptide-specific CD8+ T cells.

**Cell purification**

PBMC were prepared from fresh heparinized venous blood samples by Ficoll-Hypaque (Lymphoprep; Nyegaard, Oslo, Norway) density-gradient centrifugation. Cells were stained with APC-Alexafluor 750-conjugated anti-CD8, FITC-conjugated anti-CD45RO and PE-conjugated anti-CD28 (Caltag, Buckingham UK) and sorted using a FACSDiva cell sorter (Becton Dickinson, Oxford, UK) into subpopulations of CD28+ CD45ROhi, CD28- CD45ROhi and CD28- CD45RO- (CD45RAhi) cells. In some experiments, CD8+ CD28- CD45RO- cells were depleted of CD27+ cells by re-staining with PE-conjugated anti-CD27 (ebioscience, San Diego, USA) and re-sorting. After sorting, the purity of cell populations was checked on a FACSCalibur flow cytometer; populations were consistently >99% pure. In some experiments, CD14+ monocytes were purified from PBMC by directly-conjugated anti-CD14 magnetic microbeads and MACS columns (both Miltenyi Biotec, Surrey, UK) according to the manufacturer’s instructions. Purity of the monocyte preparation was determined using a FITC-conjugated anti-CD14 monoclonal antibody (Caltag). In blocking antibody experiments, PBMC were depleted of CD28+ cells by staining with PE-conjugated anti-CD28 and anti-CD16 followed by anti-PE magnetic microbeads and MACS columns.
Stimulation of CD8+ T cells with HCMV peptide

Sorted CD8+ T cell populations were cultured in 96 well round-bottom plates with irradiated autologous stimulator cells (50,000 monocytes or PBMC per well or 5,000 fibroblasts per well) that had been pulsed with viral peptide (40µg/ml unless indicated) and repeatedly washed. Culture was in 100µl per well RPMI-1640 medium (Sigma, Dorset, UK) supplemented with 2mM L-glutamine, 10^5 IU/L Penicillin, 100mg/L Streptomycin, 5 IU/ml human rIL-2 (National Institute for Biological Standards and Control, Potters Bar, UK), 10% Myoclone fetal calf serum (Sigma), plus either 10% human AB serum (HCMV seronegative; Blood Transfusion Service, Addenbrooke’s Hospital, Cambridge, UK) or 10% autologous heat-inactivated serum. Cultures were incubated at 37°C in 5% CO2 and every 5 days, wells were refed with 50ul of fresh medium containing supplements.

Limiting dilution analysis.

The method was as published in detail previously. Briefly, replicate microcultures (n=27) of purified responder CD8+ T cell populations were set up in 96-well round bottomed plates in which the number of responder T cells per well was progressively reduced over an appropriate range of dilutions. Irradiated autologous peptide-pulsed PBMC (50,000 per well) were used as stimulator cells, in culture medium described above. On day 14, the cells in each well were mixed and divided into three aliquots which were assayed for peptide-specific cytotoxicity in four hour 51Cr release assays against autologous or MHC mismatched lymphoblastoid B cells (4,000 per well) pulsed with viral peptide or unpulsed. Analysis of cytotoxicity assays was as published previously. Aliquots of input CD8+ T cell populations were stained with APC-conjugated peptide-MHC multimers (Promimmune) and anti-CD8 followed by flow cytometry, to quantify the input peptide-specific cells per well.
CD8+ cell phenotype analysis

Following peptide stimulation, cells were removed from culture at intervals for flow cytometry. Cells were stained with PERCP-conjugated anti-CD8, APC-conjugated peptide-MHC multimers (Promimmune) to identify antigen-specific cells, and antibodies against CD28, CD45RA, CD45RO, CD62L (from Caltag), inducible costimulator ICOS (CD278), CD27 (from ebiosciences), CD137 (4-1BB), CXCR3, CCR5 (from Becton Dickinson), CCR7 (R and D Systems, Abingdon UK) conjugated to FITC or PE, using FITC- or PE-conjugated isotype controls (Caltag; Becton Dickinson). KLRG1-Alex488 was provided by Professor Hanspeter Pircher, University of Freiburg, Germany. Staining was carried out in 50μl PBS with 2% normal mouse serum, cells were washed in PBS and resuspended in PBS with 2% paraformaldehyde prior to analysis using a FACSCalibur flow cytometer running Cellquest software (Becton Dickinson). Data were analysed using WinMDI 2.8 software (http://pingu.salk.edu/software.html).

Blocking antibody experiments

Unconjugated monoclonal antibodies against CD4, CD80, CD86 (from Caltag), CD275 (ICOSL) (from ebioscience) and CD137L (4-1BBL) (MBL, Nagoya, Japan) were dialysed to remove sodium azide using Slide-a-Lyser kits (Pierce, Rockford, Illinois, USA) according to the manufacturer’s instructions. Briefly, a 3500 MWCO cassette was pre-hydrated for 2 minutes in PBS, 0.5ml antibody injected and dialysis performed for 12 hours at 4°C before the antibody was removed. 20mg of dialysed antibody was added to cultures of peptide-pulsed PBMC which were irradiated and plated at 20,000 cells per well, to which CD28-depleted responder PBMC 10,000 per well were added in supplemented medium. Responder cell proliferation was determined by cell counting.
Plasmids

CD137L (4-1BBL)-pcDNA3 was provided by Tania Watts (University of Toronto, Canada) and CD275 (ICOSL)-pcDNA3.1 by Lieping Chen (Johns Hopkins School of Medicine, Baltimore, USA). CD80 PHR’CMV was constructed by cloning full-length CD80 into the PHR’CMV vector. CD86-pcDNA3.1 was provided by Jessica Boname and Paul Lehner (Cambridge Institute for Medical research, Cambridge UK).

Transfection of primary fibroblast lines

Autologous donor fibroblasts obtained by skin biopsy were maintained in EMEM (Sigma) plus 10% fetal calf serum. Fibroblasts were transfected using a Necleofector kit (Amaxxa, Cologne, Germany) according to the manufacturer’s instructions. Briefly, $10^6$ trypsin-detached fibroblasts were combined with 5 µg DNA in 100µl nucleofector solution and electroporated using nucleofector program U23. Cells were transferred to warm medium overnight. Cells were then detached using cell dissociation solution (Sigma) and pulsed with different concentrations of viral peptide, irradiated, washed and plated as antigen presenting cells at 5,000 cells per well with purified CD28- CD45RA$^{hi}$ responder CD8+ T cell 10,000 per well in supplemented medium. Expression of co-stimulatory molecules on transfected fibroblasts was determined by flow cytometry using PE-conjugated anti-CD80, CD86, CD275 and CD137L (all Becton Dickinson), compared to isotype controls. Control antigen presenting cells were irradiated autologous peptide-pulsed monocytes. After 10 days culture, cells in duplicate wells were counted and the peptide-specific cells were quantified by staining with multimer and anti-CD8 followed by flow cytometry, to determine the total number of antigen-specific cells per well.
Results

In response to peptide stimulation by autologous PBMC, HCMV-specific CD28- CD45RA<sup>hi</sup> CD8<sup>+</sup> cells proliferate with similarly high cloning efficiency as CD28+ CD45RO<sup>hi</sup> cells or CD28- CD45RO<sup>hi</sup> cells.

The cloning efficiency of highly purified CD28- CD45RA<sup>hi</sup> cells for proliferation and generation of cytotoxic daughter cells was assessed in limiting dilution analysis upon stimulation with irradiated autologous peptide-pulsed PBMC, in comparison to CD28+ CD45RO<sup>hi</sup> cells and CD28- CD45RO<sup>hi</sup> cells of the same subject. The proportion of peptide-specific CD8+ T cells in each input responder population was first quantified by peptide-MHC class I multimer staining using HLA-A2-NLV-pp65 or HLA B7-TPR-pp65. The cloning efficiency was similar in each of the sorted subpopulations and was in the range 20-60% (Figure 1), the same as the cloning efficiency we previously reported for the CD8+ T cell population as a whole when stimulated with irradiated peptide-pulsed autologous PBMC<sup>22</sup>. In LDA plates at the lowest input cell dilutions in which on average there was one multimer-positive cell per well at the start of culture, after 10-11 days of culture there were 88,000-380,000 antigen-specific cells per well; similar numbers of antigen-specific cells were observed in wells plated with CD28-CD45RA<sup>hi</sup> cells or CD28+ CD45RO<sup>hi</sup> cells. Without adjustment for cell death in vitro, this indicated that a single antigen-specific cell from each subpopulation had undergone at least 16 divisions. Following further depletion of CD27+ cells, the cloning efficiency of the CD28- CD27-CD45RA<sup>hi</sup> subpopulation was closely similar to that of the CD28- CD45RA<sup>hi</sup> population (data not shown). The inhibitory Killer Cell Lectin-like receptor G1 (KLRG1) has previously been suggested to identify CD8+ T cells that have reduced proliferative response to PHA or anti-CD3 stimulation<sup>23</sup>. In two HCMV-seropositive donors, almost all (>95%) of the multimer-positive CD8+ T cells in PBMC
expressed KLRG1 (data not shown). When stimulated with peptide antigen, these KLRG1+ virus-specific cells showed no apparent proliferation defect.

In response to peptide stimulation, virus-specific CD28- CD45RAhi cells upregulate CD137 (4-1BB) and CD278 (ICOS) and upregulate homing chemokine receptors

Cultures of CD28- CD45RAhi cells were harvested at intervals after stimulation with peptide-pulsed monocytes, and stained with multimers to identify antigen-specific cells and antibodies against the co-stimulatory molecules CD137 or CD278 or CD28 (Figure 2). Prior to stimulation, CD28- CD45RAhi cells did not express CD137 or CD278 or CD28. As early as day 2 in culture, CD137 was strongly expressed on a small percentage of the responder CD8+ T cells; simultaneous co-staining with multimer was inconclusive due to TCR down-regulation following peptide stimulation (data not shown). By day 5 in culture, CD137 was strongly expressed on the antigen-specific cells but on few bystander CD8+ T cells (Figure 2A,B). CD137 expression was transient and was no longer present by day 12. By day 5 in culture, CD278 was also expressed on most of the antigen-specific cells but on few bystander CD8+ T cells. CD278 expression was also transient and was no longer present by day 12 (Figure 2A,B). By days 5-7 CD28 was transiently re-expressed on a proportion of the antigen-specific cells, albeit at a level 5-10 fold less than CD28 expressed on naïve CD28+ CD45RAhi cells or CD28+ CD45ROhi cells (Figure 2A,B). By day 5 in culture, there was transient re-expression of CCR7 on most of the antigen-specific cells (Figure 2C) consistent with a previous study of CD27- CD45RAhi cells 24,25. By day 7 there was modest upregulation of CD62L on a proportion of antigen-specific cells. Upregulation of CCR5 expression on antigen-specific cells showed delayed kinetics with peak expression on day 9. Strong uniform upregulation of CXCR3 on antigen-specific cells occurred by day 5 and was sustained to day 12 (Figure 2C). Both CCR5 and CXCR3 are expressed by effector T cells
that infiltrate peripheral sites \(^{26}\) which suggests that activated CD28-CD45\(^{hi}\) cells acquire the capacity to migrate to sites of HCMV reactivation

\textit{CD137L provides co-stimulation for antigen-specific proliferation of CD28- CD45\(^{hi}\) cells whereas CD80, CD86 and CD275 do not.}

Monoclonal antibodies against individual co-stimulatory ligands on antigen presenting cells were added to irradiated peptide-pulsed autologous PBMC prior to culture with purified CD28- responder cells. Addition of anti-CD137L reduced the antigen-stimulated proliferation of responder cells compared to control antibody (Figure 3A), whereas addition of anti-CD275 or anti-CD80 or anti-CD86 had no clear effect on antigen-stimulated proliferation (Figure 3B,C), the experiment was performed with 3 further donors with similar results only anti-CD137L was able to block T cell proliferation in the range of 40-60\% depending on the donor. Autologous fibroblasts were transiently transfected to express individual costimulatory ligands, which was quantified by flow cytometry (Figure 4A,B). Untransfected fibroblasts expressed very low levels of each co-stimulatory ligand, whereas 50-80\% of transfected fibroblasts expressed CD137L, CD275, CD80 or CD86. As artificial antigen presenting cells, aliquots of transduced fibroblasts were pulsed with four dilutions (40\(\mu\text{g/ml}\) to 0.4 \(\mu\text{g/ml}\)) of viral peptide or unpulsed, and irradiated prior to culture with sorted CD28- CD45\(^{hi}\) CD8\(^{+}\) T cells. In this model system, fibroblasts transfected with CD137L were consistently able to promote the antigen-specific proliferation even at low concentrations of viral peptide, whereas untransfected fibroblasts or fibroblasts transduced with CD275, CD80 or CD86 were unable to do so even at the highest peptide concentration (Figure 4C,D). The absolute magnitude of antigen-stimulated proliferation elicited by CD137L-transduced fibroblasts was about half that observed following stimulation with autologous monocytes.
Discussion

Here we show that the cloning efficiency of CD28- CD45RA\textsuperscript{hi} CD8+ T cells in limiting dilution analysis is similar to that of CD45RO\textsuperscript{hi} CD8+ T cells. Therefore HCMV-specific CD28- CD45RA\textsuperscript{hi} cells have no intrinsic proliferation defect when stimulated by antigen; previous studies that suggested impaired proliferative responses have underestimated their true proliferative capacity, probably through sub-optimal provision of specific co-stimulation and/or exogenous IL-2. KLRG1 which has been suggested to mark cells with a reduced proliferative capacity\textsuperscript{23} but was expressed by the overwhelming majority of multimer-positive cells in two individuals and therefore does not mark cells that are unable to proliferate. This suggests that appropriate costimulation may be able to ‘rescue’ cells which otherwise exhibit reduced proliferation. Provision of CD137L on autologous, peptide-pulsed, irradiated fibroblasts costimulates proliferation of CD28- CD45RA\textsuperscript{hi} CD8+ T cells whilst costimulation provided through CD278 or CD80/86 was unable to do so. This suggests a selective use of costimulatory pathways by the CD28- CD45RA\textsuperscript{hi} CD8+ population. The reduced potency of fibroblasts compared to monocytes is not unexpected, given that fibroblasts may lack appropriate adhesion molecules and cytokine secretion as well as ligands for other additionally important costimulatory receptors.

A number of studies in mice have suggested a role for CD137 in regulating memory T cell responses. For influenza the magnitude of the primary response is not altered in CD137L KO mice early but is lower later (21-38 days) with a defect in the secondary response\textsuperscript{27}. Both Agonist mAb to CD137 and transgenic CD137L expression have been reported to stimulate the proliferation of memory T cells in
vivo \(^{28}\)
and IL-15 has been shown to up-regulate expression of CD137 and may thereby promote
survival of memory T cells in the absence of antigen \(^{29}\).

How may co-stimulation through CD137L influence the contribution of CD28- CD45RA\(^{\text{hi}}\) CD8+ T
cells to the control of virus infections? In the weeks after primary infection, virus-specific CD28-
CD45RO\(^{\text{hi}}\) cells become less numerous in peripheral blood whereas virus-specific CD28- CD45RA\(^{\text{hi}}\)
CD8+ T cells become more abundant \(^4\). CD28 expression can be modulated in-vitro with its loss
stimulated by culture in the presence of IL-2, IL-7 or IL-15 and type 1 IFN \(^{30,31}\). Purified CD45RA-
cells can re-express CD45RA during prolonged in vitro culture with appropriate cytokines (IL-7, IL-
15) \(^{15}\); in the absence of antigen re-expression of CD45RA is related to the time since last antigen
stimulation \(^{25}\). As shown using stable isotopic labeling techniques in vivo, circulating antigen-
experienced CD45RA\(^{\text{hi}}\) CD8+ T cells show reduced rates of proliferation and much reduced clearance
from the circulation compared to CD45RO\(^{\text{hi}}\) cells, consistent with a long inter-mitotic interval \(^{32}\).

Together these observations suggest that following exposure to viral antigen in vivo, activated CD28-
CD45RO\(^{\text{hi}}\) cells in the absence of antigen can, in an appropriate cytokine environment, revert to more
quiescent CD28- CD45RA\(^{\text{hi}}\) cells; the anatomical site(s) in vivo at which such reversion may occur are
unclear but may include the peripheral blood and/or the bone marrow. The physiological pathway(s) of
recirculation of CD28- CD45RA\(^{\text{hi}}\) cells are incompletely understood – it is unclear to what extent they
migrate through the uninflamed mucosa of the lung, gastro-intestinal, genital tract or breast, or through
solid organs such as liver or spleen. In the case of HCMV, reactivation of infectious virus from
latently infected monocytes and dendritic cells is dependent upon activation/differentiation of the
latently infected cell \(^{9}\). Because monocytes and dendritic cells are very widely distributed and are
recruited to sites of inflammation, HCMV reactivation could potentially occur at many different sites;
in long-term carriers, known sites of virus shedding include the throat, the cervix and breast milk.
When virus-specific CD28- CD45RA\textsuperscript{hi} CD8+ T cells encounter virus-infected cells, they can express immediate cytotoxic effector activity and secrete IFN gamma \textsuperscript{11,12,33} that enhances local antigen presentation and NK cell activation. We show that antigen stimulation also induces upregulation of CD137: therefore provided there is local CD137L co-stimulation from the virus-infected cell or a bystander cell and there is sufficient IL-2, local proliferation of virus-specific CD28- CD45RA\textsuperscript{hi} CD8+ T cells could follow. CD137L is expressed by monocytes and dendritic cells \textsuperscript{34}, and by human neurons and inflamed vessel walls \textsuperscript{35,36}. Utilization of different co-stimulatory ligands by T cells may also be influenced by HCMV itself, either in infected dendritic cells \textsuperscript{37} or in nearby cells by the action of virus-encoded IL-10 \textsuperscript{38}.

Antigen stimulation of virus-specific CD28- CD45RA\textsuperscript{hi} cells induces down regulation of CD45RA, upregulation of CD45RO \textsuperscript{4} and transient re-expression of CCR7 that may enable virus-specific cells to re-circulate to lymph nodes where (further) proliferation may take place. Subsequent time-dependent down-regulation of CCR7 and upregulation of chemokine receptors including CCR5 and CXCR3 might enable the daughter cells to migrate from lymph nodes and home to sites of inflammation where viral reactivation is occurring.

A number of human co-stimulatory molecules and their ligands have been reported, but their relative importance for activation of human antigen-experienced T cells is incompletely understood. Here we show for the first time that a well-characterized subpopulation of antigen-specific human CD8+ T cells has selective co-stimulatory ligand usage for proliferation. The cell types that express this co-stimulatory ligand CD137L including monocytes and dendritic cells may play an important role in the amplification of the virus-specific CD8+ T cell response against HCMV reactivation. More generally,
our data suggest that there is a greater functional plasticity amongst phenotypically defined subpopulations of memory T cells than is suggested by current classifications.

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Author Contribution Statement:

Edward.C.P.Waller- conceived experiments, performed research, collected, analyzed and interpreted data, drafted the manuscript.

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Mark.R.Wills - designed research, analyzed and interpreted data, drafted the manuscript.
References

Figures.

Figure 1. Cloning efficiency of sorted subpopulations of HCMV-specific CD8+ T cells in limiting dilution analysis. A) Limiting dilution analysis of sorted CD28-CD45RO- cells, CD28-CD45RO<sup>hi</sup> cells and CD28+CD45RO<sup>hi</sup> cells (donor 35). The initial number of multimer-positive cells per well was calculated from the initial number of cells per well multiplied by the initial proportion of multimer-positive cells in each subpopulation determined by flow cytometry. B) Cloning efficiency of each subpopulation in three healthy HCMV-seropositive donors; CD28-CD45RO- cells in grey, CD28-CD45RO<sup>hi</sup> cells in white, CD28+CD45RO<sup>hi</sup> cells in black.

Figure 2. Expression of co-stimulatory and homing molecules on antigen-specific CD28-CD45RA<sup>hi</sup> cells before and after stimulation with viral peptide. A) Replicate cultures of sorted CD28-CD45RO- CD8+ T cells were stimulated with peptide-pulsed autologous monocytes and harvested at intervals for flow cytometry. Expression of CD137 (at day 5), CD278 (at day 5) and CD28 (at day 7) is shown against peptide-MHC Class I multimer, gated on CD8+ T cells. B) Kinetics of expression of CD137, CD278 and CD28 following peptide stimulation (filled histograms) compared to isotype control (open histograms) gated on total input CD8+ cells (day 0) or gated on multimer-positive CD8+ T cells (days 5-12). C) Kinetics of expression of CCR7, CD62L, CCR5 and CXCR3 following peptide stimulation gated on total input CD8+ cells (day 0) or gated on multimer-positive CD8+ T cells (days 5-12).

Figure 3. Inhibition of peptide-stimulated proliferation of CD28- cells by anti-CD137L antibody. Replicate cultures of purified CD28-CD8+ T cells were stimulated with irradiated peptide-pulsed autologous PBMC in the presence or absence of antibody. Duplicate cultures were harvested at
intervals and the total cell count per well determined. A) diamond - no antibody; square - control irrelevant antibody anti-CD4; triangle - anti-CD137L. B) diamond - no antibody; square - control irrelevant antibody anti-CD4; triangle - anti-CD275. C) filled diamond - no antibody; filled square - control irrelevant antibody anti-CD4; filled triangle - anti-CD80; open square - anti-CD86; open triangle - both anti-CD80 and anti-CD86. The experiment was performed on 4 independent donors of which this is a representative experiment. The result from all 4 donors were similar antibodies to CD80, CD86 and ICOSL were unable to block proliferation while anti 41BBL was (within the range 40-60% depending on donor). anti CD4 was an irrelevant antibody control as this antibody is able to bind the antigen presenting cells and demonstrates that steric hindrance by an irrelevant antibody was not able to block co-stimulation and T cell proliferation. Anti ICOSL, CD80 and CD86 antibodies were all the same isotype (IgG1) as the blocking 41BBL antibody and act as internal isotype controls.

Figure 4. Differential co-stimulation of peptide-stimulated proliferation of CD28-CD45RA\textsuperscript{hi} cells by CD137L expressed on autologous fibroblasts. Replicate cultures of sorted CD28-CD45RO-CD8+ T cells were stimulated with autologous fibroblasts that had been transduced with individual co-stimulatory ligands and pulsed with dilutions of viral peptide (from 40 µg/ml to 0.4 µg/ml) or unpulsed, or autologous monocytes pulsed with viral peptide or unpulsed. T cells were harvested at 10 days and the number of antigen-specific cells per well was calculated from the total cell count per well multiplied by the proportion of multimer-positive cells determined by flow cytometry. A) Fibroblasts transfected with test construct stained with isotype control antibody (open histograms) or test antibody (filled histograms). B) Fibroblasts transfected with control construct stained with isotype control antibody (open histograms) or test antibody (filled histograms). C) donor 28. D) donor 26. These
experiments were performed twice on each donor and the results in both cases confirm that on 41BBL transfection was able to support T cell proliferation. At each peptide concentration, the number of antigen-specific cells per well stimulated by CD137L-transfected fibroblasts was significantly greater (p<0.05 by Student T-test) than stimulation with CD275-transfected fibroblasts or CD80/CD86-transfected fibroblasts. Error bars represent the standard deviation of the cell count from three independent wells at each peptide concentration multiplied by the proportion of antigen specific cells as determined by peptide specific MHC Class I multimers and flowcytometry.
Figure 1

A

Number of tetramer+ cells / well

CD8+CD45RA+CD28-  
0 1 2 3 4 5
100
10

CD8+CD45RO+CD28-  
0 1 2 3 4 5
100
10

CD8+CD45RO+CD28+  
0 1 2 3 4 5
100
10

B

Cloning efficiency

0% 20% 40% 60%

RA28- RO28- RO28+  
Donor 32

RA28- RO28- RO28+  
Donor 35

RA28- RO28- RO28+  
Donor 26
Figure 3

A  Anti CD137L

B  Anti CD275

C  Anti CD80/86

Cell Number / well

Days
Figure 4

A

B

CD137L  CD275  CD80  CD86

C

D

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Differential co-stimulation through CD137 (4-1BB) restores proliferation of human virus-specific "effector memory" (CD28-CD45RA hi) CD8+ T Cells

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