Blockade of PD-1 or p38 MAP Kinase signalling pathways enhances senescent human CD8⁺ T-cell proliferation by distinct pathways

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Abstract

Immune enhancement is desirable in situations where decreased immunity results in increased morbidity. We investigated whether blocking the surface inhibitory receptor PD-1 and/or p38 MAP kinase could enhance the proliferation of the effector memory CD8⁺ T-cell subset that re-expresses CD45RA (EMRA) and exhibits characteristics of senescence, which include decreased proliferation and telomerase activity but increased expression of the DNA damage response-related protein γ H2AX. Blocking of both PD-1 and p38 MAPK signalling in these cells enhanced proliferation and the increase was additive when both pathways were inhibited simultaneously in both young and old human subjects. In contrast, telomerase activity in EMRA CD8⁺ T cells was only enhanced by blocking the p38 but not the PD-1 signalling pathway, further indicating that non-overlapping signalling pathways were involved. Although blocking p38 MAPK inhibits TNF- α secretion in the EMRA population, this decrease was counteracted by the simultaneous inhibition of PD-1 signalling in these cells. Therefore, end-stage characteristics of EMRA CD8⁺ T cells are stringently controlled by distinct and reversible cell signalling events. In addition, the inhibition of PD-1 and p38 signalling pathways together may enable the enhancement of proliferation of EMRA CD8⁺ T cells without compromising their capacity for cytokine secretion.

Introduction

Human T-cell memory is maintained by episodes of repeated antigenic challenge throughout life [1, 2]. These antigenic encounters drive proliferation and differentiation of memory T cells, leading to the accumulation of highly differentiated T cells in older subjects [1, 3]. Highly differentiated T cells also accumulate in patients with malignancy [4] and those with persistent infections [3, 5, 6]. However, highly differentiated CD4⁺ and CD8⁺ T cells in humans have decreased proliferative capacity [7-9]. Therefore it is possible that the increased susceptibility to infections and malignancy during ageing may result from suboptimal maintenance of responsive pools of specific T cells due to their dysfunctional ability to divide [10, 11]. This raises the question of whether it is possible to enhance T-cell proliferation in older subjects.

Human T cells at late stages of differentiation can be identified by the loss of surface markers CD27, CD28 and CCR7 and the re-expression of CD45RA [7, 9, 12]. Furthermore highly differentiated memory T cells have relatively short telomeres [13] and have changes in cell signalling pathways including defective Akt/PKB [7, 12] and increased p38 MAP kinase (MAPK) phosphorylation that are associated with cellular senescence [7, 9, 12, 14]. These changes are particularly evident in highly differentiated effector memory T cells that re-express CD45RA (EMRA T cells) [7, 12] which are considered to be an end-stage or senescent population [15, 16]. The EMRA CD4⁺ and CD8⁺ T cells also exhibit the loss of both proliferative capacity and telomerase activity and have increased susceptibility to apoptosis [7, 9, 12, 17] and these cells increase significantly during ageing.

Two processes have been shown to induce a loss of proliferative ability in T cells. First, repeated antigenic stimulation of T cells may induce a state of functional exhaustion where proliferative activity and cytokine production are lost [18]. This immune exhaustion is initiated by external cell surface inhibitory receptors such as PD-1 and Tim3 [19, 20]. Second, repeated T-cell stimulation or genotoxic damage by agents such as reactive oxygen species can induce replicative senescence where cells lose proliferative potential [1, 8]. This process is triggered by a DNA damage response (DDR) that leads to growth arrest if the DDR is not resolved [21, 22]. It is not clear if senescence and exhaustion signaling pathways are distinct processes [14] or if they act in isolation or in concert to control the proliferation of end-stage or senescent CD8⁺ T cells.

We now demonstrate that the proliferation of highly differentiated EMRA CD8⁺ T cells can be enhanced in an additive manner by blocking p38 MAPK and PD-1 signalling together, however, other functions such as telomerase activity are uniquely regulated by the p38 MAPK pathway. Furthermore, by inhibiting both pathways simultaneously, highly differentiated T cells can be induced to exhibit increased proliferative potential while also retaining their ability to secrete cytokines such as TNF- α , that cannot be achieved by blocking either pathway alone. Nevertheless in old individuals, other mechanisms controlling proliferation may exist, since the proliferative activity of EMRA CD8⁺ T cells after blockade of PD-1 and p38 MAPK signalling does not restore proliferative responses to the level observed in young subjects.

Results

CD8⁺ EMRA T cells from young and old donors exhibit characteristics of senescent T cells

Human CD8⁺ T cells can be subdivided into 4 populations on the basis of their relative surface expression of CD45RA and CD27 molecules (Fig. 1A). Four subsets can be defined, naïve (N; CD45RA⁺CD27⁺), central memory (CM; CD45RA⁺CD27⁺) effector memory (EM; CD45RA⁺CD27⁻) and effector memory T cells that re-express CD45RA (EMRA; CD45RA⁺CD27⁻). We have previously shown that the EMRA population, when isolated from young individuals exhibited senescent characteristics [8], we now extend this to CD8⁺ T cells from old individuals. Using a panel of antibodies that defines senescent characteristics, KLRG1, CD57 and γ H2AX (Fig.1A), we find that upon overnight stimulation the CD8⁺ T-cell subsets isolated from old individuals express a greater array of senescent markers relative to young individuals (Fig. 1B). Furthermore, the EMRA population from old donors have more senescence characteristics than those isolated from young donors (Fig. 1C). However what is also evident is that differential expression of senescent features between the subsets follows a similar pattern in both young and old donors, therefore the higher levels of senescent characteristics observed are dependent on age.

A lack of proliferation is a defining feature of senescence [8]. We found that when using Ki67 staining as a readout for proliferation CD8⁺ EMRAs, from both young and old individuals, exhibit a significant proliferative impairment when compared to the less differentiated subsets after stimulation (Fig 2A). This was not thought to be

caused by a lower expression of CD3 in this subsets, as we have previously published it to be elevated [23]. However, the proliferative activity in all four subsets, especially in the naïve and EMRA populations, was decreased in the old volunteers (Fig. 2A). In addition we found that there was a significant reduction in telomerase in the EMRA population from young and old individuals when compared to the other three subsets (Fig 2B). Again the amount of telomerase activity detected in all the CD8⁺ subsets isolated from old donors was significantly less than the levels seen in young donors (Fig. 2C) which supports our previous observation that old individuals have shorter telomere lengths in all these subsets compared to the young cohort [24].

Despite the EMRA population of CD8⁺ T cells having less proliferative and telomerase activity, these cells, when isolated from both young and old donors, were significantly more multifunctional than the other subsets, defined by using a panel that measured the expression of perforin, granzyme B, IFN- γ and TNF- α simultaneously (Fig. 3A and B). It is interesting to note that the naïve CD8⁺ T cells isolated from old donors display considerably more polyfunctionality than the CM cells (Fig. 3B), suggesting that naïve cells isolated from old donors, identified by CD45RA⁺CD27⁺ expression may not be a truly naïve population. However CD8⁺ EM cells from young donors were found to be more polyfunctional based on 4 functions relative to older donors (Fig. 3B).

We next investigated each cellular function separately in each of the subsets of young and old donors (Fig. 3C). We found that despite their senescence characteristics, the percentage of the CD8⁺ EMRA T-cell population in both age groups expressed high levels of pro-inflammatory cytokines and cytotoxic molecules. However while these cells from old donors expressed a higher percentage of cytokines, they exhibited lower levels of perforin and granzyme B after stimulation. Once again, a striking observation was that the naïve CD45RA⁺CD27⁺ population showed greater capacity for both cytokine secretion and cytotoxicity than their counterparts in young subjects reinforcing the possibility that these cells in old donors are not truly naïve.

Expression of PD-1 and p38 MAPK during CD8⁺ T-cell differentiation

We next investigate mechanisms that were involved in inhibiting proliferation in donors from both age groups. PD-1 is the most investigated inhibitory receptor that is expressed by exhausted CD8⁺ T cells [25]. When we examined the expression of PD-1 on CD45RA/CD27 defined CD8⁺ T-cell subsets, we found the highest level of expression to be on the CM and EM subsets (Fig. 4A and B). These results together with their high functionality indicate that the CD8⁺ EMRA subset in young and old subjects is not an exhausted population.

Increased expression of p38 MAPK is associated with senescent T cells [26]. We found that naïve T cells express the lowest levels and EMRA T cells express significantly higher amounts of phosphorylated p38 MAPK ex vivo (Fig. 4C and D).

This supports the concept that EMRA CD8⁺ T cells are an end-stage/senescent population

Both PD-1 and p38 MAPK signalling contribute to reduced proliferation of EMRA T cells

We next investigated whether either PD-1 or p38 MAPK signalling pathways contribute to the decreased proliferative activity of EMRA CD8⁺ T cells. We isolated the four CD45RA/CD27 defined T-cell subsets from both young and old donors, which were stimulated with anti-CD3 antibody and irradiated autologous APCs in the presence of either PDL1/2 blocking antibodies, BIRB 796 or both inhibitors together. Proliferative activity was determined by staining with Ki67 antibody. Representative histograms for the EMRA subset with and without the inhibitors from young and old donors are shown in Figures 5A and C, respectively. The cumulative data from 9 young and 5 old donors are shown in Figures 5B and D, respectively. In young donors, inhibiting PD-1 signalling increased proliferative activity in CD8⁺ T cells at all four stages of differentiation. However, the inhibition of the p38 MAPK pathway was only effective at increasing proliferation in the end-stage EMRA subset (Fig. 5A and B). There was additive enhancement of proliferation in the EMRA subset when both signalling pathways were inhibited together (Fig. 5A and B). When we investigated the old group, PD-1 blockade significantly enhanced the proliferation of EM and EMRA subsets only (Fig 5C and D). In contrast, blockade of p38 MAPK was found to increase the proliferative capacity of both the naïve and EMRA subsets (Fig. 5D). Once again there was an additive enhancement in the EMRA subset when both signalling pathways were inhibited together (Fig. 5C and D).

We have recently published that while the EMRA population secretes high levels of cytokines in response to TCR stimulation, their response to CMV-specific stimulation is blunted [23]. We therefore decided to investigate the effect of PD-1 and p38 MAPK blockade in response to antigen-specific stimulation (Fig. 6). The proliferative response, measured by Ki67 of sorted CMVpp65-specific CD8⁺ CD45RA/CD27 T-cell subsets to the peptide NLV in young (n = 5) and old (n = 4) individuals followed a similar trend to that seen when using anti-CD3 (Fig. 5). Following treatment the most responsive subsets to blockade were the EMRAs in both the young (Fig. 6A and B) and old (Fig. 6B and D) donors. An additive enhancement was again observed when both blocks were used together (Fig 6B and D).

Signalling through p38 MAPK but not PD-1 pathways reduces telomerase activity of EMRA T cells

We next investigated whether p38 MAPK or PD-1 signalling were involved in the decreased telomerase activity in EMRA CD8⁺ T cells isolated from young old individuals. Our previous data indicated that p38 MAPK signalling inhibits telomerase activity in both CD4⁺ [12] and CD8⁺ [8, 27] EMRA T cells in young individuals. We stimulated CD8⁺ T-cell subsets with anti-CD3 and irradiated autologous APCs in the presence of both inhibitors separately or together as described previously. We found that incubation with BIRB 796 caused a significant increase in telomerase activity in the EMRA population isolated from young individuals, either when added alone or with anti-PDL-1/2 (Fig. 7A and B). When we examined CD8⁺ EMRA T cells from old individuals we found that p38 MAPK inhibition also increased telomerase activity, however the level of enzyme activity was not increased to the levels observed in

young subjects (Fig. 7C). Therefore, additional mechanisms may be involved in the reduced telomerase activity in these cells during ageing.

p38 MAPK signalling inhibits TNF- α secretion; blocking PD-1 reverts this effect in CD8⁺ T cells

Previous studies have shown that p38 MAPK signalling plays an essential role in the production of TNF- α in T cells [28]. The EM and EMRA CD8⁺ T cells from young donors (Fig. 8) secreted the highest levels of this cytokine after activation. As described above, both the naïve and EMRA population from old donors secreted high levels of TNF- α . PD-1 blockade had no effect on TNF- α secretion in any of the subsets in both age groups. In contrast, the inhibition of p38 MAPK signalling significantly inhibited the secretion of TNF- α in all four subsets in both young and old individuals (Fig. 8 A and B). However when p38 MAPK and PD-1 were inhibited together, the capacity for TNF- α secretion was restored in the EMRA CD8⁺ T cells in young and old donors. We found that blocking PD-1 or p38 MAPK individually or together had no consistent effect on IFN- γ , perforin or Granzyme B. Therefore the simultaneous blockade of p38 and PD-1 signalling pathways together restores proliferation and telomerase activity and also maintains the capacity for TNF- α production from EMRA of CD8⁺ T cells in young and old subjects. This cannot be achieved with blocking either molecule alone.

Discussion

Our main observation is that the CD8⁺ EMRA T-cell population is a potent effector subset that has its replicative potential and survival capacity that is rigidly controlled by non-overlapping senescence- and exhaustion-related signalling pathways. The simultaneous targeting of both the p38 MAPK (senescence) and PD-1 (exhaustion) pathways enhances their proliferative responses, to both polyclonal and antigen-specific stimuli, while allowing the retention of effector capacity. This may be of benefit during vaccination where a low proliferative potential has been shown to reduce the effectiveness of therapeutic vaccination [29].

Both p38 MAPK and PD1 have been the focus of much pharmaceutical interest, with therapies targeting the PD-1/PD-L1 axis being effective in the treatment of melanoma, renal carcinoma and non-small cell lung cancer [30]. However while treatment is generally well tolerated, a spectrum of immune-related adverse events have been experienced [30]. Furthermore, not all patients respond to PD-1/PD-L1 therapy [31]. p38 MAPK is critically involved in the induction of pro-inflammatory cytokine secretion, many pharmaceutical companies have p38 MAPK inhibitors in phase II and phase III clinical trials for the treatment of several inflammatory disorders [32-34]. However these drugs have been limited by their therapeutic index and a requirement for TNF- α to generate cytotoxic T cells. We show here that when p38 MAPK and PD-1 were inhibited together they were able to restore the capacity for TNF- α secretion in the EMRA subset, presumably owing to an unmasking of the enhancing effect on TNF- α secretion when using PD-1 blockade alone. We suggest

that p38 inhibitors could also be used for immune enhancement, providing that PD-1 signalling is inhibited at the same time.

The greatest functional effect of simultaneous PD-1 and p38 MAPK inhibition was observed in the CD8⁺ T-cell EMRA subset, where p38 MAPK is maximally expressed but PD-1 is not. However, differing PD-1 expression levels may engage distinct intracellular targets [35]. For example, PD-1 binds the SH2 domain-containing protein tyrosine phosphatases SHP-1 and SHP-2 in naïve T cells, but in exhausted cells the very high levels of PD-1 expression can recruit additional signalling molecules [35]. The varying levels of expression of PD-1 by CD8⁺ T cells that are at different stages of differentiation have implications for anti-viral therapy. For example, Hepatitis C Virus (HCV), Human Immunodeficiency Virus (HIV) and cytomegalovirus (CMV) specific CD8⁺ T-cell populations are predominantly comprised of cells at early, intermediate and late differentiation stages respectively [3] and these specific cells may therefore be differentially affected by PD-1 blockade .

The augmented levels of DNA damage observed in EMRAs may be a consequence of their highly impaired telomerase expression since telomerase can protect T cells against oxidative stress [36] in addition to telomere erosion and this enzyme is a critical regulator of the DDR [37]. p38 MAPK is activated in response to DNA damage and indeed, its expression in different CD8⁺ T-cell subsets correlates with the phosphorylation of γ H2AX, a component of DNA damage foci. Although p38 MAPK signalling has a central role in inducing senescence that results in apoptosis of the cells [7], it is also involved in pro-inflammatory cytokine production by CD4⁺

and CD8⁺ T cells including IFN- γ and TNF- α production [38]. This suggests that p38 MAPK signalling may be a key component that regulates the development of typical effector cell characteristics such as susceptibility to death but potent functional capacity.

Also evident from this study is that naïve cells isolated on the basis of cell phenotype from old individuals are not truly naïve. Unprimed naive cells are characterized by the absence of cytotoxic factors [39], however aged “naïve cells” express significant amounts of granzyme, perforin, TNF- α and IFN- γ , and have short telomeres compared to cells from young individuals [24]. We hypothesise that these cells have undergone antigen-independent homeostatic expansion to convert to a functional state similar to that of memory T cells [7, 23, 40], which raises the question about the use of phenotypic markers to analyse functional outcomes from a heterogeneous population. We also found that CD8⁺ EMRA T cells from old donors, while expressing increased levels of TNF- α and IFN- γ , display significantly reduced levels of perforin and granzyme. This loss of cytotoxic killing apparatus by senescent T cells may contribute to their ineffective immune responses to viruses, which has been observed in HIV infection [41]. We speculate that the reduced expression of cytotoxic molecules in CD8⁺ EMRA T cells from old donors may be due to a decreased production and/or responsiveness to IL-2 [42-44], that has been shown to increase the expression of perforin and granzyme and enhance cytolytic function [45]. Alternatively the higher frequency of lytic molecules produced by CD8⁺ EMRA T cells of young individuals could reflect faster immunological control the young. Epigenetic mechanisms alter chromatin structure and influence gene expression,

indeed the epigenetic control of granzyme B during CD8⁺ T-cell differentiation has recently been demonstrated [46]. They showed an increased susceptibility to nuclease digestion in the chromatin dense proximal region of the *gzmB* promoter, suggesting that *gzmB* transcription is mediated by manipulating the accessibility of the transcriptional machinery to the promoter [46]. Given that DNA damage and inflammation are both capable of altering chromatin structure [47] it is conceivable that the epigenetic control of cytokine release is less flexible in old individuals. It is also of note that while the simultaneous blockade of PD-1 and p38 MAP kinase signaling in CD8⁺ EMRA T cells from old individuals has similar effects on enhancing functional responses as observed in young subjects, the reconstituted functional responses are still lower than in the young group. This suggests that there are additional defects in the functional responses in the old group that may not be regulated by PD-1 and p38 MAPK signaling.

In addition to PD-1, there is a diverse array of other cell surface inhibitory molecules that can regulate T-cell exhaustion and gene expression profiles have identified that exhausted CD8⁺ T cells co-express up to seven inhibitory receptors [25, 48]. While PD-1 signalling is induced by cell surface interactions, p38 MAPK signalling is induced by intracellular DNA-damage associated signalling processes [14]. An exciting prospect for the future is that it may be possible to target different receptors or downstream components of exhaustion and senescence pathways to custom design a tailored T-cell response that is appropriate for a particular situation.

It is possible that blocking PD-1 and/or p38 MAPK may enhance the function of these cells in older humans [49] or patients with skin malignancy [19, 20]. However this raises the question of whether it is safe to do so. Whereas senescence may have a role in preventing malignancy, exhaustion may reduce the risk of immunopathology that results from excessive immune activation [14]. Therefore the reversal of T-cell senescence by p38 MAPK blockade carries a risk of malignant transformation of these cells while reversing the process of exhaustion may lead to excessive T-cell activity that leads to tissue damage [14]. However, while long-term blockade of p38 and or PD-1 signalling has associated risks, the short-term blockade of these signalling pathways may temporarily enhance immunity. This would be beneficial in older humans who have decreased immunity and in patients with cancer. These possibilities have to be investigated further to clarify whether it is feasible to use new strategies involving the blockade of both PD-1 and p38 MAPK to boost immunity in these subjects.

Materials and Methods

Blood sample collection and isolation

Heparinised peripheral blood samples were collected from young and old healthy donors (Young age range: 20-35, median 32 years, n= 33; old age range: 65-82, median 72 years, n= 31). Healthy was taken as donors who had not had an infection or immunisation within the last month, no known immunodeficiency or any history of chemotherapy or radiotherapy, and were not receiving systemic steroids within the last month or any other immunosuppressive medications within the last 6 months. All samples were obtained in accordance with the ethical committee of Royal Free and University College Medical School. PBMC were isolated using Ficoll hypaque [50].

Flow cytometric analysis and cell sorting

Flow cytometric analysis was carried out using the following antibodies: Live/dead fixable blue dead cell stain (Invitrogen), KLRG1 PE (2F1/KLRG1), PD-1 PE (EH12.2H7) and CD45RA Brilliant Violet 605 from Biolegend. CD8 PerCP (SK1), CD8 Alexa Fluor 700 (RPA-T8), CD27 V500 (M-T271), CD27 allophycocyanin (M-T271), CD27 allophycocyanin -H7 (M-T271), CD45RA PE-Cy7 (L48), CD57 allophycocyanin (NK-1), from BD Biosciences. For intracellular staining the following antibodies were used: IFN- γ V450 (B27), granzyme B Alexa Fluor 700 (GB11), TNF- α PE (MAb11), perforin FITC (δ G9) and p38 Alexa Fluor 488 (36/p38) all from BD Biosciences. For intranuclear staining: γ H2AX Alexa Fluor 488 (2F3, Biolegend) and Ki67 FITC (B56; BD Biosciences). Samples were processed using an LSR II (BD Biosciences) and analysed using FlowJo software (Treestar).

CD8⁺ T cells were isolated by positive selection using the VARIOMACS system (MiltenyiBiotec) according to the manufacturer's instructions. Positively selected CD8⁺ T cells were labeled with CD27 FITC (M-T271) and CD45RA allophycocyanin (HI100; BD Biosciences) and sorted using a FACSria (BD Biosciences). Multiparamter flow cytometry was performed using SPICE version 5.2, downloaded from <http://exon.niaid.nih.gov> [51].

Phospho-cytometry

The level of p38 (pT180/pY182) was analysed ex vivo using the surface markers; CD45RA BV610, CD27 V500 and CD8 AF700, PBMCs were fixed with warm Cytofix Buffer (BD Biosciences) at 37°C for 10 min. Cells were permeabilised with ice-cold Perm Buffer III (BD Biosciences) at 4°C for 30 min and incubated with the anti-p38 antibody (pT180/pY182) for 30 min at room temperature. γ H2AX (pSer139) was detected in sorted subsets following activation with 0.5 μ g/ml plate coated anti-CD3 and 5 ng/ml of rhIL-2 for 4 days, after which time the above method was used.

Proliferation assays

CD45RA/CD27 purified CD8⁺ T cells were stimulated with 0.5 μ g/ml plate coated anti-CD3 (OKT3) and irradiated APCs, as a source of co-stimulation, proliferation was assessed by staining for the cell cycle related nuclear antigen Ki67 after 4 days [50]. Irradiated APCs consisted of the remaining PBMC fraction minus the sorted population of interest.

Proliferation in response to CMV-specific stimulation was achieved through a 4 day incubation of PBMCs with 0.2 μ g/ml NLVPMVATV peptide (ProImmune), after which time the cells were stained with Ki67 as detailed above.

Measurement of telomerase activity

Isolated CD8⁺ T-cell populations (2×10^5 cells) were snap-frozen after stimulation. Telomerase activity was determined using the TRAPeze telomerase detection kit and the TRAPeze ELISA Telomerase Detection Kit (Chemicon) according to the protocol provided by the manufacturer. The absolute numbers of CD8⁺ T cells were calculated using trypan blue (Sigma). The TRAP assay was performed with samples

adjusted to 500 Ki67⁺ T cells per reaction to control for the different level of proliferation in the subsets following activation [50].

PDL-1/2 blockade and p38 MAPK inhibitor

CD8⁺ and sorted subsets CD27/CD45RA was blocked by adding 10 µg/ml each of anti-PD-L1 (29E.2A3.C6) and anti-PD-L2 (24F.10C12.G12, both from Biolegend), or 10 µg/ml each of IgG2a (Mg2a-53) or IgG2b (MPC-11) isotype control antibodies (Abcam) for 4 day stimulated with anti-CD3 (purified OKT3, 0.5 µg/ml) and irradiated APCs or with 0.2 µg/ml NLVPMVATV peptide (ProlImmune). The p38 inhibitor BIRB796 (Selleck Chemicals) was added to the 4 day cultures at a final concentration of 500nM [8, 12] using 0.1% DMSO as a control.

Measurement of TNF-α following inhibition

Different CD45RA/CD27 defined CD8⁺ T-cell subsets were sorted and 2 x10⁵ cells were cultured with 0.5 µg/ml plate coated anti-CD3 (OKT3) and 5ng/ml rhIL-2, with or without anti-PDL1/2 and BIRB796. Culture supernatants were collected at 48 h for the measurement of TNF-α using the Quantikine human TNF-α immunoassay (R&D Systems) according to the protocol provided by the manufacturer.

Statistical analysis

Graphpad Prism was used to perform statistical analysis. Statistical significance was evaluated using a repeated measures ANOVA with the Tukey correction used for post-hoc testing. Differences were considered significant when *P* was < 0.05.

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Conflict of Interest

The authors declare no financial or commercial conflicts of interest.

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Figure 1. CD8⁺ EMRA cells exhibit characteristics of senescent T cells.

(A) CD8⁺CD45RA⁺CD27⁻ T cells isolated from young and old individuals were isolated and stained for KLRG1, CD57 and γ H2AX and analysed by flow cytometry. One representative plot out of eight is shown. (B) Expression of senescence markers, KLRG1, CD57 and γ H2AX, in CD8⁺ CD45RA/CD27 T-cell subsets ex-vivo was analysed by multiparameter flow cytometry T-cell. Pie charts show the average distribution of cells showing 0, 1, 2 or 3 senescent functions within each subset from 8 young and 8 old donors. (C) Percentage of CD8⁺ T-cell subsets (N; naïve, CD45RA⁺CD27⁺; CM, central memory, CD45RA⁻CD27⁺; EM, effector memory, CD45RA⁻CD27⁻ and EMRA, effector memory T cells that re-express CD45RA, CD45RA⁺CD27⁻) expressing 3 senescent features in cells isolated from young and old individuals generated using SPICE version 5.2, downloaded from <http://exon.niaid.nih.gov>. The graph shows the mean $\pm$ SEM for 8 young (dark green) and old donors (light green) and P values were calculated using a repeated measures ANOVA with the Tukey correction used for post-hoc testing. * $p < 0.05$.

Figure 1.

Figure 2. CD8⁺ EMRA cells display functional characteristics of senescence.

(A) Proliferation of CD45RA/CD27 subsets from young and old donors was assessed by Ki67 staining following stimulation with 0.5 µg/ml anti-CD3 and irradiated APCs for 4 days. Each symbol represents a single donor, horizontal lines depict mean values. (B and C) Telomerase activity in CD8⁺ CD45RA/CD27 subsets from young and old donors was measured following a 4 day incubation with 0.5 µg/ml anti-CD3 and irradiated APCs, using the TRAPeze telomerase detection kit. (B) One representative gel is shown. (C) Graph showing the percentage expression of the mean $\pm$ SEM of relative telomerase activity, calculated as the intensity of signal corresponding to the TRAP product ladder bands compared to the internal standard (IS), from 4 young and 4 old donors. All P values were calculated using a repeated measures ANOVA with the Tukey correction used for post-hoc testing. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Figure 2.

A

B

C

Figure 3. CD8⁺ EMRA cells retain effector function.

(A) CD8⁺CD45RA⁺CD27⁻ T cells isolated from young and old individuals were stained for TNF- α , IFN- γ , perforin and granzyme B and analysed by flow cytometry. One representative plot out eight is shown. (B) Expression of IFN- γ , TNF- α , perforin and granzyme B in CD8⁺ CD45RA/CD27 T-cell subsets following 8 h-stimulation with 0.5 μ g/ml anti-CD3 was analysed by multiparameter flow cytometry. Pie charts show the average distribution of cells showing 0, 1, 2, 3 or 4 effector functions within each subset from 8 young and old donors. (C) Percentage of CD8⁺ T-cell subsets expressing TNF- α , IFN- γ , perforin and granzyme B. The graphs show the mean $\pm$ SEM for 8 young (black) and 8 old donors (grey) and P values were calculated using a repeated measures ANOVA with the Tukey's correction used for post-hoc testing. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Figure 3.

Figure 4. Expression of PD-1 and p38 MAPK during CD8⁺ T-cell differentiation.

(A and B) PD-1 expression on CD8⁺ CD45RA/CD27 defined T-cell subsets from young and old donors was measured by flow cytometry. (A) One representative plot out of 19 young and 15 old donors is shown. (B) Each symbol represents a single donor, horizontal lines depict mean values. (C and D) Phosphorylated p38 MAPK in young and old CD8⁺ CD45RA/CD27 defined T-cell subsets examined directly ex vivo by flow cytometry. (C) One representative plot out of 9 young and 9 old donors is shown. (D) Graph shows mean $\pm$ SE of phosphorylated p38, normalised in relation to the CD45RA⁺CD27⁺ subset, for 9 young and 9 old donors. All P values were calculated using a repeated measures ANOVA with the Tukey correction used for post-hoc testing. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Figure 4.

Figure 5. Signalling through both PD-1 and p38 MAPK pathways contributes to reduced proliferation the of CD8⁺ EMRA T cells.

(A and C) Representative example of Ki67 staining on CD45RA⁺CD27⁻ T cells measured by flow cytometry after 4 days stimulation with 0.5 µg/ml anti-CD3 and irradiated autologous mononuclear cells from young (A) and old (C) donors. This activation was performed in the presence of 10 µg/ml anti-PDL1/2 antibodies or 500 nM BIRB796. In control cultures, 10 µg/ml IgG2a, IgG2b or 0.1% DMSO were added individually or together. (B and D) Pooled data showing the effect of inhibition on proliferation, measured by Ki67, in CD8⁺ CD45RA/CD27 T-cell subsets that were activated as above in young (B) and old (D) donors. Graphs show the mean ± SE for single measurements of 4 young and 4 old donors and P values were calculated a repeated measures ANOVA with the Tukey correction used for post-hoc testing. * $p < 0.05$, ** $p < 0.01$.

Figure 5.

Figure 6. PD-1 and p38 MAPK signalling pathways contribute to decreased proliferation the of CMV-specific CD8⁺ EMRA T cells.

(A and C) Flow cytometry gating and representative example of Ki67 staining for CMVpp65-specific CD8⁺ CD45RA/CD27 T-cell subsets measured after 4 days stimulation with 0.2 µg/ml NLVPMVATV peptide from young (A) and old (C) donors. Stimulation was performed in the presence of 10 µg/ml anti-PDL1/2 antibodies or 500 nM BIRB796. In control cultures, 10 µg/ml IgG2a, IgG2b or 0.1% DMSO were added individually or together. (B and D) Pooled data showing the effect of inhibition on proliferation, measured by Ki67, in sorted CMVpp65-specific CD8⁺ CD45RA/CD27 T-cell subsets stimulated as above in young (B) and old (D) donors. Graphs show the mean ± SEM pooled from 5 young and 4 old donors and P values were calculated by repeated measures ANOVA with the Tukey correction used for post-hoc testing. * $p < 0.05$, ** $p < 0.01$.

Figure 6.

Figure 7. Signalling through p38 MAPK but not PD-1 pathways regulates reduced telomerase activity of CD8⁺ EMRA T cells.

(A) Representative blot for telomerase activity in CD8⁺ CD45RA/CD27 T-cell subsets on day 4 following activation with 0.5 μ g/ml anti-CD3 and irradiated autologous APCs, 10 μ g/ml anti-PDL1/2, 500 nM BIRB 796 or both molecules, using the TRAPeze and TRAPeze ELISA telomerase detection kit. The blot is representative of 4 separate experiments. (B) Telomerase activity from young donors was analysed as described above. The graph shows the mean $\pm$ SEM pooled from 4 donors. (C) CD8⁺ CD45RA/CD27 T-cell subsets from young and old donors following 4 day incubation with 0.5 μ g/ml anti-CD3 and irradiated autologous APCs with or without 500 nM BIRB 796 were analysed by TRAPeze T-cell ELISA. The graph shows the mean $\pm$ SE for single measurements from 5 donors and all P values were calculated using a repeated measures ANOVA with the Tukey correction used for post-hoc testing. * $p < 0.05$.

Figure 8. Signalling through p38 MAPK inhibits TNF- α secretion that is reversed by inhibiting the PD-1 pathway in CD8⁺ T-cell subsets. (A and B) Secretion of TNF- α by CD8⁺ CD45RA/CD27 subsets in young (A) and old (B) donors was measured by ELISA following 48 h stimulation with 0.5 μ g/ml anti-CD3 and 5 g/ml IL-2 with either 10 μ g/ml anti-PDL1/2 block, 500 nM BIRB796 or both molecules. Graph shows the mean + SE for single measurements of 5 donors and P values were calculated a repeated measures ANOVA with the Tukey correction used for post-hoc testing. * p < 0.05, ** p < 0.01.