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Differing HLA types influence inhibitory receptor signalling in CMV-specific CD8$^+$ T cells

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ABSTRACT

The dysregulated immune response to CMV constitutes a major force driving T cell immunosenescence and growing evidence suggests that it is not a benign virus in old age. We show here that the PD-1/L pathway defines a reversible defect in CMV specific CD8$^+$ T cell proliferative responses in both young and old individuals. More specifically, highly differentiated CD45RA$^+$CD27$^-$ CMV-specific CD8$^+$ T cells exhibit a proliferative deficit compared their central and effector memory counterparts, which is reversed following PD-L blockade. However, we also report that HLA-B*07/TPR specific CD8$^+$ T cells express higher levels of PD-1 than HLA-A*02/NLV specific cells and HLA-A*02 individuals show a higher proliferative response to PD-L blockade, than HLA-B*07 individuals, which we postulate may be due to the differing functional avidities for these two CMV-specific CD8$^+$ T cells populations. Nevertheless data presented here demonstrate that CMV-specific CD8$^+$ T cells can be functionally enhanced by perturbation of the PD-1/L signalling pathway, whose manipulation may provide a therapeutic modality to combat age-associated immune decline.

1. Introduction

Ageing is accompanied by a progressive, multidimensional, physiological degeneration, with immune system alterations playing a key role regulating these age-related declines [1]. Age-associated deleterious changes have been best characterised amongst the adaptive immune system, particularly among T cells, which exhibit profound age-associated alterations. Indeed, though peripheral T cell numbers do not diminish with age, the peripheral T cell pool undergoes a striking age-related remodelling, which is much more profoundly observed amongst the CD8$^+$ T cell compartment than the CD4$^+$ [2]. The proportion of naive CD8$^+$ T cells (and thus the T cell receptor (TCR) repertoire) exhibit a dramatic age-associated diminution accompanied by a large accumulation of highly differentiated T cells [3].

Such immune alterations typically associated with advanced age can also be observed in relatively young individuals who have been subjected to a high antigenic load, such as those with Human Immunodeficiency Virus (HIV) [4], cancer [5], and auto-immune diseases [6]. Among developed nations, the ubiquitous and relatively innocuous herpes virus Cytomegalovirus (CMV) appears to act as the dominant chronic stressor, and is associated with many of the same phenotypic and functional alterations to T cell immunity seen with aging. Collectively these changes, in addition to CMV infection, are known as the Immune Risk Phenotype (IRP), that have been suggested as biomarkers of immune system ageing [7,8]. The significance of CMV infection in health outcomes of elderly individuals is further highlighted by its association with deleterious responses to influenza vaccination [9], and Epstein-Barr Virus (EBV) infections [10]. The case for CMV driving immunosenescence has been further strengthened by the recent finding that CMV seropositivity acts as an independent risk factor for premature mortality amongst American adults [11].

In direct contrast to the T cell response observed towards most other pathogens, the magnitude of the CMV immune response undergoes a progressive long term expansion with age [2,12] that can dominate the T cell compartment of elderly donors [12–14]. This expansion of increasingly clonal, dysfunctional and differentiated CMV specific CD8$^+$ T cells may out-compete other T cell populations for immunological space, resulting in loss of immunological memory to previously controlled pathogens and further constrictions in the TCR repertoire [15]. Indeed, amongst aged individuals, repertoire shrinkage of clonally expanded CD8$^+$ T cells is predictive of reduced lifespan [16]. Furthermore, humoural anti-CMV responses also significantly intensify with advancing age, whose magnitude correlates with co-morbidity and functional decline [17]. Thus, the increasing weight of immune resources dedicated to the control of CMV may significantly impair immune responses amongst the aged and drive age-related immune decline.

It has been suggested that lifelong persistent CMV reactivation may drive clonal exhaustion of the most efficient and specific T

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cells so that an increased number of suboptimal cells are required to control virus infection [15]. Exhausted T cells are subject to complex layers of negative regulation by signalling through multiple inhibitory receptors that inhibit functional responses [18]. The inhibitory receptor, programmed cell death 1 (PD-1) has been shown to be the best characterised inhibitory receptor in the context of chronic infection and has a major role in regulating T cell exhaustion [18,19]. An important finding is that blockade of the PD-1 pathway has been used to reinvigorate antigen-specific T cell responses in LCMV-infected mice [19] and in humans reinvigorates HIV [20–22], HBV [23] and HCV [24,25] specific $CD8^+$ T cells. We show here that manipulation of the PD-1/L axis reinvigorates CMV specific $CD8^+$ T cell function in both young and old individuals. However our findings also indicate that HLA-A*02 and B*07 CMV-specific T cells display differing levels of PD-1 expression and proliferative response to aPD-L1/2 blockade.

2. Materials and methods

2.1. Blood sample collection and isolation

Heparinised peripheral blood samples were taken from healthy volunteers (Age range: 20–92). All samples were obtained in accordance with the ethical committee of Royal Free and University College Medical School. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll hypaque (Amersham Biosciences) and experiments performed immediately thereafter.

2.2. Identification of CMV responding donors

PBMCs were incubated with CMV-infected cell lysate prepared by infecting human embryonic lung fibroblasts with the Towne strain of CMV (European Collection of Animal Cell Cultures) at a multiplicity of infection of 2. After 5 d, the infected cells were lysed by repeated freeze–thaw cycles. PBMC were left unstimulated or stimulated with CMV lysate for 15 h at 37 °C in a humidified CO_2 atmosphere, with 5 μg/ml Brefeldin A (Sigma–Aldrich) added after 2 h. PBMCs were then stained with anti-CD4 and anti-IFNγ, with donors being considered CMV seropositive when showing a positive IFNγ response [12], with a concordance between seropositivity and a positive IFN-γ response in the blood [12].

2.3. Flow cytometric analysis

Up to five colour flow cytometric analysis was performed using the following antibodies: FITC conjugated Ki67 (B56), CD45RA APC (MEM-56), CD4 PerCP (SK7) CD8 PerCP (SK1), IFNγ APC (B27), CD27 FITC (M-T271), CD45RA PE-Cy7 (L48), all from BD Biosciences. Additionally, PE and APC- labelled HLA-A2 and HLA-B7 restricted pentamers were used to identify CMV positive individuals who may have specific $CD8^+$ T cells directed against the CMV tetramers NLVPVMTV and TPRVTGGGAM, respectively (ProImmune, Oxford, UK). PD-1 expression was analysed using clone EH12-2H7-PE, kind gift from Dr. G. Freeman, Dana-Farber Cancer Institute, Boston. The PMBCs were stimulated with 0.5 μg/

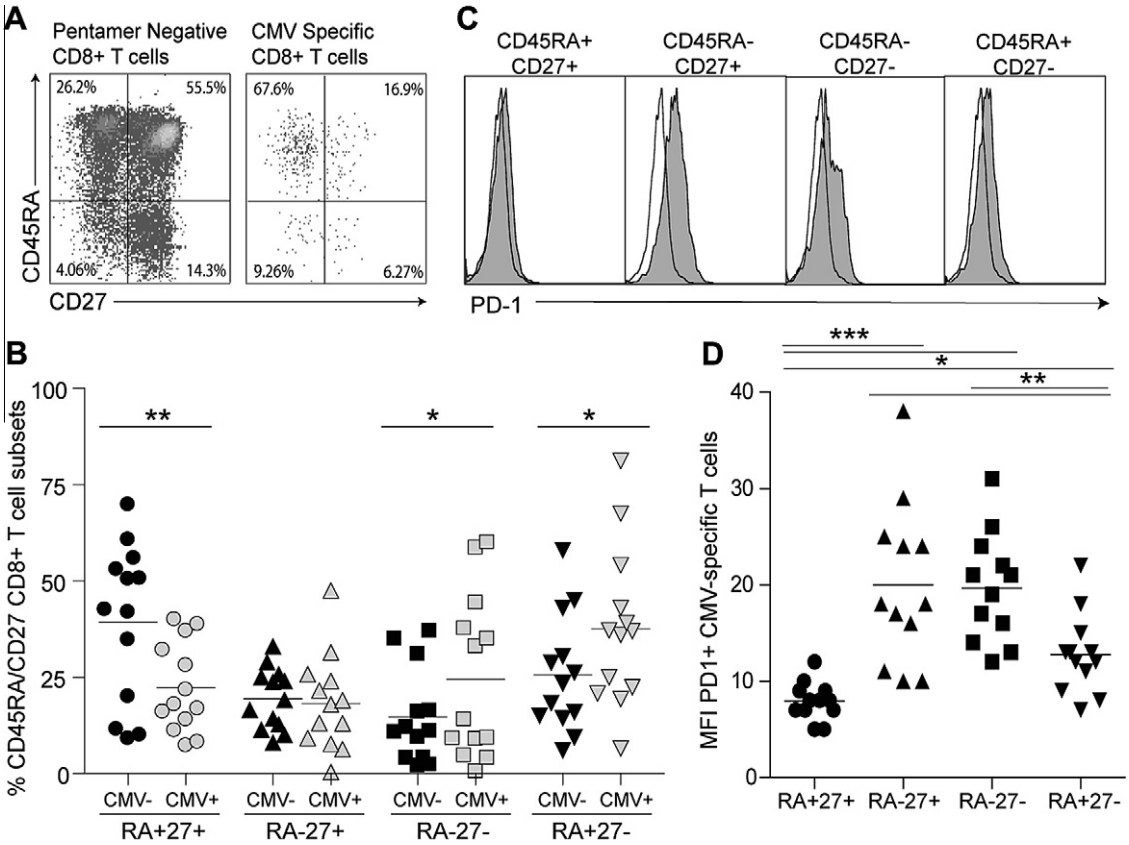

Fig. 1. CMV causes the accumulation of highly differentiated $CD8^+$ T cells. (A) Representative density plots showing the CD45RA/CD27 phenotypic profiles of $CD8^+$ pentamer-negative (left panel) or pentamer-positive (right panel) T cells. (B) Cumulative data from 13 young and old donors showing CD45RA and CD27 expression on pentamer negative (black symbols) and CMV-specific $CD8^+$ T cells (grey symbols). Horizontal lines depict mean values. *P* values were calculated using a repeated measures ANOVA. (C) Representative examples of PD-1 staining on CD45RA/CD27 CMV pentamer positive $CD8^+$ T cells. (D) Graph showing the MFI of PD-1 expression on CMV pentamer positive $CD8^+$ T cells subsets. Horizontal lines depict mean values. *P* values were calculated using a repeated measures ANOVA.

ml plate bound anti-CD3 at 37 °C for 24 h to enable PD-1 expression as described previously [26,27].

2.4. Effects of antibody blockade on PD-1 expression and proliferative responses of CD8+ NLV/TPR specific T cells

Samples were blocked using 10 μg/ml PD-L1 (29F.2A3) and PD-L2 (24F) kind gift from Dr. G. Freeman, Dana-Farber Cancer Institute, Boston or their relevant isotype control IgG2b (MPC-11) from Abcam. One μg/ml IFNαR block (Calbiochem) or IgG2a isotype control (Abcam) were also used. All blocking antibodies were azide free.

PBMCs were stimulated with 0.2 μg/ml TPRVTGGGA or NLVPMVATV peptide (ProImmune) for 3 d in the presence of inhibitory receptor blockade or relevant isotype control before staining with Ki67, a nuclear antigen associated with proliferation, to determine CMV specific CD8+ T cell proliferation. The effect of cytokine on PD-1 expression was determined by stimulating PBMCs in duplicate with 0.5 μg/ml plate coated anti-CD3 for 24 h in the presence of 500 U/ml IFNα (PeproTech EC), or IFNαR block prior to flow cytometric analysis of PD-1 expression, as detailed above.

2.5. Statistical analysis

Data analysis was performed using GraphPad Prism (GraphPad Software) with statistical significance being evaluated by either an unpaired or paired Student's *t*-test or a repeated measures ANOVA. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. CMV causes the accumulation of highly differentiated CD8+ T cells expressing high levels of PD-1

It is known that highly differentiated CD8+ T cells accumulate during ageing but what is unclear is the contribution of CMV specific T cells to this build-up of highly differentiated CD8+ T cells. Therefore the CMVpp65-specific population, defined by the HLA-A*02 and B*07 peptides, in both young and old individuals were analysed for the expression of CD45RA and CD27, markers commonly used to determine T cell phenotype (Fig. 1A). CMV-specific T cells showed significantly ($P < 0.05$) higher levels of effector memory like ($CD45RA^{-}CD27^{-}$) and highly differentiated EMRA ($CD45RA^{+}CD27^{-}$) T cells compared to the CD8+ T cell pool (Fig. 1B).

It has been suggested that lifelong persistent CMV reactivation may drive clonal exhaustion of the most efficient and specific T cells so that an increased number of suboptimal cells are required to control virus infection [15]. Therefore, reinvigorating CMV-specific CD8+ T cell functions could allow better control of CMV infection and thus impair memory inflation and the development of immunosenescence. A potential mechanism for regulating this dysfunction could be though inhibitory receptor signalling. PD-1 has been the best characterised inhibitory receptor in the context of chronic viral infections [19–22]. We and others have shown PD-1 to be differentially expressed on global CD8+ T cells with the highest level of expression found on the mid-differentiated T cells [28,29]. The expression of PD-1 on CMV pentamer positive T cells was found to display a very similar pattern (Fig. 1C), with the highest levels of PD-1 being expressed on $CD45RA^{-}CD27^{+}$

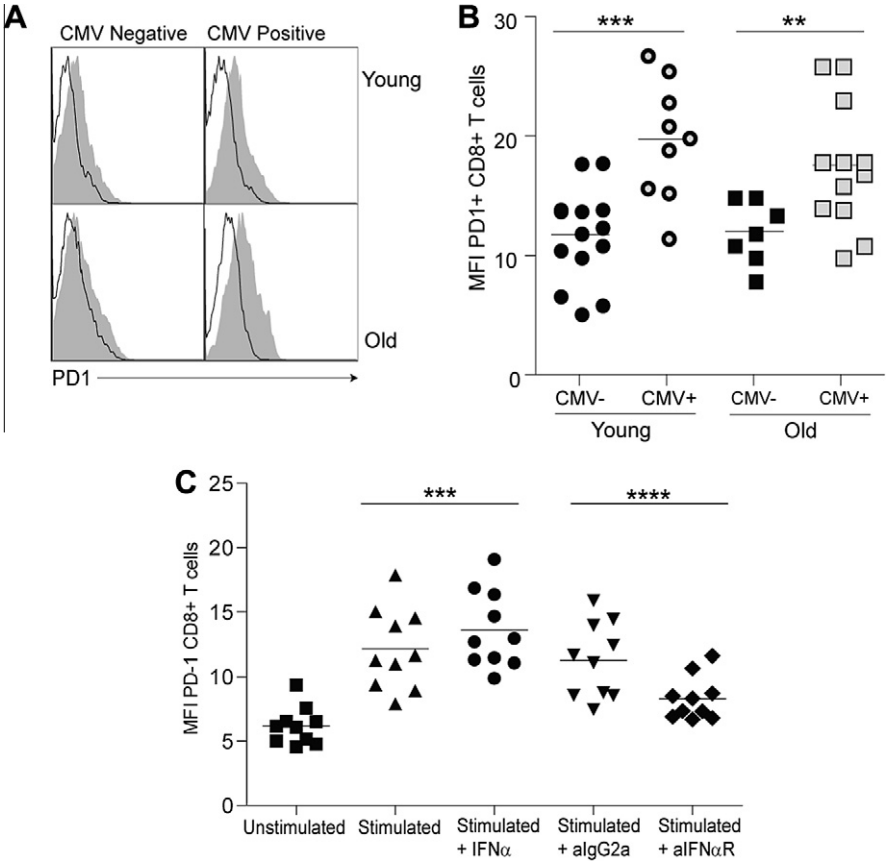

Fig. 2. PD-1 expression is higher in CMV positive individuals driven by IFNα. (A) Representative histograms showing the expression of PD-1 on CD8+ T cells from young and old CMV negative and positive donors. (B) Graph showing the expression levels of PD-1 on CMV negative (black symbols) and CMV positive (grey symbols) from young and old donors. Horizontal lines depict mean values. *P* values were calculated using a Student's *t*-test. (C) Graph showing the effect of IFNα on PD-1 expression in CD8 T cells. Horizontal lines depict mean values, and *P* values were calculated using the Student's *t*-test.

and CD45RA$^-$CD27$^-$ T cells (Fig. 1D). Furthermore it has been shown that there is no age-related increase in PD-1 expression [30–32] However we show here that PD-1 is up-regulated when young and old donors are stratified according to their CMV status, with both young and old CMV positive individuals displaying significantly higher ($P < 0.005$, $P < 0.01$ respectively) expression of PD-1 upon CD8$^+$ T cells when compared to CMV negative individuals (Fig. 2A and B).

We then sort to determine the cause of this elevated PD-1 expression in CMV positive individuals. We chose to investigated the effect of IFNα, which is secreted at high levels in response to CMV and, by virtue of its ability to induce co-stimulatory receptor loss and inhibit telomerase activity, has been suggested to be the factor driving accelerated T cell differentiation in CMV infected individuals [12,33] We found the expression of PD-1 on CD8$^+$ T cells in response to overnight stimulation with IFNα to be significantly increased ($P < 0.005$; Fig. 2C). Furthermore when a blocking antibody against IFNαR was added the amount of PD-1 decreased significantly ($P < 0.0005$; Fig. 2C).

3.2. PD-1/L blockade augments the proliferative responses of CMV specific CD8$^+$ T cells

We show here that PBMCs when stimulated for 3 d with 0.2 μg/ml CMV peptide in the presence of an anti-PD-L1/2 blocking antibody significantly increased their CD8$^+$ T cell proliferative response from both young and old individuals ($P < 0.01$, $P < 0.05$ respectively), determined by Ki67 expression when compared with its relevant isotype control (IgG2b young mean, 30.1%; PD-L young mean, 62.6%; IgG2b old mean, 20.6%; PD-L old mean, 47.7%; Fig. 3A and B). This increase in CMV-specific CD8$^+$ T cell proliferative response following PD-L blockade was not accompanied by a proportionate increase in the percentage of CD8$^+$ T cells binding the CMV tetramer in either young or old donors (IgG2b young mean, 1.1%; PD-L young mean, 1.4%; IgG2b old mean, 4.9%; PD-L old mean, 5.7%; Fig. 3C and D).

3.3. HLA-A*02 and B*07 individuals show differing levels of PD-1 expression and respond differently to PD-L blockade

An intriguing observation was that HLA-A*02 and B*07 individuals display differing levels of PD-1, with A*02 donors expressing significantly less PD-1 on their CMV-specific CD8$^+$ T cells ($P < 0.05$) than B*07 donors (Fig. 4A). This effect was not dependent on the total percentage of CMV$^+$CD8$^+$ T cells (Fig. 4B) or differentiation status of NLV-specific of TPR-specific cells (Fig. 4C), as no significant difference in either was found. This altered level of PD-1 expression between HLA-A*02 and B*07 donors was found to translate to an altered Ki67 proliferative response, with A*02 donors showing significantly higher levels of proliferation compared

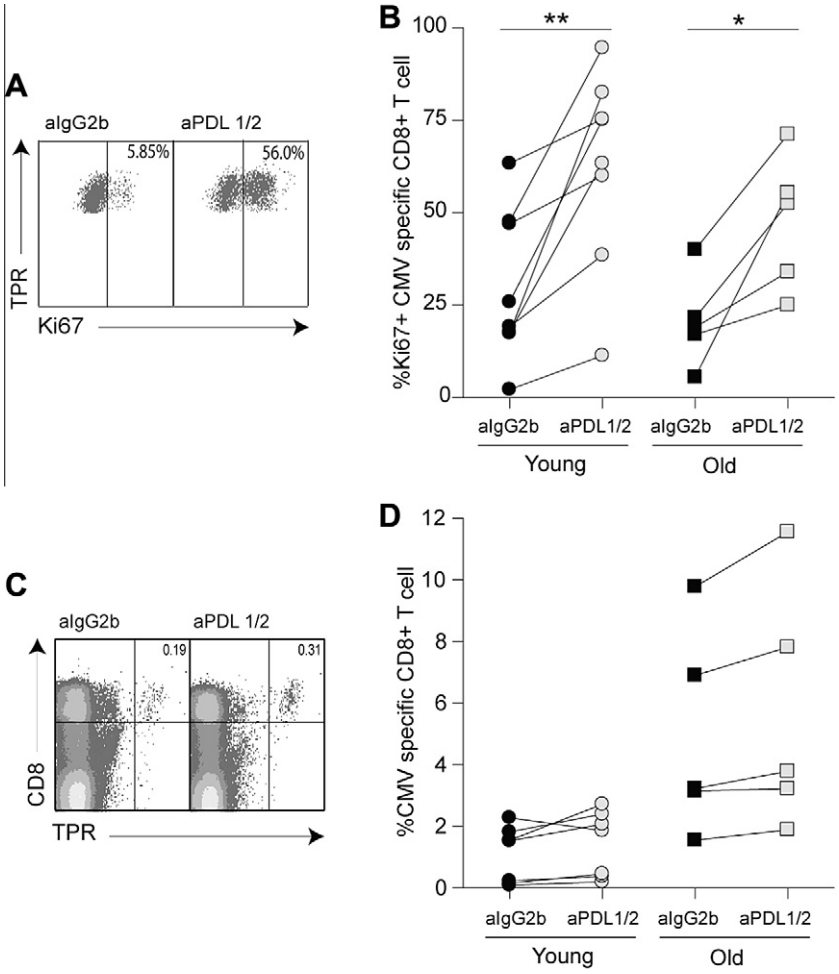

Fig. 3. Blocking PD-1/L interactions augments the proliferative responses of CMV specific CD8$^+$ T cells amongst both young and old donors. (A) Representative examples of Ki67 staining following 3 d stimulation with CMV peptides in the presence of aPD-L1/2 blocking antibody or isotype control. (B) Pooled data showing the effect of aPD-L1/2 blockade compared to the isotype control. (C) Representative dot plots showing the change in proportion of CMV-specific CD8$^+$ T cells following 3 d CMV peptide stimulation in the presence of PD-L blockade or isotype control. (D) Cumulative data illustrating the effect of aPD-L1/2 blockade on the proportion of CD8$^+$ T cells that are CMV specific. For all P values were calculated using a paired Student's t-test.

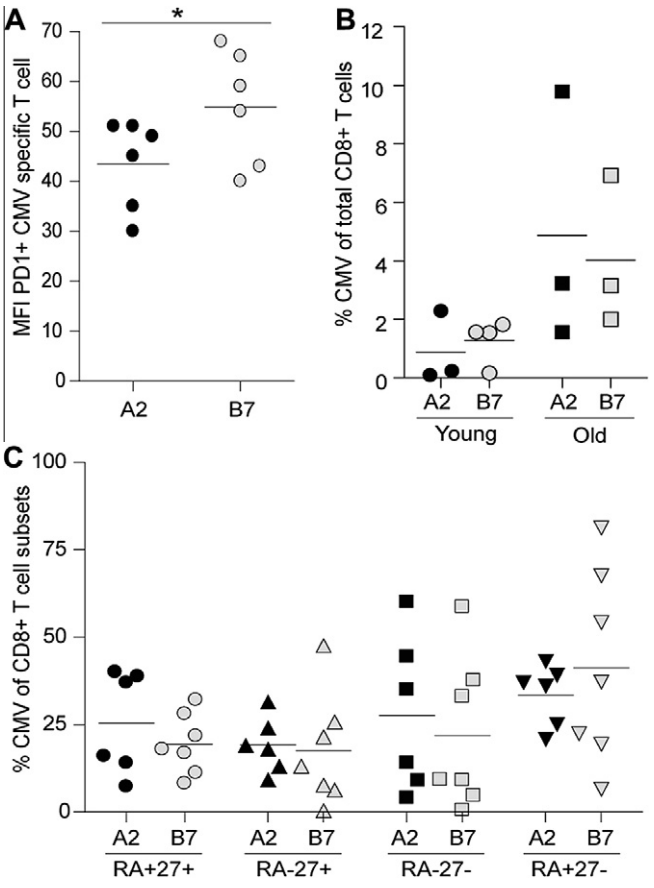

Fig. 4. Expression of PD-1 is higher on CMV-specific B*07 individuals. (A). Comparison of the expression levels of PD-1 on HLA-A*02 or B*07 CMV-specific populations. (B). Graph showing no difference in the percentage of total HLA-A*02 or B*07 CMV-specific $CD8^+$ T cells. (C). Data illustrating that there was no difference in the CD45RA/CD27 phenotypic profiles of HLA-A*02 or B*07 CMV-specific $CD8^+$ T cells. For all graphs horizontal bars depict mean values and *P* values were calculated using a Student's *t*-test.

to HLA-B*07 donors between all CD45RA/CD27 subsets (Fig. 5). Also of note was the highest fold change in both A*02 and B*07 was found to be in the highly differentiated $CD45RA^+CD27^-$ $CD8^+$ T cells, the CD45RA re-expressing memory subset exhibits a proliferative deficiency, relative to their central and effector memory counterparts, that was found to be reversible upon PD-L blockade (Fig. 5B).

4. Discussion

CMV immunosurveillance is widely considered to be maintained even in the very old [34]. However, CMV may sub-clinically reactivate with increasing frequency with age [35]. Additionally, intense immune responses to extracellular CMV in very old subjects have recently been observed, whose magnitude is associated with co-morbidity and functional decline [17]. Thus, the increased frequency and magnitude of CMV reactivation in advanced age could result in direct or indirect CMV related pathology, which may significantly contribute towards their impaired health status. It has been suggested that undiagnosed occult CMV pneumonitis followed by bacterial pneumonia may constitute a common proximate cause of death in the old [3]. Furthermore CMV has been implicated in the loss of response to vaccines in older people [9].

Infection with CMV causes plasmacytoid dendritic cells to secrete high levels of IFNα [12], which can drive T cell differentiation and may significantly contribute towards the CMV associated global differentiation of the T cell pool [36,37]. Furthermore we show here that IFNα upregulates PD-1, which may account for the T cell pool of CMV positive donors expressing enhanced levels of PD-1 compared with their CMV negative counterparts. Blocking studies revealed that IFNα alone contributes half the PD-1 expression induced by an anti-CD3 stimulus, highlighting its functional significance.

PD-1 has been shown to play a role in the dysregulation of $CD8^+$ T cell responses to CMV [20,38], so we sort to investigate whether the manipulation of the PD-1/L axis could functionally reinvigorate CMV-specific $CD8^+$ T cell, in particular their defective proliferative response which is exacerbated by advancing age [10,12,13]. We demonstrate here that CMV specific $CD8^+$ T cell proliferative responses could be enhanced by blocking PD-1/L interactions in both young and old individuals; with the highly differentiated $CD45RA^+$ $CD27^-$ $CD8^+$ T cells showing the largest increase in proliferative response compared to the less differentiated subsets. This may seem counter intuitive as the $CD45RA^+CD27^-$ subset does not express high levels of PD-1. However PD-1 is transiently up-regulated in activated T cells [39] and the low levels of PD-1 seen in this subset may represent their inability to proliferate. When combined with the changes to cell signalling in this subset it may cause the $CD45RA^+CD27^-$ T cells to be more susceptible to PD-1/L receptor blockade. However the contribution of other inhibitory receptors cannot be discounted, for the number and intensity of inhibitor receptor expression has been correlated with functional exhaustion [18]. One inhibitor receptor that has been shown to be highly expressed on $CD45RA^+CD27^-$ T cells is KLRG1 [30,40], whilst the expression of KLRG1 has not been demonstrated to be strongly associated with PD-1 co-expression during exhaustion 18, expression of KLRG1 was found on a significant fraction of exhausted $CD8^+$ T cells [18,41]. As KLRG1 is up-regulation by repetitive antigen stimulation, it is possible that its expression does not reflect T cell exhaustion but rather ongoing antigen recognition, which is thought to be the prerequisite for the development of $CD8^+$ T cell exhaustion [41,42].

We also found that the increased proliferative response of CMV-specific $CD8^+$ T cells following PD-L blockade was not accompanied by a proportional increase in the response size. This, nevertheless, concurs with PD-1 blocking studies performed in a murine LCMV model [19], suggesting that proliferation is accompanied by considerable cell death. This death may be analogous to the contraction phase of effector cell responses, which occurs following antigen clearance in acute infections. Alternatively, PD-L blockade may drive the selective expansion of less exhausted cells over their more exhausted counterparts, which are driven further along the exhaustion spectrum to death [43].

One surprising finding was that HLA-B*07/TPR specific $CD8^+$ T cells expressed higher levels of PD-1 than HLA-A*02/NLV specific cells with no difference in the CD45RA/CD27 phenotypic profiles of the CMV-specific $CD8^+$ T cells. However there was a difference in the proliferative response to PD-L blockade, with HLA-B*07 individuals showing less proliferation despite expressing more PD-1. We would like to speculate here that this may be due to the differing functional avidities for the two CMV-specific $CD8^+$ T cell populations. It has been reported that different HLA-types may equate with different T cell functional avidity for antigen during chronic viral infection [44,45]. CMV-specific $CD8^+$ T cells with high avidity expressed all effector functions upon activation *ex-vivo* compared with low avidity $CD8^+$ T cells, which exhibited no *ex-vivo* cytotoxicity and secreted minimal IFN-γ [46]. Although CMV disease is rare in immune-competent individuals, HLA type can still influence the T cell response of these individuals [47,48]. Indeed, a minimal number of $CD8^+$ CMV-specific T cells have been estimated to be needed for protection against CMV reactivation during organ transplantations [49,50]. These numbers seem to differ on the basis of

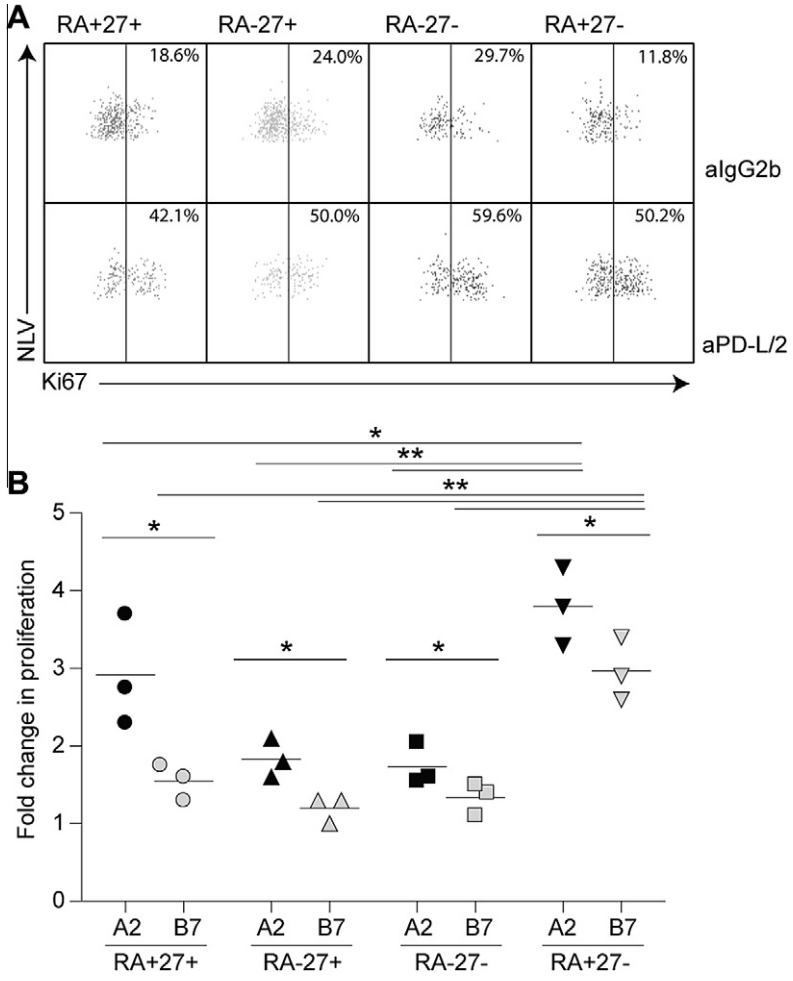

Fig. 5. Blocking PD-1/PD-L interactions augments HLA-A*02 specific responses by a greater magnitude than that of HLA-B*07. (A) Representative dot plots showing Ki67 staining on NLV$^+$ CD8$^+$ T cell subsets following 3 d stimulation in the presence of PD-L blockade or isotype control. (B) Cumulative data comparing the effects of PD-L receptor blockade on the fold increase of the proportion of NLV A2 and TPR B7 CMV specific CD8$^+$ T cells subsets expressing Ki67 defined by CD45RA/CD27. Horizontal bars depict mean values and *P* values were calculated using a repeated measures ANOVA.

HLA type [51,52]. For example, HLA-B*3501-specific CD8$^+$ T cells conferred protection from CMV reactivation with significantly lower cell numbers than HLA-A*0201-specific CD8$^+$ T cells [51]. Interestingly, individuals having both HLA-A*02 and HLA-B*07 alleles show preferential and dominant T cell numbers specific for the TPR epitope of CMVpp65 over the NLV epitope [48]. Furthermore these TPR specific T cells demonstrate preferential expansion over A2 NLV-specific T cells taken from the same individual [48]. It is therefore possible that inhibitory receptor blocking may preferentially benefit NLV-specific A2 restricted cells as their intrinsic reduced propensity to activate renders them susceptible to inhibition, whilst highly avid cells, which have a strong activation response are less likely to be inhibited by signalling through PD-1.

Why the more avid cells expressing higher levels of PD-1 should be less susceptible to PD-1/L may be squared by the finding that two subpopulations of virus specific CD8$^+$ T cells have been identified in both mouse LCMV and human HCV infections: a more terminally differentiated PD-1^{hi} subset, that responds poorly to PD-1 blockade and a less differentiated more functional PD-1^{int} population that responds well to blockade [53,54]. However it should be noted that in our study we were unable to distinguish PD-1$^{int/hi}$ CD8$^+$ T cells. The inability of PD-1^{hi} virus-specific CD8$^+$ T cells to respond to PD-L1 blockade may reflect the expression of additional inhibitory receptors on these highly exhausted cells [18]. The behaviour of PD-1^{int} and PD-1^{hi} populations of CD8$^+$ T cells specific for poorly controlled persistent viral infections such as LCMV, HCV and HIV may nevertheless, not be applicable to the well-controlled latent CMV infection. Indeed, an HBV study, reported that the more exhausted intrahepatic specific CD8$^+$ T cells, expressing higher PD-1 levels, responded more effectively than their less exhausted, lower PD-1 expressing, circulating counterparts [55]. In contrast, Nakamoto et al. [54], documented opposing results; however in this case, the disease status of the subjects was much more advanced. Furthermore, differing PD-1 expression levels may engage distinct intracellular targets [56]. The blockade of PD-L on exhausted PD-1^{int} LCMV and HCV specific exhausted CD8$^+$ T cells, but not their PD-1^{hi} counterparts, restored effector functions [53,57], while the engagement of PD-1 on resting naïve T cells, which express barely detectable levels of PD-1, effectively inhibits T cell activation [56]. This suggests that rather than increased PD-1 expression correlating with greater signalling, differing PD-1 expression levels may engage distinct pathways [56]. 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 [56].

Regardless of TPR/NLV differences the highly differentiated CD45RA$^+$CD27$^-$ CD8$^+$ T cells, which accumulate both with age and CMV infection showed the highest fold change increase in proliferative response compared to the less differentiated subsets with

PD-1/L blockade. PD-L blockade was shown to induce an increased functionality without augmenting cell numbers which may be beneficial in the context of ageing where the large numbers of CMV-specific $CD8^+$ T cells are considered a significant burden upon the immune system of the aged [3].

Authorship

R.M. and S.M.H wrote the paper, designed and performed the experiments, and analysed the data. R.M., N.E.R., S.J.G., performed experiments. A.N.A., designed the experiments.

Acknowledgments

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