Sestrins Induce Natural Killer Function In Senescent-like CD8$^+$ T Cells

B. I. Pereira[1†], R. P. H. De Maeyer[1†], L. P. Covre[1,2†], Djamel Nehar-Belaid[3†], A. Lanna[1,4], S. Ward[5], Radu Marches[3], E. S. Chambers[1], D. C. O. Gomes[2], N. E. Riddell[1,6], M. K. Maini[1], V. H. Teixeira[7], S. M. Janes[7], D. W. Gilroy[8], A. Larbi[9], N. A. Mabbott[10], Duygu Ucar[3], George A. Kuchel[11], S. M. Henson[12], J. Strid[5], J. H. Lee[13], Jacques Banchereau[3], A. N. Akbar[1*]

Affiliation:

[1] Division of Infection and Immunity, University College London, London, UK.

[2] Núcleo de Doenças Infecciosas, Universidade Federal do Espírito Santo, Vitória, Brazil.

[3] The Jackson Laboratory for Genomic Medicine, Farmington, Connecticut, USA.

[4] Nuffield Department of Medicine, University of Oxford, Oxford, UK.

[5] Department of Medicine, Imperial College London, London, UK.

[6] Faculty of Health & Medical Sciences, University of Surrey, Guildford, UK.

[7] Lungs for Living Research Centre, UCL Respiratory, University College London, London UK.

[8] Division of Medicine, University College London, London, UK.

[9] Singapore Immunology Network (SIgN), Agency for Science, Technology and Research (A*STAR), Immunos Building, Biopolis, Singapore, Singapore.

[10] Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh, UK.

[11] University of Connecticut Center on Aging, University of Connecticut, Farmington, Connecticut, USA.

[12] William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, UK.

[13] Department of Molecular & Integrative Physiology, University of Michigan, Ann Arbor, Michigan, USA.

† These authors contributed equally.

***Corresponding author:**
Professor Arne N Akbar
Division of Infection and Immunity, University College London,
Rayne Institute, 5 University Street, London, WC1E 6JF
Email: a.akbar@ucl.ac.uk

Abstract

Ageing is associated with re-modelling of the immune system to enable the maintenance of life-long immunity. In the $CD8^+$ T cell compartment, ageing results in the expansion of highly differentiated cells that exhibit characteristics of cellular senescence. Contrary to the paradigm that senescent-like $CD8^+$ T cells are dysfunctional, we identified the expression of an NKG2D/DAP12 complex with concomitant loss of TCR signalling activity in these $CD8^+$ T cells that promoted cytotoxicity against NKG2D ligand bearing targets. Immunoprecipitation and imaging cytometry showed that the NKG2D/DAP12 complex is associated with sestrin 2 (Sesn2). The genetic inhibition of Sesn2 resulted in decreased NKG2D and DAP12 expression whilst restoring TCR signaling in senescent-like $CD8^+$ T cells. Therefore, sestrins induce re-programming and re-purposing of non-proliferative senescent-like $CD8^+$ T cells to acquire broad-spectrum innate-like killing activity during ageing. This would mitigate against the global loss of immunity that may occur as life expectancy increases inexorably.

Introduction

Human life expectancy has doubled over the last 150 years. Therefore, an effective immune system must persist for on average twice as long now compared to in the mid19th century. This raises the question of whether immunity can be maintained indefinitely as human life expectancy continues to increase[1]. Since the thymus involutes from puberty onwards, its contribution to the maintenance of the T cell pool decreases considerably during ageing. Instead, antigen specific T cells are maintained in later life by repeated episodes of activation and proliferation after specific or cross-reactive antigenic challenge or by homeostatic cytokines[2,3]. This extensive proliferative activity leads ultimately to extreme functional differentiation and the development of T cell senescence through telomere erosion that is associated with decreased TCR-related signalling, telomerase activation and growth arrest[4–6]. Since replicative activity is impaired as senescence develops, mechanisms other that T cell proliferation may be required to maintain optimal immune protection during ageing.

The senescent-like characteristics exhibited by highly differentiated CD8$^+$ T cells include low proliferative activity, short telomeres, low telomerase activity and expression of senescence-associated cell surface (CD57 and KLRG1) and intracellular molecules (p38MAPkinase, γH2AX)[5,7]. These cells have also been shown to upregulate receptors associated with natural killer (NK) cells and are able to kill NK target cells[8]. However, it is not clear if the acquisition of senescence-like characteristics and NK receptor (NKR) expression by these cells are linked or controlled independently.

The sestrins are a family of stress-sensing proteins that are induced by convergent pathways induced by cellular senescence and low nutrient availability that inhibit TCR activation and proliferation in CD4+ T cells in both mice and humans[9]. We now show that in CD8+ T cells, sestrins also inhibit expression of TCR signaling molecules (LAT, ZAP70, LCK) while concomitantly inducing NK receptors (NKR) including the inhibitory killer-cell lectin-like receptor G1 (KLRG1) and NKG2A and activating NKRs such as NKG2D, in both mice and humans. Furthermore, we show that the sestrins regulate the association of NKG2D with its

innate scaffold DAP12 that converts it into a direct activating receptor that induces cytotoxicity and cytokine secretion towards target cells bearing NKG2D ligands, independently of the TCR. Collectively, these data challenge the concept that senescent-like CD8+ T cells are a defective end-stage population. Instead we conclude that these cells while non-proliferative are re-programmed during differentiation to recognize and kill via both TCR[10] as well as NKR recognition mechanisms, a process regulated by the sestrins. This may be an adaptation that compensates for the decreased thymic output, that narrows the T cell repertoire[11–14] and decreases NK cell function that occurs in older people[15]. The repurposing of senescent-like CD8+ T cells to mediate innate-like functional activity may be crucial to mitigate against the increased burden of tumours and stromal senescent cells that accumulate in tissues during ageing[16,17].

Results

Human CD8$^+$ TEMRA cells upregulate NK machinery concomitantly with decreased TCR complex expression

We first examined the breadth of NKR expression by human CD8$^+$ T cell subsets at different stages of differentiation defined by the relative expression of surface CD27 and CD45RA using Affymetrix U133 plus 2 microarrays[18] (**Fig. 1A and Fig. S1A**). We compared gene expression in central memory (CD27$^+$CD45RA$^-$), effector memory (CD27$^-$CD45RA$^-$) and terminal effector memory (CD27$^-$CD45RA$^+$) relative to the naïve (CD27$^+$CD45RA$^+$) CD8$^+$ T cell subset. We identified differentially expressed genes (**Table S1**) based on a minimum of a 2-fold change with a *p* value of < 0.05 and a false-discovery rate (FDR) for multiple comparisons of < 0.05%[19]. Hierarchical clustering of a selection of genes of interest (listed in **Table 1**) revealed a transcriptional signature that clearly distinguished T_{EMRA} (CD27$^-$CD45RA$^+$) from naïve (CD27$^+$CD45RA$^+$) CD8$^+$ T cells (**Fig. 1B, C**). This signature included downregulated genes, which were mostly involved in co-stimulation (*Cd28, Cd27*), TCR signaling (*Trac, Cd3e, Cd3g,*

Lck, Lat, Plcg1), and proliferation and cell cycle control (*Ccne1, Ccnd3*). Compared to naïve cells, highly differentiated $CD8^+$ T cells upregulated genes encoding the transcription factors *Zeb2* (fold change: 10.75, $p < 0.0001$) and *Plzf* (*Zbtb16* fold change: 6.48, $p < 0.0001$) in line with previous studies showing that these molecules can regulate memory T cell differentiation and transcriptional control of terminal differentiation[20,21] and the development of innate-like features in T cells in mice[22,23]. However, *Plzf* (*Zbtb16*) was also increased in central memory and effector memory $CD8^+$ T cells compared to naïve populations (7.7- and 4.8-fold respectively; **Table 1**). Concomitantly, highly differentiated $CD8^+$ T cells upregulated genes encoding for NKRs, innate signalling adaptors (*Tyrobp*), and molecules involved in effector functions such as cytotoxicity (*Gzma/b*, *Prf*, *Fasl*, *Itgav*, and *Itgb1*)[24,25]. Highly differentiated cells also upregulated chemokine receptors associated with NK cell migration into tissues (*Cx3cr1*, *S1pr5*, and *Cmklr1*)[26,27] Collectively these findings corroborate that terminal differentiation of $CD8^+$ T cells is associated with a transcriptional program that promotes cytotoxic effector function at the expense of proliferative potential[5]. Furthermore, such a marked increase in genes related to NK cell function at the level of migration, recognition and cytotoxicity supported the notion that highly differentiated $CD8^+$ T cells may become multi-functional by enabling effector action through a mechanism independent of the TCR.

To validate the increased expression of NKRs on $CD8^+$ T_{EMRA} cells relative to naïve cells, we used flow cytometry. We analyzed the cell surface expression of several NKRs on $CD8^+$ T cell populations in PBMCs isolated from healthy donors across different ages (n = 22; median age = 52; range = 25-83). Irrespective of age, the expression of NKRs on non-NK cells was most elevated on $CD3^+CD8^+CD27^-CD45RA^+$ T_{EMRA} cells (**Fig. 1D, S1A**) that have been defined as senescent-like[5,18]. Co-incident with the gene expression analysis, the repertoire of NKRs expressed on $CD8^+$ T_{EMRA} cells was diverse and included both activating (NKG2D, NKG2C and KIR2DS) and inhibitory (NKG2A, KIR2DL/KIR3DL and CD244) receptors, as well as the maturation markers KLRG1 and CD57 (**Fig. 1D, S1C**).

To confirm decreased expression of TCR machinery in senescent-like CD8$^+$ T_{EMRA} cells, we isolated CD8$^+$ T cell subsets by their relative expression of CD27 and CD28 that enabled the isolation of sufficient cells for functional analyses. Previous studies demonstrated a sequential loss of CD28 and CD27 co-stimulatory receptors on CD8$^+$ T cells as they transition from naïve (CD27$^+$CD28$^+$) to intermediate (CD27$^+$CD28$^-$) and finally to a terminal or senescent-like (CD27$^-$CD28$^-$) phenotype[28,29]. The CD27$^-$CD28$^-$ CD8$^+$ T cell subset contains the EMRA (CD27$^-$CD45RA$^+$) population and also some effector memory (CD27$^+$CD28$^-$) cells (**Fig. S1B**). These cells also exhibit increased NKR expression (**Fig. S2A**). It has been shown that senescent-like CD8$^+$ T cells, either identified as CD27$^-$CD28$^-$[30,31] or CD27$^-$CD45RA$^+$[32] increase during ageing and exhibit markers of senescence[5,6,9,33]. We found that senescent-like CD4$^+$ T cells, defined by simultaneous loss of CD27 and CD28, also acquired the expression of NKRs, but to a lower degree than their CD8$^+$ counterparts (**Fig. S2B**). Western Blot analysis showed a significant downregulation of LCK, LAT and PLCγ1, but conversely increased Zap70 expression in senescent-like (CD27$^-$CD28$^-$) compared to naïve CD8$^+$ T cells (**Fig. 1E**). Together, these findings extend previous studies showing an increased expression of NK-lineage receptors on CD8$^+$ T cells with characteristics of terminal differentiation and senescence[8,34,35].

Individual T_{EMRA} CD8$^+$ T cells express NK receptors, cytotoxic machinery and characteristics of senescence

It was not clear if the expression of NK receptors, cytotoxicity-related molecules and senescence markers that we found in CD8$^+$ T_{EMRA} cells were all present on the same individual cells. To clarify this, we investigated the transcriptomes of ~62k purified CD8$^+$ T cells from six healthy older adult donors by single cell RNA-sequencing (scRNA-seq). These cells were separated based on their IL-7R protein expression, with IL-7R^{lo} cells being enriched for T_{EMRA} populations. This resulted in 12 samples (six CD8$^+$ IL7R$^+$ and six CD8$^+$ IL7R$^-$ T cells) (**Fig. 2A**). CD8$^+$ IL7R$^+$ and CD8$^+$ IL7R$^-$ samples yielded an average of 6199 cells (SD 1582) and 4192 cells (SD 1269) per donor respectively with an average of 1043 and 1011 genes per cell,

respectively (**Fig. S3A-B**). After discarding hybrid transcriptomes (multiplets) using Scrublet[36] (see Methods), raw data from the 12 samples were combined. scRNA-seq profiles that passed the quality control (**Fig. S3C**) were then corrected for technical batch effects (e.g., 10X run) using BBKNN[37]. Unbiased clustering followed by a two-dimensional uniform manifold approximation and projection (UMAP)[38] on the corrected data, revealed 13 distinct clusters (**Fig. 2B**). Cluster assignments were independent of 10X batch (**Fig. S3D**) and donor (**Fig. S3E**) effects. IL7R^{+} and IL7R^{-} groups were associated with distinct set of clusters (**Fig. 2C**). The number of cells within each cluster varied from 9,263 to 915 (**Fig. S3F**) and their respective IL7R mRNA expression was confirmed (**Fig. S3G**). We then assigned these clusters to cell types based on differential analysis comparing expression values among cells from a given cluster to all other cells (**Supplementary Table 3**). For selected genes, including lineage (e.g., *CD3E*, *CD8A* or *CD4*), naïve (e.g., *CD27*, *CCR7*, *SELL* or *CD28*) and effector (e.g., *KLRG1*, *PRF1* or *GZMB*) markers, expression values in each cell are shown in **Fig. 2D.** The populations studied uniformly expressed CD3 and CD8 but not CD4 transcripts (**Fig. 2D**).

The inspection of the expression patterns of naïve and effector markers allowed the definition of C0, C4 and C8 as naïve, and C1, C2 and C6 as T_{EMRA} CD8^{+} T cell associated clusters (Fig. 3A). These six selected clusters were then extracted and a second round of clustering was performed (Fig. 3B), which confirmed distinct transcriptomic profiles of the naive and T_{EMRA} compartments (**Fig. 3B, Fig. S4A**) and confirms enrichment of the T_{EMRA} compartment in IL-7R^{-} sorted cells (**Fig. S4B**). The naive CD8^{+} T cells were characterized by the upregulation of *CD27*, *CD28*, CCR7 and *SELL*, while T_{EMRA} CD8^{+} T cells showed an upregulation of these genes but had increased expression of *KLRG1*, *PRF1* or *GZMB* relative to the naïve clusters (**Fig. S4C**).

We first investigated the expression of NK-associated genes, within naïve and T_{EMRA} compartments and found an increase of NK-associated genes including FCGR3A (CD16), *FCRL6* (NK-related receptor) and *TYROBP* (DAP12; functional NK adaptor molecule; **Fig. 3C**) in the latter, confirming our previous results (**Fig. 1**). We further investigated the relative level

of expression of 15 NK-related genes and showed that they were significantly enhanced in the T_{EMRA} compared to the naïve $CD8^+$ T cell population (**Fig. 3D; Supplementary Table 2)**. We next assessed the expression of senescence-related genes (e.g., *B3GAT1*, *CDKN1A* and *CDKN2A*) and showed that they were increased in $CD8^+$ T_{EMRA} compared to naïve populations (**Fig. 3E**). We constructed a senescence score based on the average expression of 27 senescence associated genes (**Supplementary Table 2**) and confirmed that the T_{EMRA} population expressed multiple characteristics of senescent cells (**Fig. 3F**). Although we found that LCK, PLCγ1 and LAT were reproducibly decreased in senescent-like $CD8^+$ T cells at the protein level (Fig. 1E) this was not observed at the single cell transcriptional level (not shown). This suggests that the expression of these molecules in the senescent-like CD8 subset may be regulated by post translational modification. Collectively our results indicate that the same highly differentiated $CD8^+$ T cells, that have characteristics of cellular senescence, also express a range of NK receptors, NK adaptors and cytotoxic machinery.

Senescent-like $CD8^+$ T cells mediate TCR-independent cytotoxicity through NKG2D and DAP12

Given the upregulation of a wide NK receptor phenotype in senescent-like $CD8^+$ T cells we investigated whether these cells were able to mediate NK-like functions independently of TCR/MHC interactions. For this purpose, we used a classical NK target cell, the MHC class I-deficient K562 tumour cell line for *in vitro* cytotoxicity assays. Using the degranulation marker CD107a as a surrogate for cytotoxicity[39], we observed that highly differentiated $CD8^+$ T cells responded to K562 target cells to the same extent as NK cells (**Fig. 4A**). We confirmed that the senescent-like $CD8^+$ T cell population had cytotoxic activity in a direct functional assay (**Fig.S5A-B**). To specifically address whether NKG2D could mediate cytotoxic functions of $CD8^+$ T cells, we used an MHC class I-deficient tumour cell line (C1R) transfected with the NKG2D ligand MICA*008 (C1R-MICA). The untransfected, MICA-deficient C1R was used as a control (**Fig. 4B**)[40]. To determine the specificity of NKG2D mediated killing, we used a small interfering RNA (siRNA) for NKG2D or a scrambled siRNA control to knock down NKG2D

expression in $CD28^-$ $CD8^+$ T cells (**Fig. 4C**). siCtrl and siNKG2D $CD8^+$ T cells were then co-cultured with C1R or C1R-MICA target cells for 6 hours. siCtrl transfected T cells showed increased degranulation when cultured with C1R-MICA as compared to control C1R cells (**Fig. 4D**). However, this cytotoxicity towards C1R-MICA was significantly inhibited in siNKG2D transfected $CD8^+$ T cells (**Fig. 4D**). Collectively, these findings indicate that $CD8^+$ T cells upregulate and utilise NKG2D to kill tumour cells lacking MHC class I and expressing NKG2D ligands. Therefore, these cells may have a functional role in immune surveillance by using TCR-independent, NK-like recognition mechanisms.

Although NKG2D is expressed across all subsets of $CD8^+$ T cells, the acquisition of NKR dependent cytotoxic functions only occurs in the highly differentiated populations (**Fig. 4A**). Expression of NKG2D on the cell surface requires its association with adaptor proteins to stabilise the immunoreceptor complex and provide it with signalling activity[41]. NKG2D is known to associate with two adaptor molecules, DAP10 and DAP12. The former contains an YxxM-motif which activates PI3K signalling[42,43]. DAP12, however, has an ITAM-motif that can recruit and activate ZAP70/Syk protein kinases directly triggering cytokine release and cytotoxicity[44–46]. In human $CD8^+$ T cells, NKG2D is predominantly associated with DAP10 (**Fig. S5C**) allowing it to act as a TCR co-stimulatory signal[7], whereas its association with DAP12 is less well understood. Our transcriptomic data showed that the transcript for DAP12 (*Tyrobp*) was strongly induced in senescent-like $CD8^+$ T cells while DAP10 expression was relatively unchanged (**Fig. 1A, Fig 3C and Table 1**). Increased DAP12 expression was observed in highly differentiated $CD27^-CD28^-CD8^+$ T cells by Western Blot analysis (**Fig. 4E**) and by intracellular flow cytometry (**Fig. 4F**). We therefore hypothesised that NK killing activity in senescent-like $CD8^+$ T cells was mediated by NKG2D stimulation in association with DAP12. To investigate this further we immunoprecipitated NKG2D from freshly isolated $CD8^+CD28^+$ or $CD8^+CD28^-$ T cell subsets. DAP12 was found in the $CD28^-$ population only, confirming the association of DAP12 with NKG2D in highly differentiated $CD8^+$ T cells (**Fig. 4G**).

We next investigated whether NKG2D ligation induced the phosphorylation of ZAP70/Syk in highly differentiated $CD8^+$ T cells (**Fig. 4H**). As expected, CD3 ligation induced ZAP70/Syk phosphorylation, but NKG2D stimulation had a greater effect. Thus, senescent-like $CD8^+$ T cells can be activated through stimulation of both the TCR and NKG2D but show an increased propensity to respond to the latter. This was further highlighted by the fact that NKG2D ligation alone was sufficient to induce IFN-γ secretion and granzyme B expression in senescent-like ($CD27^-CD28^-$) $CD8^+$ T cells (**Fig. 4I**). Finally, we repeated cytotoxic assays with C1R-MICA or the C1R control cell lines, using $CD28^-CD8^+$ T cells transfected with siRNA to DAP12. Silencing of DAP12 significantly impaired the cytolytic degranulation of $CD8^+$ T cells towards C1R-MICA as compared to the scrambled siRNA control (**Fig. 4J**). Together these observations indicate that in senescent-like $CD8^+$ T cells, DAP12 expression is upregulated strongly and is necessary and sufficient to convert NKG2D into a direct stimulatory receptor. Although NKG2D is constitutively expressed on all subsets of $CD8^+$ T cells, the absence of DAP12 in less differentiated cells explains why NKG2D ligation is insufficient to induce cytokine secretion and degranulation in these subsets. DAP12 has been shown to couple with different activating receptors in addition to NKG2D in NK cells, including activating KIRs (KIR2DS), NKp44 and NKG2C[48] that are also overexpressed in senescent-like $CD8^+$ T cells (**Fig. 1B-D, 3C-D**). Therefore, DAP12 expression may confer (in part) multiple NK-like functions to senescent-like $CD8^+$ T cells.

Senescent-like $CD8^+$ T cells express elevated levels of sestrins that impair proximal TCR signalling and modulate the expression of NK cell adaptors

We investigated whether the reduced expression of components of the CD3/TCR complex (**Fig. 1C, 1E**) compromised the efficiency of proximal TCR signaling in senescent-like $CD8^+$ T cells. We previously showed that these cells have reduced proliferative activity after TCR stimulation[5,7]. Indeed, we observed impairment in phosphorylation of CD3ζ after anti-CD3 activation as shown by phospho-flow cytometry (**Fig. 5A**). Although the expression of total Zap70 was increased (**Fig. 1E**), its phosphorylation following anti-CD3 activation was impaired

in $CD28^-CD27^-$ relative to $CD28^+CD27^+$ $CD8^+$ T cells (**Fig. 5B**). According to the canonical model of TCR signaling, activation of LCK is one of the first events to occur after TCR ligation, leading to the recruitment and phosphorylation of CD3ζ, Zap70 and the assembly of the LAT signalosome[49]. The observation that highly differentiated/senescent-like $CD8^+$ T cells have significantly reduced LCK expression (**Fig. 1E**) may explain the impaired phosphorylation of CD3ζ after TCR activation. However, the conserved expression of total ZAP70 suggests that this Syk-family tyrosine kinase may be important for the activation of alternative (TCR-independent) pathways in highly differentiated $CD8^+$ T cells.

It was demonstrated recently that the sestrins, a family of stress-sensing proteins, induce senescent characteristics in $CD4^+$ T cell by forming a TCR-inhibitory complex with AMP kinase (AMPK) and the MAP kinases[9]. We investigated if highly differentiated $CD8^+$ T cells similarly expressed sestrins and whether these molecules were pivotal in the expression of either NKR or TCR expression by senescent-like $CD8^+$ T cells. We found that $CD27^-CD28^-CD8^+$ T cells exhibited increased sestrin 1 and 2 expression by flow cytometry (**Fig. 5C-D**) and by western blot (**Fig. 5E**). In fact, sestrin 2 is upregulated in total $CD8^+$ T cells in people over 65 years of age (**Fig. S5D**). In addition, senescent-like $CD8^+$ T cells, much like their $CD4^+$ counterparts[9], had increased levels of an activated MAP kinase, Jnk (**Fig. 5E**). In immunoprecipitation experiments we found that DAP12, sestrin 2 and Jnk were associated with NKG2D (**Fig. 6A**). Furthermore, imaging cytometry confirmed that sestrin 2, DAP12 and phospho-Jnk co-localise in senescent-like, but not naïve, $CD8^+$ T cells (**Fig. 6B-D**). This together with our previous observations[9] strongly suggest that sestrin 2 is associated with the formation of the functional NKG2D/DAP12/Jnk complex in these cells.

We next investigated whether the sestrins were directly involved in regulating the expression of NKG2D in senescent-like human $CD8^+$ T cells. We transduced isolated human $CD28^-CD8^+$ T cells with lentiviral vectors containing inhibitory shRNA against sestrin 1,2 and 3 (**Fig. 6D**). We found that this significantly reduced NKG2D expression (**Fig. 6E**) indicating the direct involvement of sestrins in NKG2D expression in these cells. Blocking Jnk either

pharmacologically, or using siRNA (not shown), yielded similar results to sestrin inhibition by knocking down NKG2D (Fig. 6F). Indeed, Jnk blockade increased the frequency of CD28+ cells and restored TCR-mediated signalling as detected by CD3-activated Lck phosphorylation, indicating a reconstitution of T cell related function at the expense of NK related activity (Fig. 6F). Together with our previous studies[9] this indicates that sestrins may act through Jnk to induce NKG2D expression in senescent-like CD8+ T cells.

Yellow fever virus (YFV) vaccination induces upregulation of NKR by YFV-specific CD8$^+$ T cells

We questioned if the upregulation of NKRs by CD8$^+$ T cells occurred exclusively as a result of cellular senescence or if these receptors were expressed at earlier stages of an immune response and maintained as cells differentiated towards senescence in response to antigenic stimulation *in vivo*. To this end, we mined existing, publicly available gene expression data generated by Akondy *et al.* using a cohort of individuals vaccinated against yellow fever[50]. Effector CD8$^+$ T cells, defined as YFV-tetramer$^+$ cells 14 days post-vaccine, and memory CD8$^+$ T cells (YFV-tetramer$^+$ 4-13 years post-vaccine) were compared to naïve (tetramer$^-$) cells by RNA-seq. Cytotoxic mediators such as FasL, Perforin (*Prf1*) and granzymes (*GzmA, GzmB*) were highly expressed in effector and memory compared to naïve CD8 T cells. Additionally, there was a significant upregulation of multiple NK receptors on antigen-specific CD8$^+$ T cells, including many of the KIRs, CD16, CD57 and NKG2A, as well as chemokine receptors (*S1pr5, Cmklr1 and Cx3cr1*) and NK adaptor proteins such as DAP12 (*Tyrobp*) (**Fig. S6**). Interestingly, the upregulation of these molecules occurred on YFV specific CD8$^+$ T cells during the effector phase of the response and is maintained on these cells in the long-term memory phase. YFV-specific memory CD8$^+$ T cells lacked markers of senescence like CD57, expressed CD27 and CD28 and were polyfunctional. Most importantly, unlike the T_{EMRA} subset, they exhibited excellent proliferative potential *in vitro* suggesting that they were not terminally differentiated[51].

This indicates that NKR expression on $CD8^+$ T cells is not only limited to senescent populations but is also a feature of antigen-experienced T cells. YFV-specific effector and memory $CD8^+$ T cell cells did show some downregulation of the TCR signalosome (**Fig. S6**) but this was not as profound as observed in highly differentiated $CD8^+$ T cells (**Fig. 1E**). Of note, Sestrin 2 was upregulated in the YFV specific $CD8^+$ T cells during both the effector and memory phases of the response (**Fig. S6**).

Sestrins regulate NK function of $CD8^+$ T cells in vivo

We next investigated if sestrins directly regulated the expression of NKR expression by $CD8^+$ T cells *in vivo*. We previously showed that sestrins regulate decreased $CD4^+$ T cell function identically in both aged mice and humans[9]. We investigated young wild type (Y WT, ~6 weeks), old wild type (O WT; ~18 months), old sestrin 1 knockout mice (O $Sesn1^{-/-}$; ~18 months) and old sestrin 2 knockout mice (O $Sesn2^{-/-}$; ~18 months). Since NKR were induced on specific $CD8^+$ T cells after activation (see above) we vaccinated these groups of mice against methylated BSA (mBSA) and subsequently re-challenged them to induce a delayed-type hypersensitivity response in the footpad as an index of successful immune induction (**Fig. S7A**). All groups of mice mounted a DTH response to mBSA re-challenge, however the DTH response in the old groups reached a higher peak of swelling compared to young WT mice (**Fig. S7B**). The response resolved more slowly in old compared to young WT mice, while Sesn knockout mice resolved better than their old WT counterparts (**Fig. S7B-C**). Spleen weights post-challenge were equivalent in all groups (**Fig. S7D).**

Following the DTH response there were no changes in the proportions of splenic NK cells, iNKT cells, $CD4^+$ and $CD8^+$ T cells between any of the old and young groups (**Fig. 7A-B**). We did however note that, compared to Y WT mice, effector ($CD44^+CD62L^-$) cells expanded while naïve ($CD44^-CD62L^+$) $CD8^+$ T cells were decreased in O WT mice, but not in the $Sesn1^{-/-}$ and $Sesn2^{-/-}$ animals (**Fig. S7E-F**).

We observed that expression of NKG2D, DAP12 as well as NKG2A/C/E and Ly49 were significantly higher on $CD8^+$ T cells from old compared to young mice (**Fig. 7C-D, G-H, S8**). However, the absence of sestrin 1 or 2 in old mice prevented the age-induced upregulation of these NK receptors (**Fig. 7C, S8**). This suggests that these receptors are modulated by the sestrins. Importantly, sestrin deficiency did not alter NKG2D (and NKG2A/C/E) expression in *bona fide* NK cells or iNKT cells in any group (**Fig. 7E-F**) indicating that sestrins uniquely regulate NKR in $CD8^+$ T cells. Finally, we examined the effect of sestrin deficiency, and subsequent NKR deficiency, on *in vivo* killing of NK targets. To do this we challenged NK-depleted aged WT mice and NK-depleted aged $Sesn1^{-/-}Sesn2^{-/-}Sesn3^{+/-}$ mice with differentially labelled $Rae\text{-}1^+$ and $Rae\text{-}1^-$ target cells, the former being targets for NKG2D-bearing cells as Rae-1 is the murine equivalent of MICA/B (**Fig. 7I**). Six hours post-injection, we retrieved more $Rae\text{-}1^+$ target cells from the spleens of knockout mice compared to WT controls (**Fig. 7J**) and observed decreased specific killing of these targets in the knockouts (**Fig. 7K**). Together these data show that the sestrins regulate both NKG2D and DAP12 expression and thereby confer NK-like function through NKG2D in highly differentiated/senescent-like $CD27^-CD28^-CD8^+$ T cells *in vivo*.

Discussion

The overall aim of this work was to understand how T cell related immunity is maintained during ageing despite the constraints imposed on the immune system by thymic involution and the development of replicative senescence in cells that undergo repeated episodes of stimulation throughout life. We provide phenotypic, functional and mechanistic data to support the hypothesis that as $CD8^+$ T cells differentiate towards an end stage, they undergo a sestrin-dependent shift towards NK-like functionality. We propose that these cells undergo a defined reprogramming to be able to utilise machinery required to recognise and respond to NK targets while at the same time losing their TCR-dependent potential for activation. This would prevent the loss of these cells through replicative senescence driven by lifelong antigenic re-

stimulation while repurposing them to exhibit broad NK activity. This is particularly relevant for T cells that are specific for persistent pathogens such as cytomegalovirus (CMV) and Epstein-Barr Virus (EBV)[52–54]. Our data suggest that long term immunity against these viruses during ageing may be maintained in part by specific T cells that have NK-like function and is antigen independent. The key finding here is that this process is regulated by the sestrins.

T cell expansion after activation takes time before reaching sufficient numbers for optimal immunity. During this expansion, the host is vulnerable to infections that spread rapidly and/or cause severe pathology and this is prevented in part by NK cells that lyse infected target cells and secrete cytokines without prior activation. NK cell numbers generally increase during ageing with a shift from an undifferentiated $CD56^{bright}$ to a differentiated $CD56^{dim}$ phenotype. However, these cells have reduced cytotoxicity and a decreased capacity to secrete cytokines such as IFN-γ, MIP-1α and IL-8. This may lead to decreased immune protection, especially during the early stages of infection in older subjects. The development of NK activity by highly differentiated/senescent-like T cells that accumulate during ageing may compensate in part for the age-associated decrease in NK function.

Senescent-like T cells in both $CD4^+$ and $CD8^+$ compartments concomitantly decrease their expression of key components of the TCR signalosome including LCK, ZAP70 and PLCγ1. We showed previously that the inhibition of sestrins enhances the expression of TCR signalling molecules suggesting that they are a pivotal switch that regulates TCR vs NKR related function in senescent-like T cells. While long term sestrin blockade may be dangerous as it would enhance the proliferation of senescent-like T cells that harbour DNA damage, temporary sestrin blockade could be exploited to increase antigen specific T cell numbers to boost vaccine responsiveness during ageing as suggested previously[9]. This raises the question of when sestrins and NKR expression occurs on T cells after vaccination. Data relating to this was provided by an important series of studies where gene expression was investigated in yellow fever virus specific $CD8^+$ T cells after vaccination of previously non-immunized individuals[50,51]. It was found that both NKR and sestrin expression were

upregulated in the effector phase of the response (weeks) and maintained in the memory cells (years; see **Fig. S6**).

Other studies support the notion that altered TCR signalling pathways may predispose cells to develop unconventional functions that are not restricted to the TCR/MHC interaction[55–57]. Furthermore, it has been suggested that the suppression of TCR signalling with acquired responsiveness to innate stimuli is a characteristic that defines innate-like cells[58]. Our observations suggest that senescent-like $CD8^+$ T cells should be included as part of the innate lymphoid cell family albeit a population that retains the ability to transition between innate and TCR dependent functions that is regulated by the sestrins. Like in $CD4^+$ T cells, it does involve Jnk MAPK[6]. It must be said that, over and above an increase in NK mediators and overlap between sestrins and the NKG2D/DAP12 complex, the exact mechanism by which sestrins orchestrate the TCR to NKR switch is unclear. While the investigation of sestrin deficient animals showed that these molecules do not regulate NKR expression by *bona fide* NK cells or iNKT cells, these studies should be extended to other cell types including γδT cell populations.

These findings raise questions about the biological significance of such changes and the possible advantage of generating T cells with NK-like characteristics. The acquisition of NKRs by $CD8^+$ T cells may be an adaptation to broaden their capacity for immune surveillance by utilising different recognition systems which would compensate for the decreased output of naïve T cells during ageing[35]. In addition to their capacity to recognise and kill in an MHC-dependent manner, albeit to a lesser extent due to the downregulation of the TCR signalosome, the ability to kill MHCI-deficient and NKG2D-ligand bearing cells may be important for immune surveillance of transformed, stressed, and senescent cells[16,17]. Given the propensity to develop an increased burden of tumours and infections with age, the expansion of these NK-like $CD8^+$ T cells would be an advantageous adaptation and it has been recently suggested that the accumulation of these cells is a predictor of successful ageing[10]. It has been shown that senescent cells (non-lymphoid and lymphoid) are

421 inflammatory and increase in many organs during ageing[59,60]. Furthermore, their removal
422 enhances organ function and retards age-related functional decline[60,61]. It was shown recently
423 that senescent cells can be recognized and killed by NK cells[16,63]. Furthermore, NKR
424 expressing $CD8^+$ T cells can also kill senescent fibroblasts[15]. Therefore, a novel role for NKR
425 on senescent T cells may be in the surveillance and killing of senescent tissue cells. This
426 would identify a co-evolving system where senescent $CD8^+$ T cells are able to recognize and
427 kill different senescent cell types in different tissues during ageing.

428

Figure legends

Fig. 1: Transcriptional signature of human senescent-like CD8$^+$ T cells.

A) High purity CD8$^+$ T cells were isolated from PBMCs of 6 healthy donors and sorted by Fluorescence Activated Cell Sorting (FACS) into naïve (CD27$^+$CD45RA$^+$), central memory (CD27$^+$CD45RA$^-$), effector memory (CD27$^-$ CD45RA$^-$) and terminally differentiated/senescent-like (CD27$^-$ CD45RA$^+$) CD8$^+$ T cells. Numbers in gates represent percentages of cells in each subset from a representative donor. **B)** Gene expression was analysed using Affymetrix U133 plus 2 microarrays. Heat map comparing the expression of selected genes of interest between naïve (CD27$^+$CD45RA$^+$) and highly differentiated/senescent-like CD8$^+$ T cells (CD45RA$^+$CD27$^-$) showing downregulated (in yellow) and upregulated genes (in blue). **C)** The relative fold-change (log_{10}) of differentially expressed genes of interest in CD8$^+$ T cell subsets compared to naïve CD8$^+$ T cells. The list of genes of interest is shown in **Table 1** and the complete list of differentially expressed genes from the whole-transcriptome analysis (≥2-fold change, p<0.05, FDR<0.05%) is available in **Table S1**. **D)** The expression of NK cell receptors (NKR) on CD8$^+$ T cells was assessed by flow cytometry on PBMCs from 22 healthy donors (median age = 52, range 25-83). Total CD8$^+$ T cells were stratified into four subsets according to CD27/CD45RA expression as shown in Fig. S1A. **E)** Human CD8$^+$ T cells were freshly isolated from PBMCs using magnetic activated cell sorting according to CD27 and CD28 expression into CD27+CD28+, CD27+28- and CD27-28- as shown in Fig. S1B Representative immunoblot of the proximal TCR components LCK, PLCγ1, LAT and Zap70. Summary data (n=4) of LCK, Zap70, PLCγ1 and LAT expression normalized to the loading control (GAPDH) and presented relative to the basal expression in CD27+28+ cells set as 1. **D)** Two-way ANOVA with Dunnett's post-test correction and **E)** one-way ANOVA with Tukey's correction (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

Fig. 2: Single cell RNA-seq (scRNA-seq) shows distinct phenotypic differences between naïve and T_{EMRA} CD8$^+$ T cells

A) Overview of the scRNA-seq processing pipeline. Raw data (n=82,061 sorted CD8$^+$ T cells) from six healthy older adult donors (six IL7R$^+$ and six IL7R$^-$ CD8$^+$ T cell samples) were first cleaned from the multiplets, using Scrublet[36], then merged, resulting in a dataset containing 62,343 cells. After batch correction using BBKNN[37], the Scanpy[66] -based pipeline was ran (see Methods section). **B)** UMAP plot representing the putative identity of each cluster. Each colour represents a cluster. **C)** UMAP plot representing IL7R$^+$ (in green) and IL7R$^-$ (in purple) CD8$^+$ T cells groups. **D)** UMAP plots representing expression values of selected genes. Other aliases or CD numbers of some genes are shown in brackets.

Fig. 3: The cellular distribution of NK and senescence markers within naïve and Temra compartments

A) Highlighted clusters were considered as naïve (C0, C4 and C8) and T_{EMRA} (C1, C2 and C6) compartments. **B)** A second round of clustering on the selected clusters (n = 39,634) was performed. UMAP plots representing the expression values of **C)** NK and **E)** senescence-associated genes are shown. Other aliases or CD numbers of some genes are shown in brackets. **D)** NK and **F)** senescence scores were calculated based on the average normalized expression of each gene across naïve and T_{EMRA} compartments (gene lists in Supplementary Table 2).

Fig. 4: NKG2D associates with DAP12 in senescent-like $CD8^+$ T cells endowing them with innate-like functions.

A) High purity human $CD8^+$ T cell subsets defined by the expression of CD27/CD28 were freshly isolated from PBMCs by FACS (n = 5) and cultured with K562 cells (E:T ratio 2:1) in a 6-hour incubation assay. The expression of CD107a, as a correlate of the cytotoxic activity, was assessed by flow cytometry on the indicated subsets of $CD8^+$ T cells and on NK cells, as positive controls**. B)** Representative FACS plot of the percentage of MICA/B$^+$ cells in C1R cells transduced with MICA*008 (C1R-MICA) or the control cell line (C1R), lacking the expression of MICA. **C)** Representative histogram of NKG2D expression on $CD8^+$ T cells after transfection with siRNA for NKG2D (black) or siRNA control (grey), determined 36 hours after transfection using flow cytometry. Numbers indicate MFI of NKG2D. **D)** Summary data (n = 4) of CD107a expression on $CD8^+$ T cells transfected with siRNA NKG2D or control siRNA and cultured with C1R-MICA or C1R (E:T ratio 2:1) in a 6-hour incubation assay. *Ex vivo* expression of DAP12 on $CD8^+$ T cell subsets and NK cells as controls, determined by **E)** western blot (n = 5) and **F)** flow cytometry (n = 12). **G)** Whole-cell lysates from purified human $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells were immunoprecipitated with anti-NKG2D and blotted for DAP12. Detection of light chain IgG (IgGL) served as a loading control. Results are representative of 2 independent experiments. **H)** Phosphorylation of Zap70(Tyr319)/Syk(Tyr352) in freshly isolated CD27-28- CD8+ T cells after anti-CD3 (OKT3, 10 µg/mL) and anti-NKG2D (1D11, 5 µg/mL) stimulation. Numbers indicate the relative expression (normalized to GAPDH). Representative of 2 experiments. **I)** Granzyme B expression (left) and IFN-γ secretion (right) after NKG2D ligation (n = 5). **J)** Human $CD8^+$ T cells, transfected with siRNA DAP12 or control siRNA were cultured with C1R-MICA*008/ C1R cells and the expression of CD107a was assessed by flow cytometry (n = 4). Statistical significance determined with Kruskal-Wallis test in A) Friedman test with Dunn´s correction in F), two-way ANOVA with Bonferroni correction

in D) and J) and one-way ANOVA with Tukey's in I) (*p <0.05, **p <0.01, ***p <0.001).

Fig. 5: Senescent-like CD8$^+$ T cells express elevated levels of sestrins and display impaired TCR signalling.

The efficiency of TCR proximal signaling was assessed in the 3 subsets using phospho-flow cytometry, after TCR crosslinking with anti-CD3 (OKT3, 10 µg/mL). Representative histograms and summary data (n = 8) of **A)** CD3ζ and **B)** Zap70/Syk phosphorylation are shown. Numbers represent the mean fluorescence intensity (MFI) for each subset. Light grey histograms represent unstimulated controls. Summary results presented as the MFI relative to that of CD27$^+$CD28$^+$ T cells, set as 1. **C)** Sestrin 1 and **D)** Sestrin 2 expression in CD8$^+$ T cell subsets determined by flow cytometry (n = 10). **E)** A representative donor for the *ex vivo* expression of Sestrin 2 and phospho-Jnk (T183/Y185) on CD8$^+$ T cell subsets, determined by western blot. Densitometry data from western blots for all donors is also shown (n = 3-4). Statistical significance determined with ANOVA with Friedman test and Dunn's post-test correction **A-B)**, repeated measures one-way ANOVA with Tukey's multiple comparisons test in **C-E)** (*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001).

Fig. 6: Sestrins regulate DAP12 and NKG2D expression in CD8$^+$ T cells.

A) Whole-cell lysates from purified human CD8$^+$CD28$^+$ and CD8$^+$CD28$^-$T cells were immunoprecipitated with anti-NKG2D and blotted for DAP12, Sesn2 and P-Jnk (T183/Y185). Detection of the IgG light chain (IgGL) in immunoprecipitate blots (IP) served as loading control. Results are representative of 2 independent experiments. **B)** Representative images of Sesn2 (AF488, green), DAP12 (PE, red) and P-Jnk (T183/Y185, AF647, yellow) single stain controls as well as CD27$^+$CD28$^+$ (DP) and CD27$^-$CD28$^-$ (DN) CD8$^+$ T cells stained for both markers on the ImageStreamX Mk2. Nuclei are stained with DAPI (blue). Scale bars – 7 µm. **C)** Overlap of Sesn2 and DAP12 or P-Jnk in CD28$^+$ and CD28$^-$ CD8$^+$ T cells was enumerated based on a Bright Detail Similarity (BDS) score. Values exceeding 2 were considered as being overlapping. Data are normalized to the DP subset for each donor (n = 6). **D-E)** Isolated human CD8$^+$CD28$^-$ T cells were transduced with control (shCtrl) or anti-sestrin (shSesn) vectors. **D)** Representative western blot for Sesn2 and DAP12. **E)** Flow cytometry data for NKG2D expression. Results are presented relative to cells transduced with shCtrl for each donor, set as 1 (n = 3). **F)** CD8$^+$ T cells were subjected to Jnk inhibition using siRNA or the inhibitor (SP-600125, 10 µM). The frequency of CD28$^+$ and NKG2D$^+$ cells was evaluated, as well as the phosphorylation of LCK following anti-CD3 stimulation. Paired Student's *t* tests in C-F) (*p <0.05, **p <0.01, ***p <0.001).

Fig. 7: Sestrins induce an age-dependent NK phenotype in CD8$^+$ T cells and regulate NK function *in vivo*

A) Polychromatic flow cytometry was used to identify NK1.1$^+$ NK cells (violet), TCRβ$^+$CD1d tetramer reactive iNKT cells (purple), TCRβ$^+$CD3$^+$ CD4$^+$ (blue) and CD8$^+$ (red) T cells. **B)** Quantification of these cell types as a proportion of total splenocytes (n = 3 per group). **C)** Representative pseudocolour density plots for all groups of mice showing CD44 vs NKG2D expression. Frequencies of parent gates are shown in the top right-hand corner. The frequency of NKG2D expression in **D)** CD8$^+$ T cells, **E)** NK cells, and **F)** invariant NKT cells was assessed by flow cytometry (n = 3 per group). **G-H)** DAP12 expression was assessed in CD8$^+$ T cells from the peritoneum of mice following a DTH challenge. Representative histograms for DAP12 expression on total CD8$^+$ T cells are shown in **G)** and data for all mice (n = 3 per group, n = 1 for young WT) are shown in **H)**. Legend under D) applicable to B-H. **I)** Rae-1$^-$ cells (light green) and Rae-1$^+$ cells (dark green) were stained with low and high concentrations of CFSE respectively. Both were mixed at equal ratios and 2x10^7 total cells were injected i.v. into the tail vein of old WT and old Sesn$^{-/-}$ mice subjected to NK depletion 24 h earlier. Mice were left for 6 hours post-challenge before sacrifice. CFSE-labeled cells were examined in spleens, and the ratio of CFSE^{hi} and CFSE^{lo} used to determine NKG2D-mediated killing by the mice. Readouts of *in vivo* cytotoxicity are shown as **J)** the proportion of Rae-1$^+$ cells retrieved in spleens from WT and Sesn$^{-/-}$ mice (n = 3 per group) and **K)** the overall lysis of Rae-1$^+$ cells (n = 3 per group). Statistical significance determined with two-way ANOVA with Tukey's multiple comparisons test in **B)**; one-way ANOVA with Tukey's multiple comparisons test in **D-F, H)**; unpaired Student's *t* tests in **J-K)**. (*$p < 0.05$, **$p < 0.01$).

Figure S1: Senescent-like CD8$^+$ T cells gating and NKR expression

A) Representative FACS plots showing NKR expression in peripheral blood lymphocytes, specifically focusing on CD8$^+$ T cell subsets stratified by the expression of CD27/CD45RA in healthy donors. **B)** Flow cytometry gating of CD8$^+$ T cells to confirm CD27 and CD28 expression in subpopulations based on CD27/CD45RA gating. **C)** Confirmation of expression of certain NKRs on CD8$^+$ T cell subsets. Numbers in quadrants represent percentages of cells in each subset. Numbers above the histograms indicate the MFI.

Figure S2: NKR Protein expression in CD8$^+$ and CD4$^+$ T cells defined by CD27/CD28

The expression of NK cell receptors (NKR) on **A)** CD8$^+$ and **B)** CD4$^+$ T cells was assessed by flow cytometry on PBMCs from 22 healthy donors (median age = 52, range 25-83). Total CD8$^+$ and CD4$^+$ T cells were stratified into three subsets according to CD27/CD28 expression as shown in Fig. S1A.

Figure S3: scRNA-seq method and quality control.

A) Number of cells per individual (n=12). IL7R+ (n=6, in green) and IL7R- (n=6; in purple). **B)** Number of genes per distribution across the IL7R+ (in green) and IL7R- (in purple) cells. **C)** Number of cells before (light orange) and after (light blue) filtration (i.e. doublet removal and other filtration steps that are described in Methods), within each individual. **D)** Bar plot highlighting the cell abundances across clusters (n=13) for 10X run batches (upper panel) and $IL7R^+$ and $IL7R^-$ groups (lower panel) after BBKNN batch effect correction. **E)** Bar plot highlighting the individual (n=12) cell abundances across clusters (n = 13) after BBKNN batch effect correction. Each color represents an individual. **F)** Number of cells in each cluster. **G)** Violin plot showing the IL7R expression (as defined by scRNA-seq) across the 13 clusters.

Figure S4: scRNA-seq comparison of re-clustered T cells.

A) Dotplot showing the genes that are modulated in naïve (top genes in red) and Temras (top genes in green) compartments. The scores (y-axis) were defined using the Scanpy function (sc.tl.rank_genes_groups), based on Wilcoxon statistical test. FC= Fold change. Naïve (C0, C4 and C8) and T_{EMRA} (C1, C2 and C6) compartments were extracted, a second round of clustering on the selected clusters (n = 39,634) was performed (as in Fig. 3) and UMAP plots highlighting **B)** *IL7R* groups (*IL7R*$^+$ in green, *IL7R*$^-$ in purple, as defined by flow sorting) and **C)** of representative genes are shown.

Figure S5: TCR-independent cytotoxicity and DAP10 expression in $CD8^+$ T cell subsets.

Calcein-release cytotoxicity assays were performed on $CD8^+$ T cell subsets defined by CD27/CD28 expression and NK cells isolated by FACS. **A)** Titration curve of varying effector to target (E:T) ratios using $CD27^-CD28^-CD8^+$ T cells (red triangles) and NK cells (black circles). Non-linear regression (5-parameter asymmetric) was performed (n = 3, mean ± sd). **B)** Isolated $CD8^+$ subsets and NK cells were used at a fixed E:T ratio of 20:1. Cytotoxicity was assessed over a period of six hours (n = 3 per group mean ± sd). **C)** Expression of DAP10 on human NK cells, and $CD8^+$ T cell subsets defined by CD27 and CD28. Mean fluorescence intensity is shown (n = 4 for T cell, n = 3 NK cells). **D)** Sestrin 2 expression was determined by flow cytometry on total $CD3^+CD8^+$ T cells from young (<35 years, n = 5) and old (>65 years, n = 4) donors. MFIs are shown. Welch's t test, * $p < 0.05$.

Figure S6: Antigen-specific cells generated during vaccination exhibit an NK phenotype.

Data mined from Akondy *et al.* (GSE100745)[45] showing the relative fold-change (log_2) of differentially expressed genes of interest in YFV-tetramer$^+$ effector (14 days post-vaccination,

black bars, n = 3) or memory (4-12 years post-vaccination, red bars, n = 5) compared to naïve (n = 6) $CD8^+$ T cells.

Figure S7: Characterization of immune cell subsets and in murine delayed type hypersensitivity model.

A) Graphical representation of the study design for a mouse model of mBSA driven delayed type hypersensitivity. **B)** A time course of paw size (normalized to the contralateral, PBS control paw) following DTH challenge (time = 0 h) and **C)** the overall response assessed as the integration of the time course data (n=4-10 per group). **D)** Spleens were weighed immediately after harvest. **E)** Polychromatic flow cytometry was used to identify $CD44^-CD62L^+$ naïve (grey), $CD44^+CD62L^+$ central (blue), and $CD44^+CD62L^-$ effector (red) cells. **F)** Quantification of these cell types as a proportion of total splenic $CD8^+$ T cells (n = 3 per group). The legend shown underneath C-D**)** is applicable to all panels in this figure. One-way ANOVA with Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure S8: $CD8^+$ T cell subsets and NKR expression in a murine DTH model.

Polychromatic flow cytometry was performed on mouse splenocytes following an mBSA recall challenge. **A)** Expression of NKRs on total splenic $CD8^+$ T cells (n = 3 per group). One-way ANOVA with Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$.

Gene Symbol	Name	Description	Fold Change CM/N	EM/N	SEN/N	P value
NK cell receptors						
NCAM1	CD56	Neural cell adhesion molecule, NK cell marker	1.62	3.9	**42.07**	**0.000013**
FCGR3A /// FCGR3B *	CD16	Receptor for the Fc region of IgG; Antibody-dependent cellular cytotoxicity	-1.99	1.3	**26.31**	**1.73E-07**
B3GAT1	CD57	Marker of replicative senescence	1.05	2.5	8.86	0.000002
CD244 (2B4)	CD244 (2B4)	SLAM receptor; costimulation and inhibition in NK cells; co-inhibition in T cells	2.57	1.59	**23.64**	**0.000003**
CD226 (DNAM-1)	DNAM-1	Regulation of cytotoxicity in both NK and T cells	3.79	5.89	3.96	0.002281
KLRD1	CD94	C-type lectin NK cell receptor, dimerizes with NKG2 family of receptors	2.96	4.66	7.31	7.41E-07
KLRC1 /// KLRC2 *	NKG2A///NKG2C*	NKG2A inhibitory and NKG2C activating receptor, both recognize HLA-E	2.82	4.32	5.47	0.000027
KLRC3	NKG2E	Activating receptor	-1.5	1.34	7.82	0.000712
KLRC4	NKG2F	Activating receptor	-1.14	1.16	2.15	0.011495
*KLRC4-KLRK1 /// KLRK1**	NKG2D*	Activating receptor, regulation cytotoxocity and cytokine secretion	-1.32	-1.59	1.02	0.860846
KLRG1	KLRG1	Inhibitory receptor; marker of terminal differentiation	4.91	2.27	4.56	0.000391
KLRB1	CD161	Marker of Th17 phenotype; highly expressed on MAIT cells	6.06	3.94	5.63	0.002317
KLRF1	Nkp80	Activating receptor; marker of functional maturity in both NK and CD8 T cells	1.15	1.65	**39.13**	**0.000016**
NCR1 (NKp46)	NKp46	Natural Cytotoxicity Receptor	-1.15	1.29	2.25	2.79E-07
NCR2 (NKp44)	NKp44	Natural Cytotoxicity Receptor	1.16	-1.16	2.2	0.01141
NCR3 (NKp30)	NKp30	Natural Cytotoxicity Receptor	2.97	2.05	1.88	0.041509
KIR2DL1-3	KIR2DL	Killer cell Immunoglobulin-like receptor (inhibitory)	-1.18	2.26	**23.78**	**0.000001**
KIR2DS 1-5	KIR2DS	Killer cell Immunoglobulin-like receptor (stimulatory)	-1.1	2.05	**18.01**	**9.79E-07**
KIR3DL1-3	KIR3DL	Killer cell Immunoglobulin-like receptor (inhibitory)	1.15	2.48	**18.2**	**6.47E-07**
KIR3DS1-3	KIR3DS	Killer cell Immunoglobulin-like receptor (stimulatory)	-1.23	1.75	**16.19**	**0.000198**
Costimulatory and homing receptors						
CD27	CD27	Costimulatory Receptor	-1.93	-7.85	**-26.53**	**0.000001**
CD28	CD28	Costimulatory Receptor	-1.05	-1.26	**-15.48**	**0.000004**
CD274	PD-L1	T cell exhaustion	1.33	2.02	-1.95	0.01804
CCR7	CCR7	Secondary lymphoid organ homing receptor	-2.77	-2.59	-3.88	0.000568
CD69	CD69	Activation marker	1.06	-1.24	-1.17	0.271801
SELL	CD62	Adhesion molecule, migration to secondary lymphoid organs	-2.21	-3.71	-4.65	0.000024
T cell signaling transduction						
TCR-alpha	TCR-alpha	TCR complex	-1.05	-2.32	**-8.43**	**0.005636**
CD3D, CD3E, CD3G	CD3 complex	CD3 complex	1.02	1	-1.29	0.002293
LCK	LCK	Lymphocyte protein tyrosine kinase, TCR signaling	-1.25	-1.96	-1.78	0.000107
LAT	LAT	Lynker for activation of T cells, TCR signaling	-1.56	-1.14	-2.15	0.002927
PLCG1	PLCG1	Phospholipase gamma, TCR signaling	-1.52	-2.55	-2.11	0.000117
ZAP70	ZAP70	Syk-family protein tyrosine kinase, TCR signaling	1	-1.05	1	0.861184
HCST	DAP10	YxxM-Motif adaptor molecule, constitutive expression on CD8 T cells	1.28	1.39	1.55	0.000151
Innate signaling molecules						
TYROBP	DAP12	ITAM containing activating signaling adaptor, recruits Zap70/Syk kinases	1.64	2.59	**20.98**	**0.000004**
SYK	SYK	Syk-family protein tyrosine kinase, analogous of Zap70, BCR signaling	-1.28	1.1	**17.33**	**0.000099**
SH2D1A	SAP	SH2-domain containing molecule, binds SLAM receptors	1.17	-1.05	1.06	0.341073
SH2D1B	EAT-2	SH2-domain containing molecule, binds SLAM receptors	1.01	-1.08	19.2	0.000107
Transcription factors						
EOMES	EOMES	T-box transcription factor, cytotoxic effector differentiation and maturation	1.58	1.61	1.81	0.000789
TBX21	Tbet	T-box transcription factor, cytotoxic effector differentiation and maturation	-1.09	1.26	1.31	0.005497
FOXO1	FOXO1	Survival, proliferation and differentiation of T cells	-1.26	-1.75	-2.47	0.000031
FOXO3 /// FOXO3B	FOXO3	Survival, proliferation and differentiation of T cells	1.18	1.5	1.48	0.001226
FOXO4	FOXO4	Survival, proliferation and differentiation of T cells	-1.08	-1.03	1.43	0.065459
FOXP3	FOXP3	Development and function of regulatory T cells	2.78	2.36	-1.14	0.618522
PRDM1	Blimp-1	T cell effector differentiation and terminal maturation	2.97	3.61	3.16	0.002186
RORC	RORC	Th17 lineage differentiation	9.11	5.85	1.11	0.786225
ZBTB16	PLZF	NKT cell effector differentiation	7.65	4.78	**6.48**	**0.000081**
ZEB2	ZEB2	Cytotoxic T cell terminal differentiation	2.57	5.5	**10.75**	**0.000039**
Cytokine receptors						
IL2RA	IL2R	IL2 receptor	2.65	3.54	2.8	0.021623
IL7R	IL7R	IL7 receptor	1.16	-1.91	-5.06	0.001062
IL12RB1-2	IL12R	IL12 receptor	1.09	-1.01	1.58	0.173214
IL15RA	IL15R	IL15 receptor	1.39	1.6	1.47	0.066267
IL18R1	IL18R1	IL18 receptor	2.76	2.85	3.04	0.000252
IL18RAP	IL18RAP	IL18 receptor acessory protei	8.72	10.51	**11.54**	**0.000092**
Cytotoxicity						
GZMA	Granzyme A	Cyototoxicity	3.83	3.53	4.61	0.000159
GZMB	Granzyme B	Cyototoxicity	1.41	1.61	1.46	0.004983
GZMH	Granzyme H	Cyototoxicity	2.51	4.55	3.93	0.00122
GZMK	Granzyme K	Cyototoxicity	3.59	-1.04	-2.01	0.030512
PRF1	Perforin	Cyototoxicity	2.42	4.05	8.29	0.00001
Proliferation and cell cycle control						
CCND3	Cyclin D3	Cell cycle progression	-3.04	-3.43	-2.99	0.002381
CCNE1	Cyclin E1	Cell cycle progression	-2.09	-2.33	-3.21	0.000071
CDKN1A	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	3.63	3.99	4.52	0.000515
CDKN2A	CDKN2A	Cyclin-dependent kinase inhibitor 2A (p16)	2.48	2.73	2.53	0.000035
CDKN2B	CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	5.11	5.92	4.39	0.000601

Table 1: List of selected genes of interest

Numbers denote mean fold changes in gene expression of central memory (CM, $CD27^+CD45RA^+$), effector memory (EM, $CD27^-CD45RA^-$) and senescent-like (SEN, $CD27^-CD45RA^+$) $CD8^+$ T cell subsets as compared to naïve ($CD27^+CD45RA^+$) $CD8^+$ T cells. The

most significant changes are highlighted in bold. Asterisks (*) indicate the inability of Affymetrix microarrays to distinguish between two genes.

Antibody	Conjugate	Clone	Isotype	Manufacturer	Catalogue #	Dilution
Surface markers						
Human						
CD3	BUV395	UCHT1	Mouse IgG1	BD	563546	1:100
CD3	BV711	UCHT1	Mouse IgG1	BD	563725	1:100
CD4	PerCP/Cy5.5	SK3	Mouse IgG1	Biolegend	344608	1:50
CD4	FITC	RPA-T4	Mouse IgG1	BD	555346	1:100
CD8	BV421	RPA-T8	Mouse IgG1	Biolegend	301036	1:50
CD8	BUV737	SK1	Mouse IgG1	BD	612754	1:100
CD27	BV786	L128	Mouse IgG1	BD	563327	1:50
CD28	BV510	T44	Mouse IgG1	Biolegend	302936	1:50
CD45RA	BV605	HI100	Mouse IgG2b	Biolegend	304134	1:50
CD16	APC	VEP13	Mouse IgG1	Miltenyi Biotec	130-091-246	1:10
CD56	FITC	HCD56	Mouse IgG1	Biolegend	318304	1:20
CD56	BUV395	NCAM16.2	Mouse IgG2b	BD	563554	1:100
CD57	FITC	HNK-1	Mouse IgM	BD	333169	1:20
CD57	PE/Dazzle	HNK-1	Mouse IgM	Biolegend	359620	1:100
KLRG1	PE	2F1	Syrian Hamster	Biolegend	138408	1:100
NKG2A	AF700	131411	Mouse IgG2a	R&D Systems	FAB1059N	1:20
NKG2C	APC	134591	Mouse IgG1	R&D Systems	FAB138A	1:20
NKG2D	PE	149810	Mouse IgG1	R&D Systems	FAB139P	1:20
NKG2D	PE/Cy7	1D11	Mouse IgG1	Biolegend	320812	1:20
NKp30	PE	AF29	Mouse IgG1	Miltenyi Biotec	130-099-706	1:20
NKp44	PE	2.29	Mouse IgG1	Miltenyi Biotec	130-092-480	1:20
CD244 (2B4)	PE	C1.7	Mouse IgG1	Biolegend	329508	1:20
CD161	PE	HP-3G10	Mouse IgG1	Biolegend	339904	1:20
KIR2DL2/3	PE	DX27	Mouse IgG1	Biolegend	312603	1:20
KIR2DL1/S1/S3/S5	PE	LB2	Mouse IgG1	Biolegend	339505	1:20
KIR3DL1 (NKB1)	PE	DX9	Mouse IgG1	Biolegend	312707	1:20
iNKT	APC	6B11	Mouse IgG1	Miltenyi Biotec	130-094-839	1:20
TCR αβ-1	FITC	WT31	Mouse IgG1	BD	347773	1:10
TCR Vα7.2	APC/Cy7	3C10	Mouse IgG1	Biolegend	351714	1:100
Mouse						
CD3	Pacific Blue	17A2	Rat IgG2b	Biolegend	100214	1:100
CD3	BV605	17A2	Rat IgG2b	Biolegend	100237	1:100
CD4	BV510	RM4-5	Rat IgG2a	BD	563106	1:100
CD4	PerCP/Cy5.5	RM4-5	Rat IgG2a	BD	550954	1:100
CD8α	BUV737	53-6.7	Rat IgG2a	BD	564297	1:100
CD8α	APC-H7	53-6.7	Rat IgG2a	BD	560247	1:100
TCRβ	PE/Cy7	H57-597	Armenian Hamster IgG2	BD	560729	1:80
CD44	PE/Cy7	IM7	Rat IgG2b	Biolegend	103030	1:50
CD62L	PE	MEL-14	Rat IgG2a	Biolegend	104408	1:50
NK1.1	AF700	PK136	Mouse IgG2a	BD	560515	1:100
CD1d Pentamer	PE	N/A	N/A	ProImmune	E001-2A-G	1:200
NKp46	BV421	29A1.4	Rat IgG2a	Biolegend	137612	1:80
NKG2A/C/E	APC	20d5	Rat IgG2a	BD	564383	1:50
NKG2D	BV711	CX5	Rat IgG1	BD	563694	1:50
Ly49C/F/I/H	FITC	14B11	Syrian Hamster IgG	Biolegend	108205	1:100
KLRG1	BV605	2F1/KLRG1	Syrian Hamster IgG	Biolegend	138419	1:100
KLRG1	PE/Dazzle 594	2F1/KLRG1	Syrian Hamster IgG	Biolegend	138424	1:100
Intracellular markers						
IL-2	FITC	MQ1-17H12	Mouse IgG1	BD	511408	1:10
IFN-γ	PE/Cy7	B27	Mouse IgG1	BD	557643	1:20
TNF-α	APC	MAb11	Mouse IgG1	BD	340534	1:10
Granzyme B	AF700	GB11	Mouse IgG1	BD	560213	1:10
DAP10	Unconjugated	FL-93	Rabbit IgG	Santa Cruz	sc-25623	1:100
DAP12	PE	406288	Mouse IgG1	R&D Systems	IC5240P	1:10
Sesn1	Unconjugated	EPR1930(2)	Rabbit	Abcam	ab134091	1:100
Sesn2	Unconjugated	Polyclonal	Rabbit	Abcam	ab135597	1:100
Sesn2	Unconjugated	D1B6	Rabbit IgG	Cell Signaling Technologies	8487S	1:100
Anti-rabbit	FITC	Polyclonal	Goat IgG	ThermoFisher	31635	1:1000
Anti-rabbit	AF647	Polyclonal	Goat IgG	ThermoFisher	A-21245	1:1000
PhosphoFlow						
P-CD3ζ (CD247) (pY142)	AF647	K5	Mouse IgG1	BD	558489	1:20
P-Zap70/Syk (pY319/pY352)	PE	17A	Mouse IgG1	BD	557881	1:20
Viability dyes						
Zombie NIR	NIR	N/A	N/A	Biolegend	423106	1:1000
Zombie UV	UV	N/A	N/A	Biolegend	423108	1:1000
Zombie Green	FITC	N/A	N/A	Biolegend	423112	1:1000

Table 2: List of antibodies used in Flow cytometry.

Excel File (Table S1)

Table S1: Complete list of differentially regulated genes in sorted CD8⁺ T cell subsets

Gene Symbol	Name/Alias	Description
NK related		
NKG7	NKG7	Natural killer cell granule protein 7
GNLY	Granulysin	Granulysin, NK-lysin
FCGR3A	CD16	Fcγ Receptor 3a, CD16a
FCRL6	FcR-like 6	Fc Receptor-like protein 6
KLRD1	CD94	Killer cell lectin-like receptor subfamily D, member 1. Dimerises with NKG2 family of receptors
KLRB1	NK1.1, CD161	Killer cell lectin-like receptor subfamily B, member 1. CD161
KLRG1	2F1, CLEC15A	Killer cell lectin-like receptor subfamily G, member 1.
TYROBP	DAP12	Tyro protein kinase-binding protein, DAP12. NKG2D signaling adaptor.
KLRK1	NKG2D, CD314	Killer cell lectin-like receptor subfamily K, member 1. NKG2D. Activating NK receptor.
KLRC1	NKG2A, CD159a	Killer cell lectin-like receptor subfamily C, member 1. NKG2A. Inhibitory NK receptor.
KLRC2	NKG2C, CD159c	Killer cell lectin-like receptor subfamily C, member 2. NKG2C. Activating NK receptor.
KLRF1	NKp80, CLEC5C	Killer cell lectin-like receptor subfamily F, member 1. Activatin coreceptor.
HCST	DAP10	Haematopoietic cell signal transducer. NKG2D signalig adaptor.
NCAM1	CD56	Neural cell adhesion molecule 1. NK marker.
KIR2DL1	CD158a	Killer cell immunoglobulin-like receptor 2DL1. ITIM-linked inhibitory NK receptor.
Senescence related		
SESN1	Sesn1, PA26	Sestrin 1
SESN2	Sesn2, HI95	Sestrin 2
SESN3	Sesn3	Sestrin 3
KLRG1	KLRG1	Killer cell lectin-like receptor subfamily G, member 1.
B3GAT1	CD57	Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase 1.
ATM	ATM	ATM Ser/Thr kinase, Ataxia Telangiectasia Mutated. DNA damage response protein.
ATR	ATR	ATR Ser/Thr kinase, Ataxia Telangiectasia and Rad3-related protein. DNA damage response protein.
RELA	NKFKB3, p65	RELA proto-oncogene, NFKB subunit.
RELB	RelB	RELB proto-oncogene, NFKB subunit.
MTOR	mTOR	Mechanistic Target of Rapamycin.
PRKAA1	AMPKa1	AMP-activated protein kinase, catalytic subunit alpha 1.
MYC	c-Myc	MYC proto-oncogene, BHLH transcription factor.
RAD1	Rad1	RAD1 Checkpoint DNA Exonuclease, cell cycle checkpoint and DNA damage checkpoint protein.
RAD17	Rad17	RAD17 Checkpoint Clamp Loader Component, cell cycle checkpoint protein.
RAD50	Rad50	RAD50 Double Strand Break Repair Protein, DNA damage response protein.
MAPKAPK2	MK-2	Mitogen-ActivatedProtein Kinase-Activated Protein Kinase 2.
MRE11A	MRE11, HNGS1	MRE11 Homologue, double strand break repair nuclease.
TNF	TNFA, TNF-alpha	Tumour Necrosis Factor alpha, pro-inflammatory cytokine and SASP component.
HMGB1	SBP-1	High Mobility Group Box 1.
CDC25A	CDC25a	Cell Division Cycle 25A. Cell cycle protein.
MKI67	Ki-67	Marker of Proliferation, Ki-67.
CCND1	Cyclin D1, BCL1	Cyclin D1, cell cycle protein.
CCNE1	Cyclin E1	Cyclin E1, cell cycle protein.
CDKN2A	p14, p16, INK4A, ARF	Cyclin-dependent Kinase Inhibitor 2A, cell cycle protein and DNA damage checkpoint protein.

Table S2: Gene list for NK and Senescence used to establish Fig.3D scores

Excel File (Table S2)

Table S3: Complete list (Top 100) of differentially regulated genes in clusters generated by scRNA-seq, defined as naïve and T_{EMRA}

631 Excel File (Table S3)

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Materials and Methods

Study design

The study protocol was approved by the Ethical Committee of the Royal Free and University College London Medical School (Research Ethics number: 11/0473). Written informed consent was obtained from all study participants. Donors did not have any co-morbidity, were not on any immunosuppressive drugs, and retained physical mobility and lifestyle independence. For analyses involving the $CD8^+$ T cell, $IL7R^+/IL7R^-$ single cell dataset studies were conducted following approval by the Institutional Review Board (IRB) of the University of Connecticut Health Center (IRB 14-194J-3). After receiving informed consent, blood samples were obtained from 6 healthy old (65+ yr) research volunteers residing in the Greater Hartford, CT, region using services of the University of Connecticut Center on Aging Recruitment and Community Outreach Research Core and following previously published screening criteria (PMID:28904110).

Cell isolation and transfection

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient (Ficoll–Hypaque, Amersham Biosciences, UK) from heparinized blood of healthy donors (n = 22, 26-83 years). Untouched NK and $CD8^+$ T cells were freshly isolated by magnetic activated cell sorting (MACS, Miltenyi Biotec, UK) using a negative selection procedure. For microarray analysis, high-purity $CD8^+$ T cell subsets were sorted on the basis of CD27 and CD45RA expression[18], using a FACSAria (BD Biosciences, UK) flow cytometer. For functional assays, $CD8^+$ T cell subsets were freshly isolated according to CD27/CD28 expression by magnetic activated cell sorting (MACS, Miltenyi Biotec, UK), which identified analogous subsets but provided higher yields of viable cells (> 95% purity) as previously described[6,9]. Double negative cells were obtained by complete negative isolation. We found that <1% of cells within these isolated populations expressed iNKT markers. Mucosal associated invariant T cells

(MAIT) cells express TCR Vα7.2 and these cells constitute ~5% of the peripheral CD8+ T cells pool in humans[64,65]. We found 4% (range 1-7.5%) of these cells in isolated $CD28^+$ $CD27^+$, 3% (range 1-6.4) in the isolated $CD28^-CD28^+$ and 2.9% (range 1-4.9%) in the isolated $CD28^-CD27^-$ $CD8^+$ T cells populations. The results obtained are therefore unlikely to be due to contaminating iNKT or MAIT cells in our $CD8^+$ T cells populations.

Where indicated, freshly purified human $CD8^+$ T cells were transfected with small interfering RNA (siRNA) for NKG2D (Santa Cruz Biotechnology, sc-42948) or DAP12 (sc-35172) by electroporation using the Amaxa Human NK Cell Nucleofector Kit and Nucleofector technology (Lonza), according to the manufacturer's instructions. A scrambled control siRNA (sc-37007; Santa Cruz) was used throughout. Efficiency of siRNA transfection was confirmed by measuring the expression of the protein of interest using flow cytometry, 36-48 hours after transfection.

Microarray data acquisition

Cells purified by FACS were stimulated for 2 hours with 0.5 µg/ml plate-coated anti-CD3 (OKT3) and 5 ng/ml rhIL-2 before RNA isolation using the ARCTURUS PicoPure Isolation Kit (ThermoFisher). The concentration of small quantities of RNA was determined using Nanodrop. Linear amplification of 10 ng of total RNA was performed using the Ovation Biotin RNA amplification and labelling system (NuGEN). Fragmented, labelled cDNA was hybridized to Affymetrix U133 plus 2 arrays.

Single cell RNA sequencing

Sample processing: all samples were processed within one hour from venipuncture.

Cell Sorting: PBMCs were isolated from fresh whole blood using Ficoll-Paque Plus (GE) density gradient centrifugation. For cell sorting, we used fluorochrome-labeled antibodies specific for CD3 (UCHT1), CD27 (M-T271) (Biolegend), CD4 (RPA-T4), CD19 (HIB19), IgD (IA6-2), CD127 (HIL-7R-M21) (BD Biosciences), and CD8 (SCF121Thy2D3) (Beckman-

Coulter). $CD8^+IL7R^+$ ($CD8^+CD127^+$) and $CD8^+IL7R^-$ ($CD8^+CD127^-$) T cells were sorted from the $CD19^-CD3^+CD4^-$ fraction. Cell sorting was performed using FACSAria Fusion (BD).

Blood preparation for single cell RNA sequencing (scRNA-seq): PBMCs were thawed quickly at 37°C and transferred to DMEM supplemented with 10% FBS. Cells were spun down at 400 g, for 10 min. Cells were washed once with 1 x PBS supplemented with 0.04% BSA and finally re-suspended in 1 x PBS with 0.04% BSA. Viability was determined using trypan blue staining and measured on a Countess FLII and samples with <80% viability were discarded. 12,000 cells were loaded for capture onto the Chromium System using the v2 single cell reagent kit (10X Genomics). Following capture and lysis, cDNA was synthesized and amplified (12 cycles) as per manufacturer's protocol (10X Genomics). The amplified cDNA was used to construct an Illumina sequencing library and sequenced on a single lane of a HiSeq 4000.

Single cell Raw data processing and data combination: Illumina basecall files (*.bcl) were converted to fastqs using cellranger v2.1.0, which uses bcl2fastq v2.17.1.14. FASTQ files were then aligned to hg19 genome and transcriptome using the cellranger v2.1.0 pipeline, which generates a gene - cell expression matrix. The samples were merged together using cellranger aggr from cellranger, which aggregates outputs from multiple runs, normalizing them to the same sequencing depth (normalize=mapped) and then re-computing the gene-barcode matrices and analysis on the combined data (See scripts here: https://github.com/dnehar/Temra-IL7R-Senescence).

Scrublet for multiplet prediction and removal: Generally, we expected about 2 to 8% of the cells to be hybrid transcriptomes or multiplets, occurring when two or more cells are captured within the same microfluidic droplet and are tagged with the same barcode. Such artifactual multiplets can confound downstream analyses. We applied Scrublet[36] python package to remove the putative multiplets. Scrublet assigns each measured transcriptome a 'multiplet score', which indicates the probability of being a hybrid transcriptome. Multiplet scores were determined for each individual (using the raw data), and 0.7% - 10.7% highest

scoring cells were tagged as multiplets after visual inspection of doublet score distributions and excluded from the further analysis.

Single cell processing, clustering and cell type classification: The aggregated matrices were fed into the Python-based ScanPy[66] workflow (https://scanpy.readthedocs.io/en/stable/), which includes preprocessing, visualization, clustering and differential expression testing. The pipeline we used was inspired by The Seurat[67] R package workflow.

Quality control and cell-filtering: We applied the following filtering parameters: (i) all genes that were not detected in ≥ 3 cells were discarded, (ii) cells with less than 400 total unique transcripts were removed prior to downstream analysis, (iii) cells in which > 20% of the transcripts mapped to the mitochondrial genes were filtered out, as this can be a marker of poor-quality cells and (iv) cells displaying a unique gene counts > 2,500 genes were considered outliers and discarded.

Data normalization: After discarding unwanted cells from the dataset, we normalized the data. Library-size normalization was performed based on gene expression for each barcode by scaling the total number of transcripts per cell to 10,000. We log-transformed the data and then regressed out using the total number of genes and the fraction of mitochondrial transcript content per cell. 1202 highly variable genes (HVG) were identified using filter_genes_dispersion scanpy function and used to perform the principal component analysis (PCA).

Linear dimensional reduction using PCA and graph-based clustering: Dimensionality reduction was carried out in SCANPY via principal component analysis followed by Louvain clustering UMAP visualization using the top 40 significant components (PCs).

Finding marker genes/evaluation of cluster identity: To annotate the cell type of each single cell transcriptome, we used both differential expression analysis between clusters and classification based on putative marker gene expression. We applied the 'tl.rank_genes_groups' scanpy function to perform differential analyses, comparing each

cluster to the rest of the cell using Wilcoxon test (Supplementary Table 3). We only considerate clusters that showed a distinct trancriptomic programs.

Batch effect correction: We performed a batch (10X genomics batch) correction using BBKNN (https://github.com/Teichlab/bbknn). More details about the parameters used can be found as a Jupyter notebook here: https://github.com/dnehar/Temra-IL7R-Senescence.

NK and Senescence scores:

Gene lists (Table S3) were used to score NK or senescence expression in naïve and Temras CD8 T cells. To do so, we calculated the mean expression for each cell, within each cluster using the h5ad object (adata), as follow:

```
adata.obs['NK_score] = adata.X[:,NK_markers].mean(1).
```

The scores were then plotted, as shown in Fig .3D.

Lentiviral transduction

Sestrin knockdown in human $CD8^+$ T cells was achieved using a lentiviral transduction system as described previously[9].

The pHIV1-SIREN-GFP system used for knockdown of gene expression possesses a U6-shRNA cassette to drive shRNA expression and a GFP reporter gene that is controlled by a PGK promoter5. The following siRNA sequences were used for gene knockdowns: CCTAAGGTTAAGTCGCCCTCG (shCTRL), CCAGGACCAATGGTAGACAAA (shSesn1), CCGAAGAATGTACAACCTCTT (shSesn2) and CAGTTCTCTAGTGTCAAAGTT (shSesn3). VSV-g pseudotyped lentiviral particles were produced, concentrated and titrated in HEK293 cells as described[9].

Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 µg/ml gentamicin, 2 mM L-glutamine (all from Invitrogen) and 0.5 ng/ml anti-mycoplasma (Bio-Rad) at 37 °C in a humidified 5% CO_2 incubator. Purified human highly differentiated $CD28^-CD8^+$ T cells were activated in the

presence of plate-bound anti-CD3 (purified OKT3, 0.5 µg/ml) plus rhIL-2 (R&D Systems, 10 ng/ml), and then transduced with pHIV1-Siren lentiviral particles (multiplicity of infection (MOI) = 10) 72 h after activation.

Flow cytometry and Phospho-flow

Multi-parameter flow cytometry was used for phenotypic and functional analyses of PBMC. For analysis of surface markers, staining was performed at 4°C for 30 min in the presence of saturating concentrations of antibodies (listed in **Table 2**) and a live/dead fixable Near-Infrared stain (Thermo Scientific, L10119). For intracellular analysis of cytokine secretion, cytotoxic granule expression, and sestrin 1, sestrin 2, DAP12, and DAP10 expression, cells were fixed and permeabilized with the Fix & Perm® Kit (Invitrogen, Life Technologies, UK), before incubation with indicated antibodies or the respective isotype controls. For imaging cytometry, samples were acquired on an Amnis ImageStreamX Mk2 using INSPIRE software, magnification 60X. Data were analysed using IDEAS v6.2 software (*Amnis*). Co-localization of signals was determined on a single cell basis using bright detail similarity (BDS) score analysis. Co-localization was considered with BDS ≥ 2.0.

For Phospho-Flow cytometry, after staining for surface markers, $CD8^+$ T cells were stimulated with anti-CD3 (purified OKT3, 10 µg/mL) for 30 minutes on ice, followed by crosslinking with goat anti–mouse IgG antibody during 30 minutes on ice. Cells were then transferred to an incubator at 37°C, and stimulation was terminated after 10 minutes, with immediate fixation with Cytofix Buffer (PBS containing 4% paraformaldehyde, BD Biosciences) followed by permeabilization with ice-cold Perm Buffer III (PBS containing 90% methanol, BD Biosciences) and staining with antibodies for phospho-proteins (listed in **Table 2**) for 30 minutes at room temperature. Samples were acquired on a LSR II flow cytometer (BD Biosciences) and analysed using FlowJo software (TreeStar).

Cytotoxic assays - CD107a degranulation assay

Freshly isolated NK and $CD8^+$ T cell subsets were incubated at 37°C for 6 h with K562 or C1R-MICA/C1R cells, at a fixed effector to target (E:T) ratio of 2:1, in the presence of APC-conjugated CD107a antibody (BD Biosciences), as previously described[68]. Brefeldin A (1µg/ml; Sigma-Aldrich) and Monensin (1 µg/ml; Sigma-Aldrich) were added in the final 5h-incubation period. Effector cells incubated alone in the presence phorbol-12-myristate-13-acetate (PMA, 50 ng/ml, Sigma-Aldrich) with ionomycin, (250 ng/ml, Sigma-Aldrich) were used as positive control whereas medium alone served as unstimulated (US) control. After incubation, cells were stained for surface markers for 30 min on ice, followed by intracellular detection of cytokines (TNF-α and IFN-γ) and CD107a expression and analysed by flow cytometry.

Cell lines

K562 (human erythroleukemic) cell line was purchased from the European Collection of Cell cultures (ECCAC, UK) and cultured in 25 cm2 flasks (Nunc) in complete RPMI-1640. B-lymphoblastoid cell lines, C1R and C1R transfected with MICA*008 (C1RMICA) were kindly provided by Professor Antoine Toubert (INSERM UMR1160, Paris) and maintained in complete RPMI-1640 in the presence of the aminoglycoside antibiotic G-418 (Sigma, G8168) for selection of transfected cells[40].

Western blotting

Human $CD8^+$ T cell subsets purified using immunomagnetic separation (MACS) according to CD27/CD28 expression were stimulated with anti-CD3 (purified OKT3, 10 µg/mL) or anti-NKG2D (1D11, 10 µg/mL) before lysis. Cells were normalized by equal cell number, harvested and lysed in ice-cold Radio-Immunoprecipitation Assay (RIPA) buffer (Sigma-Aldrich, UK), supplemented with protease and phosphatase inhibitors (GE Healthcare, Amersham, UK), during 30 minutes on ice. Cell lysates were processed for immunoblot analysis as described[6].

Immunoprecipitation

Human $CD8^+$ T cells were separated into $CD28^+/CD28^-$ fractions (to obtain sufficient number of cells for analysis) and stimulated with anti-NKG2D (1D11, 10 μg/mL) or isotype control, for 30 minutes at 4 °C. Lysates from $1x10^7$ cells were prepared with ice-cold HNGT buffer (50 mM HEPES, pH 7.5, 150 mM EDTA, 10 mM sodium pyrophosphate, 100 mM sodium orthovanadate, 100 mM sodium fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride), for 30 minutes on ice. Cell lysates were incubated overnight at 4°C with anti-NKG2D antibody (clone 5C6, Santa Cruz) or control antibody, followed by precipitation with 10 μL of pre-washed protein A/G agarose beads (sc-2003, Santa Cruz) for 3 h at 4°C on a rotary shaker. After extensive washing in HGNT buffer, supernatants were recovered and processed for immunoblot analysis, as described above. Co-Immunoprecipitated proteins were detected after incubation with primary antibodies followed by incubation with mouse anti-rabbit IgG (conformation-specific antibody; L27A9; Cell Signaling) or mouse anti-rabbit IgG light chain (L57A3; Cell Signaling) and by a secondary anti-mouse IgG antibody (7076; Cell Signaling).

Animal housing

$Sesn1^{-/-}$ and $Sesn2^{-/-}$ mice were described previously[9]. The mouse ageing study was performed at the University of Michigan, where the animal procedures were approved by the Institutional Animal Care & Use Committee and overseen by the Unit for Laboratory Animal Medicine. All mice were rested for at least 10 days before being used for *in vivo* studies. Animals were housed under standard conditions, maintained in a 12 h/12 h light/dark cycle at 22 ^oC ± 1 ^oC and given food and tap water *ad libitum* in accordance with United Kingdom Home Office regulations (PPL-P69E3D849) and the NIH guideline.

Murine delayed type hypersensitivity model

Knockout and age-matched (18-month-old) C57BL66J WT control mice were imported from the University of Michigan. Young (~6 weeks) WT mice were purchased separately from *Charles River*. All mice were male. The methylated BSA (mBSA) delayed type hypersensitivity

model was performed as described previously[69]. Mice were sensitized at the base of the tail with a 50 µl injection of mBSA in Freund's complete adjuvant (20 mg/ml solution of mBSA in saline emulsified with an equal volume of Freund's adjuvant containing 4 mg/ml *Mycobacterium tuberculosis* H37Ra, *Sigma Aldrich*). An immune response was evoked 14 days later by subplantar challenge with 50 µl of mBSA in saline (1 mg/ml). The contralateral paw received a saline-only injection and served as a control. The immune response is reported as the difference in paw swelling between left and right paws as determined using callipers (POCO2, Kroeplin). Mice were sacrificed 7 days post-challenge, according to Schedule 1, using an increasing concentration of CO_2. Death was confirmed by cervical dislocation. Spleens and inguinal lymph nodes were obtained, weighed and dispersed through a 70 µm followed by a 35 µm sterile cell sieve (*Becton Dickinson*) to yield single cell suspensions. Cell numbers were enumerated by haemocytometer and up to 10^6 cells were used for polychromatic flow cytometry.

In vivo cytotoxicity

24-month-old knockout ($Sesn1^{-/-}Sesn2^{-/-}Sesn3^{+/+}$) males were imported from the University of Michigan. Age-matched wild type female C57Bl/6J mice were purchased from Envigo. Natural killer cells were depleted by intraperitoneal injection of 100 µg anti-NK1.1 antibody (PK136, BioXCell) 24 hours before cell challenge. The high Rae-1 expressing myeloma cell line 5TGM was labeled with 5 µM CFSE, while splenocytes stained with 0.5 µM were used as Rae-1^- controls. Both cell types were mixed at equal ratios and $2x10^7$ were co-injected i.v. Mice were left for 6 hours before being sacrificed. As a measure of Rae-1 directed killing, the ratio of $CFSE^{hi}$ compared to $CFSE^{lo}$ was used to determine Rae-1^+ cell retrieval and specific lysis.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.00. Tests were used to determine data distribution and depending on the normality of the data, comparisons were performed using the Student *t* test (for two groups, parametric) or the non-parametric Mann–

Whitney U test (for two groups, unpaired) and the Wilcoxon signed rank test (for two groups, paired) with two-tailed *P* values unless otherwise stated. When comparing more than two groups, we used one-way ANOVA (parametric, > 2 groups, unpaired), repeated measures ANOVA (parametric, > 2 groups, paired), Kruskal–Wallis (non-parametric, > 2 groups, unpaired) or Friedman (non-parametric, > 2 groups, paired) tests with post-correction for multiple comparisons, as appropriate. The two-way ANOVA test was used to compare the effects of two independent variables between groups. Linear regression analysis was performed to generate lines of best fit and correlations between variables were analysed using Pearson´s or Spearman's rank correlation coefficient (r). Differences were considered significant when p <0.05 (*), p <0.01 (**), p <0.001(***) and p <0.0001 (****). Data are presented as means ± standard error of the mean (SEM) unless otherwise stated.

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Author contributions: B.I.P., L.P.C and R.P.H.D. designed and performed the experiments, analysed the data and wrote the manuscript. DNB designed and analysed the single cell RNA-seq data under the supervision of JB and DU. RM performed all the experiments with the healthy older adult subjects. GAK recruited all the healthy older adult donors subjects in Farmington. CT. A.L., E.S.C. and N.E.R. designed and performed experiments. S.W. and J.S. designed and performed *in vivo* cytotoxicity studies. S.M.H. and A.N.A. designed and performed the microarray studies. N.A.M., V.H.T. and S.M.J. analysed the microarray and RNA-seq data. D.C.O.G., D.W.G., J.H.L. and M.K.M. facilitated mouse experiments. D.E. designed and provided the lentiviral vectors. A.N.A. designed the experiments and reviewed and edited the manuscript and organized the collaborative infrastructure.

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