Revised manuscript

CMV-specific T-cell responses at older ages: broad responses with a large central memory component may be key to long-term survival

Short title: Ageing, CMV-specific T-cells, and long-term survival

Martha Bajwa[1,*], Serena Vita[4,*], Rosanna Vescovini[5], Martin Larsen[6,7], Paolo Sansoni[5], Nadia Terrazzini[8], Stefano Caserta[2], David Thomas[1], Kevin A. Davies[1], Helen Smith[3], and Florian Kern[1]

[1]Department of Clinical and Experimental Medicine, [2]Department of Global Health and Infection, [3]Department of Primary Care and Public Health, Brighton and Sussex Medical School, Brighton, United Kingdom;

[4]Institute Pasteur, Cenci-Bolognetti Foundation, Department of Public Health and Infectious Diseases, University Sapienza of Rome, Rome, Italy.

[5]Dipartimento di Clinica Sperimentale, Università di Parma, Parma, Italy.

[6]Inserm UMR-S1135, Centre d'Immunologie et des Maladies Infectieuses (CIMI-Paris), Paris, France.

[7]AP-HP, Groupement Hospitalier Pitié-Salpêtrière, Département d'Immunologie, Paris, France.

[8]School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton, United Kingdom;

Footnotes:

Conflict of interest statement:

MB: No COI; SV: No COI; RV: No COI; ML: No COI; PS: No COI; NT: No COI; SC: No COI; DT: No COI; KD: No COI; HS: No COI; FK: FK is a named owner/inventor on a

patent describing protein-spanning peptide pools for T-cell stimulation (EP1257290 B1). Reagents covered by this patent were used in this study.

Funding statement:

This work was funded by the Dunhill Medical Trust, UK (Grant Nr. R107/0209)

Meeting presentations:

None

Corresponding author information:

Prof. F. Kern
Brighton and Sussex Medical School
Medical Research Building
University of Sussex
Biology Road
Brighton, BN1 9PS
Tel.: +44 1273 877671
E-mail: f.kern@bsms.ac.uk

Abstract

Cytomegalovirus (CMV) infection sometimes causes large expansions of CMV-specific T-cells, particularly in older people. This is believed to undermine immunity to other pathogens and to accelerate immunosenescence. While multiple different CMV proteins are recognized, most publications on age-related T-cell expansions have focused on dominant target proteins, UL83 or UL123, and the T-cell activation marker, IFN-γ. We were concerned that this narrow approach might have skewed our understanding of CMV-specific immunity at older ages. We have, therefore, widened the scope of analysis to include *in vitro*-induced T-cell responses to 19 frequently recognized CMV proteins in young and older healthy volunteers and a group of oldest old, long-term survivors (>85 years of age). Polychromatic flow-cytometry was used to analyze T-cell activation markers (CD107, CD154, IL-2, TNF, IFN-γ) and memory phenotype (CD27, CD45RA). The older had on average larger T-cell responses than the young, but, interestingly, response size differences were relatively smaller when all activation markers were considered rather than IFN-γ or TNF alone. The oldest old recognized more proteins on average than the other groups and had even bigger T-cell responses than the older with a significantly larger central memory CD4 T-cell component. (191 words)

Introduction

T-cells have a central role in containing virus infections and T-cell immunity to CMV has been repeatedly characterized [1-4]. As aged T-cells die, the thymus gland replenishes the T-cell pool with fresh, naïve cells, but thymic output is reduced by 90% in 20 year-olds, and by 99% in 70 year-olds, compared to newborns [5]. However, T-cell numbers do not decline significantly as we age because memory T-cell proliferation compensates the lack of fresh naïve cells.

Cytomegalovirus (CMV) infection is uniquely effective in driving compensatory memory T-cell proliferation; other herpes viruses have been implicated in this process to a much lesser degree [6, 7]. Frequent reactivation of latent CMV is thought to repetitively stimulate the T-cell compartment, driving up its size over time [8]. Very large CMV-specific T-cell responses observed in older people have created the paradigm of CMV-induced T-cell 'memory inflation' [9]. Some researchers suspect that these T-cell expansions undermine immune responsiveness by skewing the T-cell receptor repertoire towards CMV [2, 10, 11]. However, the studies exploring the relationship between immunosenescence and the size of the CMV-specific T-cell response in humans to date have considered a limited number of recognized target proteins. By focusing on specific proteins or even individual epitopes and single functions (e.g. IFN-γ) they have created a fragmented picture of CMV specific T-cell immunity in older life [10-16].

We intended to assess whether CMV infection leads to a large increase of CMV-specific T-cells in older people by measuring T-cell responsiveness to a wider, more representative range of proteins than previously studied and also including several functional response read-outs. We used protein-spanning peptide pools for CMV antigen-specific stimulation [17-23]. Target protein selection was based on our previous work showing that the full size of the response to CMV (represented by 213 protein-spanning peptide pools) can be extrapolated from the responses to 19 proteins, including 6 dominant CD4 and 15 dominant CD8 T-cell targets [24].

The increase of T-cell response size was smaller in the CD8 but larger in the CD4 compartment in older compared to young participants than previously reported. In addition, our analysis of response breadth and T-cell memory compartments across all

protein-specific responses provides new insight into the changes that occur in older people and a potential signature of successful ageing.

Methods

Ethics statement

This study was approved by the UK National Research Ethics Service (NRES) (09/H1102/84) and the University of Parma Ethics Committee. Written informed consent was obtained from all participants. The study was conducted in agreement with the Declaration of Helsinki.

Participants

Fifty-five healthy volunteers referred to as 'young' (19-35 years, including university students and staff) and 131 healthy volunteers referred to as 'older' (60-85 years, recruited by general medical practitioners) were recruited in Brighton (UK). Twenty-two additional individuals, referred to as 'oldest old' (85-102 years) with known T-cell responsiveness to CMV were recruited at Parma University Hospital (Italy) as a comparison group of particularly advanced age. Exclusion criteria were designed to select generally healthy young and older individuals but in the oldest old the presence of cerebrovascular and/or cardiovascular disease (heart failure, TIA, AMI) was accepted, as this is representative of such an advanced age. Details of all inclusion/exclusion criteria and demographics for CMV- participants are provided in the online supplement. Demographics for CMV+ participants are shown in **Table 1**. Venous blood was collected in sodium-heparin plasma tubes (BD, Oxford, UK). Only CMV+ individuals (52.6% of the older and 47.3% of the young participants) were selected for the analysis of CMV-specific T-cell responsiveness. The proportion of Non-White among young participants ranged from 20-33%, depending on the analysis. There were no statistically significant differences between White and Non-White British participants with respect to response size distribution.

CMV serology

CMV IgG serology (Architect CMV IgG, Abbot, Maidenhead, UK) was performed at the Brighton and Sussex University Hospital Trust (BSUHT) virology laboratory.

Peripheral blood mononuclear cell (PBMC) Isolation and activation

PBMCs were isolated by density gradient centrifugation (Ficoll-Hypaque, PLUS Healthcare, Buckinghamshire, UK) as described previously [25]. PBMCs were resuspended at $5x10^6$ cells/mL in complete RPMI (Fisher Scientific, Loughborough, UK) containing 10% fetal calf serum (Fisher). For each tube 200 µL of PBMC suspension was incubated with peptide pools dissolved in DMSO (Sigma-Aldrich, Gillingham, UK), DMSO alone as a negative control, or Staphylococcus enteroxin B (SEB, Sigma) as a positive control, and Monensin (BD) for 2 hours in a standard incubator (37°C, humidified 5% CO_2 atmosphere) before addition of BFA (Sigma) for the remaining incubation time of 14 hours. More details are provided in the online supplement.

CMV Peptide Pools

Peptides (15 amino acids length, 11 amino acids overlap between adjacent peptides) spanning the entire amino acid sequence of 19 CMV proteins were prepared by solid-phase synthesis using the same protein sequences as previously published [24]. Quality control included mass spectroscopy and HPLC. Peptide purity was generally >80%. One peptide pool per protein was generated ('Pepmix', JPT Peptide Technologies, Berlin, Germany) save for UL48, for which two pools were required. Pools were arranged in 16 stimulation pools, of which 12 contained one protein (frequent responses) and 4 contained 2 proteins each (as they elicited less frequent responses) **(Table 2)**. Freeze-dried pools were stored at -80°C.

Antibodies and cell staining

We used the following fluorescence-conjugated monoclonal antibodies; anti-CD3-v500, anti-CD8-Allophyocyanine(APC)-H7, anti-CD27-Phycoerythrine(PE), IL-2-Fluoresceine-iso-thio-cyanate(FITC), TNF-α- Alexa 700, CD107a-APC (all BD Biosciences, Oxford, UK), anti-CD4-Peridinin chlorophyll(PerCP), anti-IFN-γ PE-Cy7(Cyanine 7), anti-CD154 Pacific-Blue (BioLegend, Cambridge, UK), anti-CD45RA-ECD (Beckman Coulter, UK) and Yellow live-dead stain (Invitrogen, Paisley, UK). Cells were stained on the surface and intracellularly as described previously [25] (see online supplement for details).

Data analysis and gating strategy

FlowJo-v9.x software (TreeStar Inc., Ashland, USA) was used for analysis. After identifying CD4 and CD8 T-cells, individual gates were set on activation marker-positive events (**Supplementary Fig. S1**) and then combined using FlowJo's Boolean gate function. All subset frequencies were computed based on the frequencies of individual non-overlapping Boolean subsets after background subtraction. Responses were considered positive if they were identifiable by at least one activation marker, formed a visible cluster on inspection, and included $\geq$ 0.01% (1/10,000 T-cells) of the reference population. The analysis of target protein recognition profiles and total CMV-specific responses *excluded* participants whose responses had not been tested with the complete set of 19 peptide-pools (e.g. for lack of sufficient material).

Absolute T-cell counts

Absolute T-cell counts (cells/nL of whole blood) were determined in most, but not all, UK participants. They were computed by multiplying the percentage of CD3 T-cells among white blood cells with the white blood cell count (wbc) obtained with a Sysmex Counter (Sysmex, UK) (see online supplement for details).

T-cell polyfunctionality

The polyfunctionality index was calculated as previously described [26] (see online supplement for details).

Statistical analysis

SPSS v22 software (IBM, London, UK) was used for statistical analyses. The Chi-square test was used to compare protein recognition between cohorts. Histograms, Q/Q plots, and the Kolmogorov-Smirnov test were used to determine data distribution. Non-parametric tests (Mann-Whitney) were used to compare groups. T-cell frequencies were log-transformed where appropriate for normalizing distribution or improving data presentation. P-values ≤0.05 were considered significant for single endpoints. Multiple end-point correction (Bonferroni) was applied when appropriate ($p \leq 0.05/n$, where n is the number of endpoints).

Results

T-cell response size is unrelated to protein recognition frequency

Size and phenotype of T-cell responses to 19 CMV proteins were analyzed in young, older, and oldest old participants (**Table 1**). Activated T-cells were quantified using five simultaneous read-outs, IL-2, IFN-γ, TNF, CD107a and CD154. As previously reported, the average size of the T-cell response to a given CMV protein was unrelated to the proportion of individuals recognizing it (**Fig. 1A-B)** [24]**.**

CMV-specific T-cell response breadth is increased in the oldest old

The frequencies of T-cells recognizing specific target proteins were not significantly different between young and older participants, however, significant differences existed between the older and oldest old with respect to several proteins (**Fig. 2A**). The number of target proteins recognized per individual seemed somewhat bigger in the older compared to the young participants but this was not statistically significant. However, the oldest old had significantly broader responses than the older participants (**Fig. 2B**). The oldest old were considered examples of exceptional ageing and compared only with the older whose age was within normal expectation (direct comparisons between the oldest old and the young did not appear useful).

The median frequency of CMV-specific TNF-producing CD4 T-cells is 4.9 times higher in older than young participants

We initially compared responses between young and older participants as this was considered to reflect average ageing. As a global measure of T-cell responsiveness to CMV, without bias to selected proteins, we first compared the summed response to the 19 proteins ('total response') among CD4 and CD8 T-cells and then the responses to the two most frequently recognized proteins for CD4 (UL83, UL55) and CD8 T-cells (UL83, UL123) **(Fig. 3)**. Response size comparisons were based on the combined readout (cells were considered activated if at least one activation marker was positive), IFN-γ alone (the most commonly used T-cell activation marker) or TNF alone. The difference of the total CD4 T-cell response between the young and the older (**Fig. 3A**) was statistically significant only when IFN-γ or TNF were considered alone, but not

when the combined read-out was used. In older participants, the median of the total CD4 T-cell response was 3.2, 4.5, and 4.9-fold higher than in the young group for the combined read-out, IFN-γ, and TNF, respectively. UL83-specific responses were significantly larger in the older group for each of the read-outs; unlike UL55-specific responses, which were not significantly different for any read-out. In CD8 T-cells a similar pattern was observed but increases were generally smaller (**Fig. 3B**). Medians for the total CD8 T-cell response were 2.1, 2.3, and 2.3-fold higher in the older than in the young group for the combined read-out, IFN-γ, and TNF, respectively. CD8 T-cell responses to UL83 were also significantly larger in older compared with young participants (any read-out), but no significant difference was observed with respect to UL123 ('IE-1').

Interestingly, total response size differences (all 19 proteins) between the oldest old and the older (aged 85-103) were significant for all read-outs for both CD4 and CD8 T-cells **(Fig. 3A-B, left).** This might be explained in part by the higher average number of proteins recognized in the oldest old (**Fig. 2B**). For both CD4 and CD8 T-cells, UL83-specific responses were also significantly different between these groups for the combined read-out and IFN-γ, but not TNF. The UL55-specific CD4 T cell response was significantly higher in the oldest old group using the combined read out. No direct comparison between oldest old and young participants was made.

Absolute T-cell counts may conceal the increasing CMV bias of the T-cell repertoire in the older

The corresponding response size differences in terms of absolute T-cell counts (cells/nL of blood) between young and older participants were less conspicuous and statistically significant only for UL83-specific CD4 T-cells (**Supplementary Fig. S2A-B**). At the same time a general decline of CD4 and, particularly, CD8 T-cell numbers (statistically significant) was observed in the older group **(Supplementary Fig. S2C-D)**. Absolute T-cell counts, therefore, underestimated age-related increases in CMV-specific response dominance that were, however, revealed by the CMV-responsive fractions of CD4 or CD8 T-cells **(Fig. 3A-B)**. Absolute T-cell counts were not available for the oldest old.

CMV-specific CD4 T-cells arise predominantly from the T_{EM} compartment in young and older but from the T_{CM} compartment in the oldest old

The distributions of CMV-specific CD4 and CD8 T-cells among the memory compartments defined by CD45RA and CD27 expression were evaluated in all individuals (CD45RA+/CD27+ = 'naïve' or T_{NA}; CD45RA-/CD27+ = 'central memory' or T_{CM}; CD45RA-/CD27- = 'effector memory' or T_{EM}; CD45RA+/CD27- = 'revertant' or T_{EMRA}) (**Supplementary Fig. S3A**). This distribution changes subject to age and CMV-status (**Supplementary Fig. S3B**) [27], and in CMV+ individuals is also related to the size of CMV-specific T-cell responses [28]. The quantitative contribution of these compartments to the total CMV-specific T-cell response was determined across all 19 target proteins. In young and older participants, the largest proportion of the CD4 T-cell response arose from the T_{EM} compartment (**Fig. 3C**), whereas in CD8 T-cells an equally large or even larger contribution originated from the T_{EMRA} compartment (particularly in the older) (**Fig. 3D**). Surprisingly, in the oldest old, among CMV-specific CD4 T-cells, the T_{CM} compartment was dominant (**Fig. 3C**). Note that differences between the sizes of corresponding memory compartments in different age groups in **Fig. 3C-D** (for example the CD4 T_{CM} compartment in the older versus the oldest old participants) reflect the overall response size differences between these age groups and show to what extent these differences are located in each memory compartment. However, relative changes of the contribution that each memory compartments makes to the whole response (i.e. all four compartments together) are more easily appreciated when frequencies are normalized, in which case a significant increase is visible for oldest old versus older participants in the CD4 T_{CM} compartment and a significant decrease in older compared with young participants in the CD8 T_{NA} compartment **(Supplementary Fig. S4A)**. T-cell memory compartment distributions were also expressed in absolute counts (limited to young and older participants) showing a very similar pattern as when expressed as fractions of CD4 or CD8 T-cells (**supplementary Fig. S4B**).

The entire CD4 and CD8 T-cell memory compartments (irrespective of antigen specificity) also showed larger central memory components in the oldest old than the older but differences were not significant. The most striking difference compared with

CMV-specific T-cells alone was the much smaller relative size of the T_{EMRA} compartment. In older participants, the T_{EMRA} compartment dominated the CD8 T-cell repertoire whereas in the oldest old the T_{EM} compartment was dominant (**Supplementary Fig. S4C, top and bottom)**.

We finally tested T-cell polyfunctionality across the memory compartments; it was generally highest in the T_{EM} compartment in CD4 and CD8 T-cells in all three groups, however, in the oldest old, despite a general decline of polyfunctionality, the CD8 T_{NA} compartment was more polyfunctional than in older participants (**Supplementary Fig. 5).**

Discussion

Our study explored whether the CMV-specific T-cell response is generally inflated in older people. It provides a more definitive answer than previous work, which has focused on select antigens, individual peptides/MHC-multimers, and often single effector read-outs. While CMV-specific T-cell responses were on average larger in older than in young people, our data provides compelling evidence that the size of such differences depends strongly on how the comparison is made; be it with respect to individual proteins, or a range of proteins, be it with respect to single activation markers, or a combination of activation markers. Response size differences were more pronounced when the analysis was focused on single effector read-outs (IFN-γ, TNF), but less striking when all read-outs were considered simultaneously. This demonstrates that differences in functional profiles between individuals, or groups of individuals, may appear as differences in response size if single activation markers are used as read-out. While 2.1-fold and 3.2-fold higher median frequencies of CMV-specific CD8 and CD4 T-cells, respectively, in older compared to young people (considering all T-cell targets and read-outs) clearly show a considerable age-related response size increase, it remains unclear if this is enough to significantly undermine immunity in older people. An increase of CMV-specific pro-inflammatory T-cells, however, might have a more profound effect on the immune system. When considering TNF-producing T-cells only, the difference between young and older was 'only' 2.3 fold for CD8 T-cells but, surprisingly, 4.9 fold for CD4 T-cells (a similar pattern was seen for IFN-γ producing T-cells). It appears, therefore, that the effect of ageing (within normal bounds) on CMV-specific T-cell numbers has been somewhat overestimated with regard to the CD8 but underestimated with respect to the CD4 compartment. In any case, our work has clarified that a huge increase in TNF-producing CMV-specific T-cells does indeed occur in the average CMV+ older person.

Pourgheysari et al. previously reported significant expansions of CMV-specific CD4 T-cells in older people, however, using a CMV lysate for stimulation. Based on TNF production they found a little more than a doubling in older compared to younger people, which is less than half the difference found in the present study. This discrepancy could be explained, first, by the fact that the 'young' people examined by

Pourgheysari were up to 50 years old compared with up to 35 years in our study, and second, that CMV lysate (made from CMV-infected fibroblasts) does not stimulate T-cells as effectively as protein-spanning peptide pools [24].

The oldest old represented a group of exceptional, successfully aged people. They recognized more proteins on average than the older participants (see Fig. 2B) and their summed responses to all proteins were much larger, irrespective of read-out. Future research will determine whether increased response breadth contributes to successful ageing, is a by-product of it, or possibly the result of lifestyle factors contributing to longevity. Interestingly, the role of UL83 as an unusual protein in regards to driving CMV-specific T-cell expansions was confirmed by the observation of an even larger difference in response size between the oldest old and older than between the older and the young. Whether very large UL83-specific T-cell responses are harmful, helpful, or maybe neither, remains unclear. Unlike the young and older, who were predominantly White British, the oldest old were White Italian. Both population samples belong to the same major ethnicity (Caucasoid), however, the frequencies of some HLA alleles vary between UK and Italian populations according to the online HLA-allele database, www.allelefrequencies.net [29]. It may be that HLA-type or other genetic factors have affected response breadth and/or size somewhat but it is very unlikely that they would explain the full extent of the differences we have observed.

By quantifying the contribution of the different T-cell memory compartments to the overall CMV-specific response in a summative way *across all 19 target proteins*, our study significantly extends previous reports [10, 28, 30]. This comprehensive evaluation demonstrated that both in young and older participants the bulk of the CMV-specific T-cell response arises from the T_{EM} compartment in CD4 T-cells, and to a similar extent from the T_{EM} and T_{EMRA} compartments in CD8 T-cells. In the oldest old 'survivors', however, a large contribution to the CD8 T-cell response size and the largest contribution to the CD4 T-cell response size originated from the T_{CM} compartment. This raises the question, does an increase of this compartment occur as a result of successful ageing or is it a survival advantage during the process of ageing? The latter

would support the idea that a long-lived T_{CM} pool provides improved protection from infection as a result of its ability to proliferate upon antigen re-exposure [31]. Interestingly, it was recently shown that the live attenuated VZV vaccine, Zostavax, boosts polyfunctional central memory CD4 T-cells in individuals aged 55-65 [32]. It is, therefore, tempting to speculate that expansion of the T_{CM} compartment both in terms of CMV-specific T-cells, but also generally, reflects natural boosting by exposure to real infections. However, this observation and potential consequences for vaccine design would need to be assessed in future studies.

Importantly, the definition of T-cell memory compartments by CD45RA versus CD27 expression is not precise, e.g. T-cells in the naïve compartment would not be expected to produce IFN-γ after overnight stimulation, indicating a more advanced phenotype. Nonetheless these, and similar subset definitions based on two markers (e.g. CD45RA and CCR7) provide good overall subset discrimination and are widely used in the field [28, 33]. Interestingly, stem cell memory T-cells (T_{SCM}) are antigen-experienced and, like naïve cells, express CD45RA and CD27. It is possible, therefore, that the oldest old, have accumulated CMV-specific T_{SCM} [34] potentially contributing to protection.

Using the same cohorts, we recently reported that polyfunctionality was on the whole reduced in the oldest old [35], however, we did not examine differences between T-cell memory subsets. Our current analysis confirmed that polyfunctionality in the oldest old is generally lower than in older individuals but also showed a slight increase of polyfunctionality in CD8 T_{NA} cells. This agrees with a recent report by others showing increased polyfunctionality among CD8 T_{NA} (but, interestingly, not T_{SCM}) cells in an older compared to a younger participant group [36]. Age-wise, this older group was in between our older and oldest old groups.

Importantly, our present work shows that age-related expansions of the CMV-specific T-cell response can only be fully appreciated if a representative range of proteins and several functional read-outs are considered in combination, allowing an assessment of response breadth both in regards to target proteins and functionality. We also previously demonstrated striking differences between individuals regarding CMV protein dominance and response hierarchies [37], providing additional reason to use many

target proteins in parallel for this kind of work. In conclusion, our current and previous findings combined suggest that a possible ‘signature’ of successful ageing might include a broad CMV-specific T-cell response with a large central memory component but overall moderate polyfunctionality (thus avoiding unnecessary ‘collateral’ tissue damage) [35]. We believe that our work will be useful in informing the design of future studies in this field.

Acknowledgements

The National Institutes of Health Research (NIHR) kindly assisted us with participant recruitment through the Primary Care Research Network (PCRN).

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492
493

Figure Legends

Fig. 1. The frequency of target protein recognition is unrelated to T-cell response size. PBMC from CMV+ participants were stimulated overnight with 19 CMV protein-derived overlapping peptide-pools. Activated T cells were identified by flow-cytometry. **(A)** Bars represent all age groups and indicate the fraction of individuals recognizing individual proteins with respect to CD4 and CD8 T-cells. Proteins are ordered by decreasing frequency of recognition. **(B)** The sizes of CD4 and CD8 T-cell responses (Log10 transformed fractions) across all age groups are shown for all proteins in the same order as under (A).

Fig. 2. The breadth of the CMV-specific T-cell response is not significantly different between young and older participants but strongly increased in the oldest old. PBMC from CMV+ participants were stimulated over night with 19 CMV protein-derived overlapping peptide-pools. Activated T cells were identified by flow-cytometry. **(A)** A comparison of response breadth between the young (white bars) and older (grey bars) individuals revealed no significant differences in terms of protein recognition frequencies (CMV proteins are ordered by decreasing frequency of recognition in the older group), however, there were several significant differences between the older and the oldest old (dark grey bars) (Bonferroni multiple end-point correction, significance threshold set to p=0.003, significant differences indicated by asterisks). (**B)** The number of recognized CMV target proteins (between 1 and 15) was computed separately for CD4 and CD8 T-cells in the young (left) and older (middle), and oldest old (right), suggesting a mild (non-significant) trend for higher response counts in older compared to young participants, but showing a significant difference between older and oldest old. Cross-bars show median and interquartile range.

Fig. 3. Age-related increases in T-cell response size depend on target protein-specificity and functional response read-out. PBMC from CMV+ participants were stimulated over night with 19 CMV protein-derived overlapping peptide-pools. Activated T cells were identified by flow-cytometry. While our study focused on 'average' ageing,

525 i.e. differences between young and older participants, oldest old participants are shown
526 as examples of unusually succesful ageing. **(A, B)** The fractions of all cells displaying at
527 least one activation marker ('combined read out'), IFN-γ, or TNF are shown. Diagrams
528 show the CMV-specific T-cell response size (log-transformed fractions of CD4 or CD8
529 T-cells) for all 19 proteins combined (left panels) and the most frequently recognized
530 CMV proteins in the UK cohort **(A)** for CD4 T-cells with respect to UL83 (middle) and
531 UL55 (right), **(B)** for CD8 T-cells with respect to UL83 (middle) and UL123 (right).
532 Statistical significance levels are indicated. The main study end-point was the increase
533 in CMV-specific T-cell response size between young and older people (combined read-
534 out in connection with all 19 proteins); the significance level of $p \leq 0.05$ was not adjusted.
535 **(C,D)** T-cell memory compartment distributions defined by the expression of CD27 and
536 CD45RA (CD45RA+/CD27+ = 'naïve' or T_{NA}; CD45RA-/CD27+ = 'central memory' or
537 T_{CM}; CD45RA-/CD27- = 'effector memory' or T_{EM}; CD45RA+/CD27- = 'revertant' or
538 T_{EMRA}) showed significant differences between young and older participants among
539 CMV-specific T_{CM} CD4 T-cells and CD8 revertant (T_{EMRA}) T-cells. Compared with older
540 participants, the oldest old displayed a striking and significant increase of the CD4
541 central memory (T_{CM}) compartment (Mann-Whitney test, significance threshold set to at
542 $p \leq 0.0125$, Bonferroni correction for 4 end-points). No direct comparison was made
543 between the young and oldest old participants. Boxplots show minimum, maximum,
544 median, interquartile range, and outliers ("o").
545

546 **Tables**

547

548 **Table 1. CMV+ participant demographics**

Parameter	**'young'**	**'older'**	**'oldest old'**
Total n	26	69	22
Age range (mean ± STD)	19 – 35 (23.3±4.2)	60 – 85 (69.0±7.5)	85-103 (95.9±5.9)
Females	18 (69 %)	35 (51 %)	16 (73%)
Males	8 (31 %)	34 (49 %)	6 (27%)
White (British or Italian)	18 (69%)	69 (100 %)	22 (100%)
Non-White British[a]	8 (31%)	0 (0%)	n.a.

549 [a]Non-white British young adults included 1 Syrian, 2 Indian, 1 Sri-Lankan,
550 1Bangladeshi, 1 Malaysian, 1 White/Asian and 1 Black African/Asian participants.
551

552

553

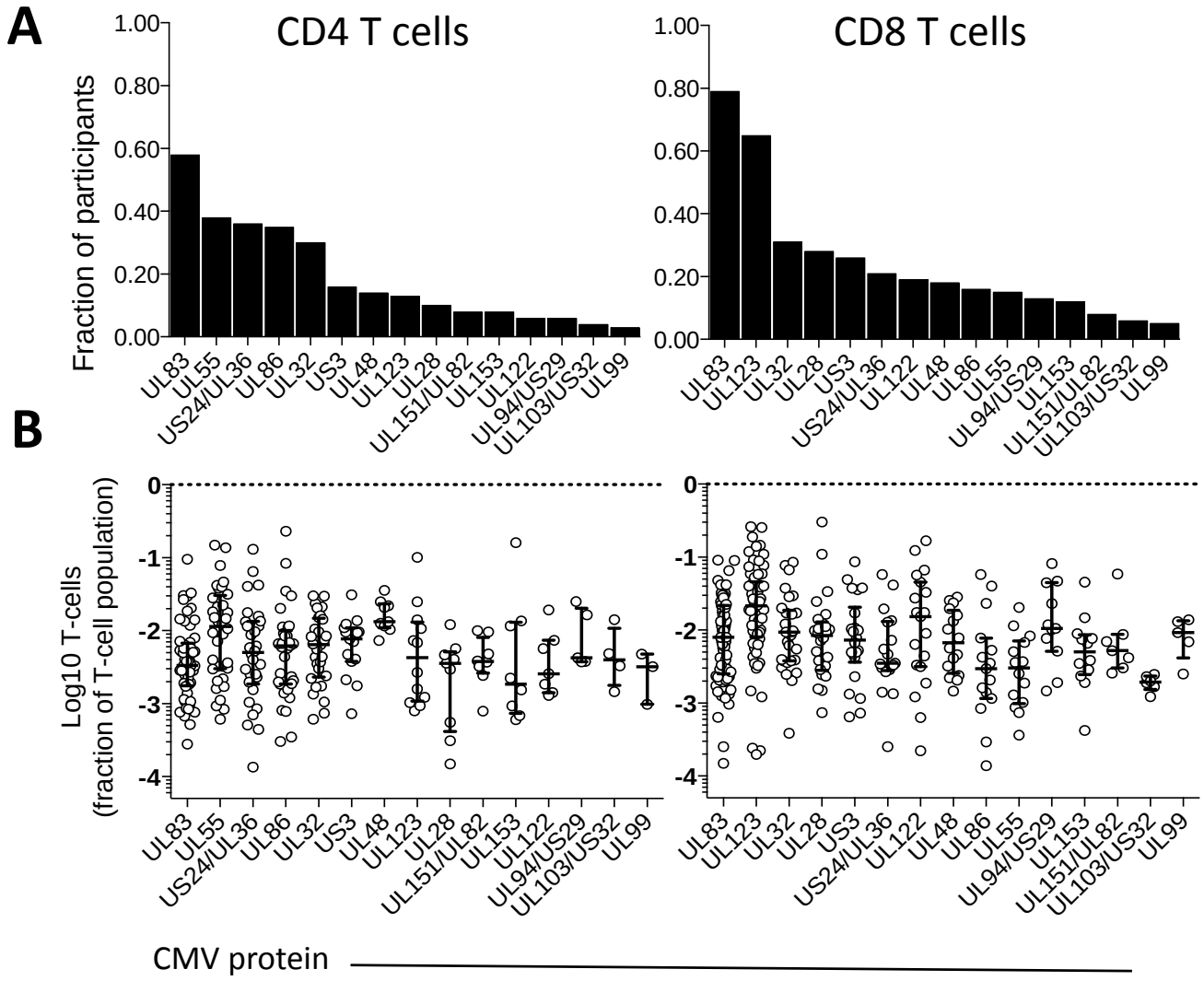
554

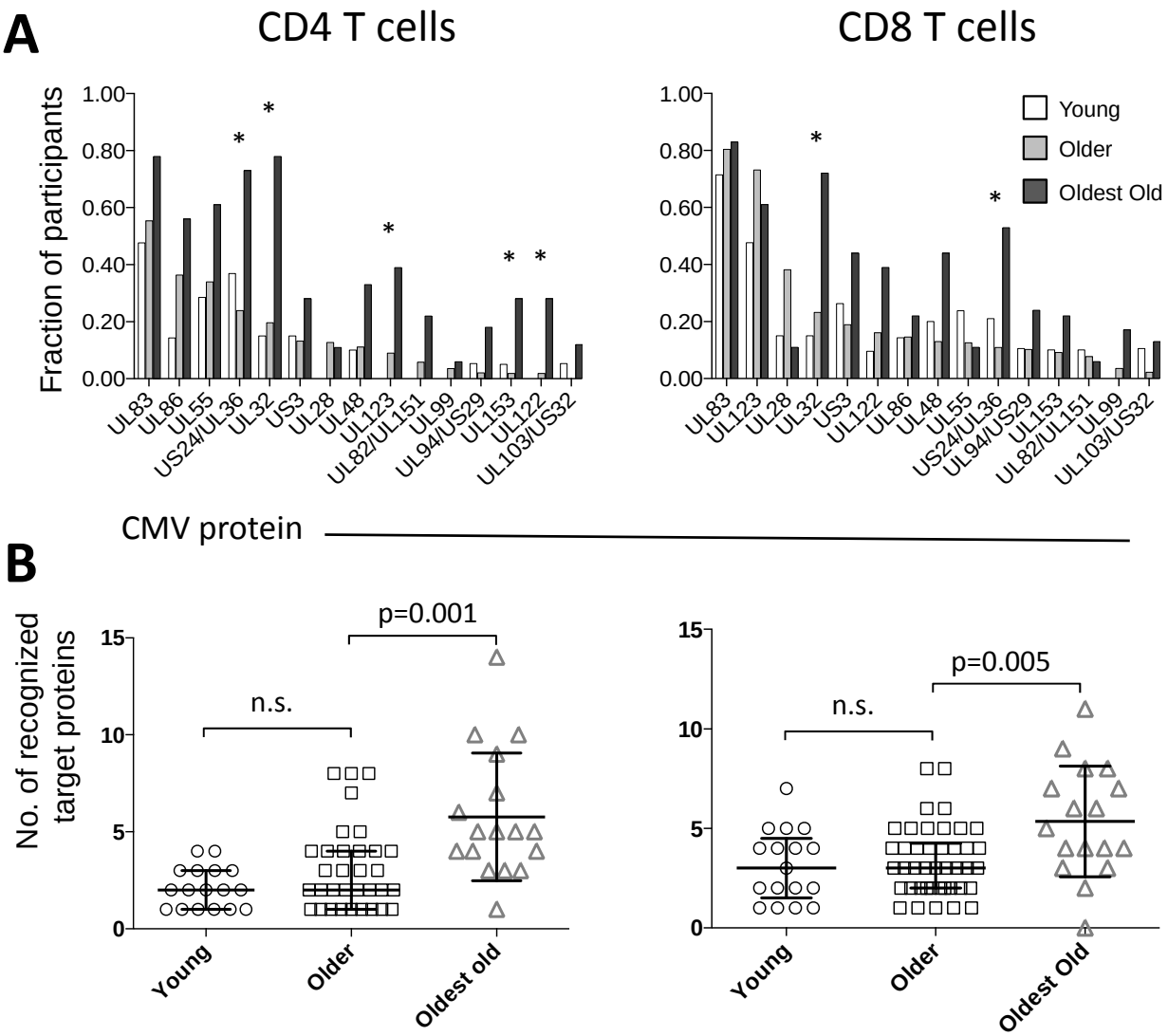
555 **Table 2: CMV peptide-pools used for stimulation**

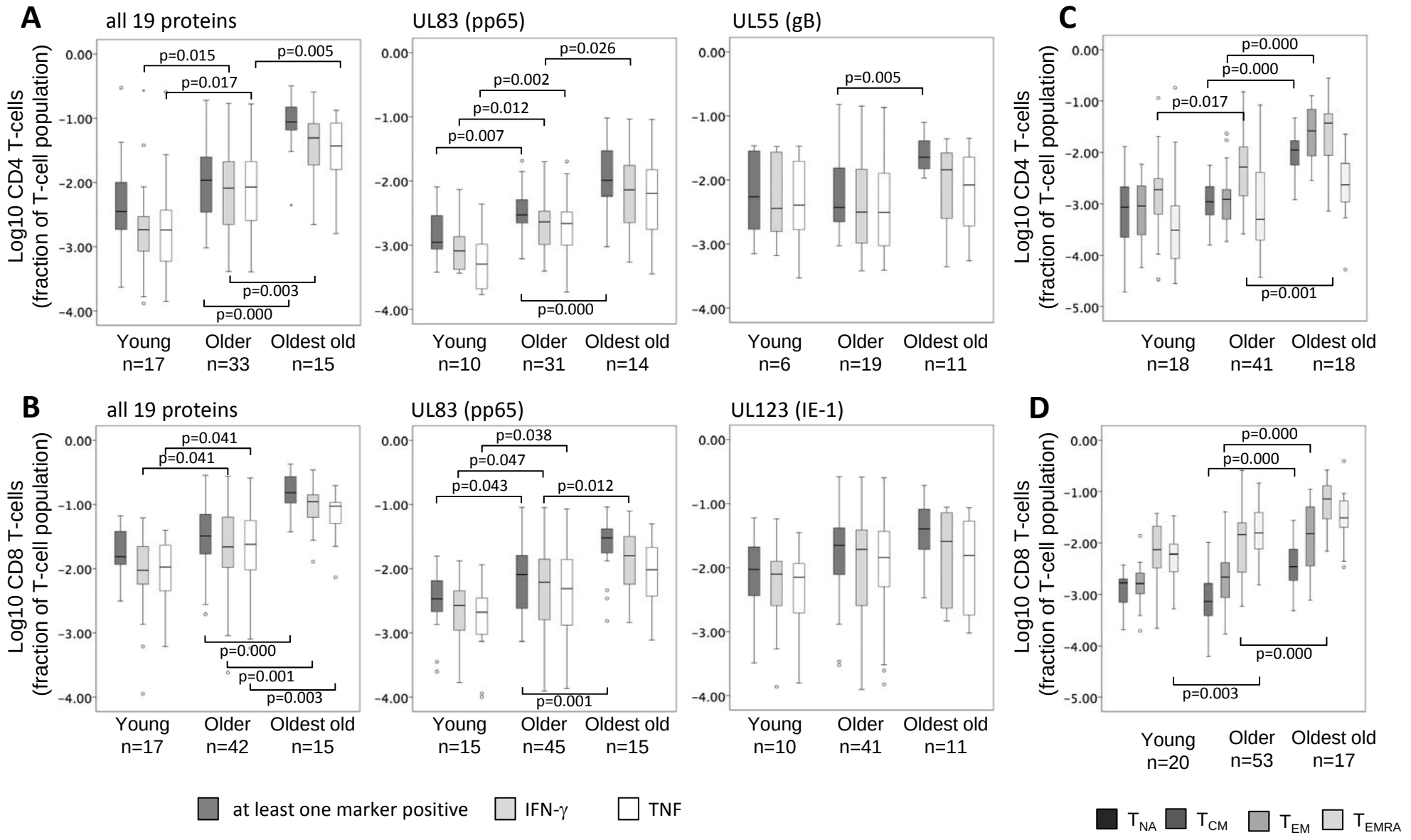
Protein(s)	No. of Peptides
UL55	224
UL83	138
UL86	340
UL122	120
UL123	143
UL99	45
UL153	67
UL32	260
UL28	92
UL48A[a]	281
UL48B[a]	281
US3	44
UL151& UL82	219 (82 &137)
UL94 & US29	197 (84 &113)
UL103 & US32	103 (60 & 43)
US24 & UL36	240 (123 &117)

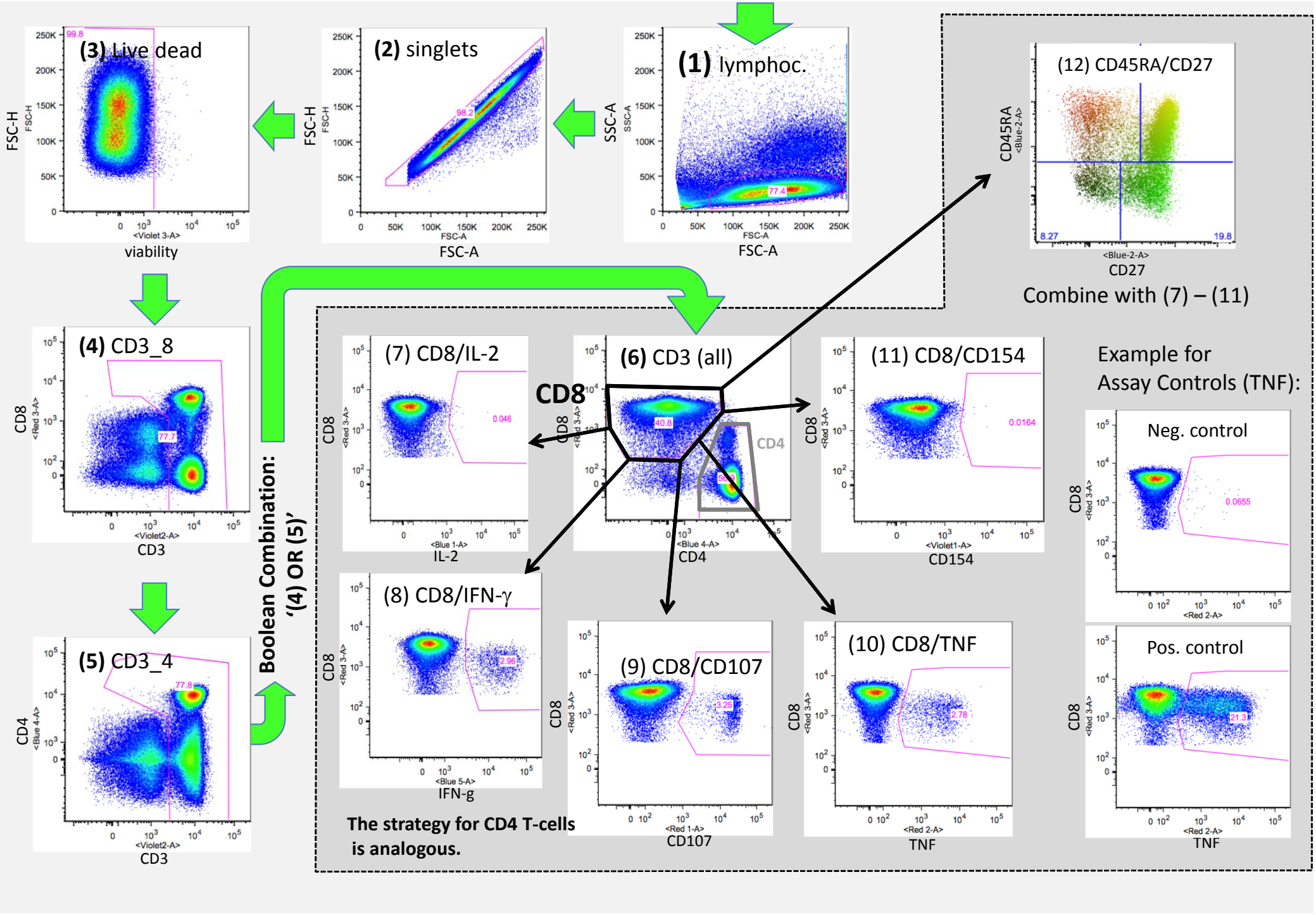
556 [a] UL48 was divided into two pools (UL48A and UL48B), however, results were combined

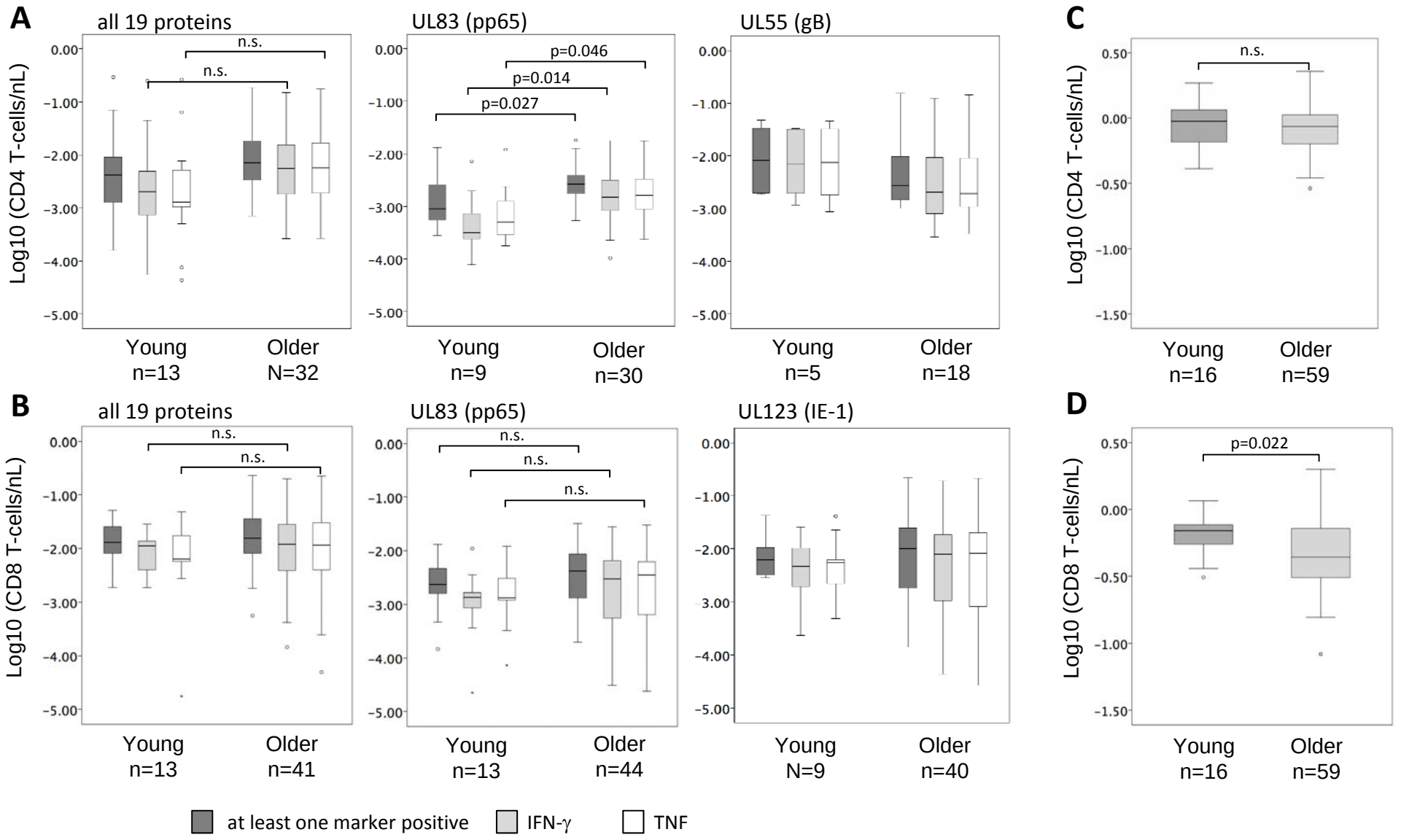
557

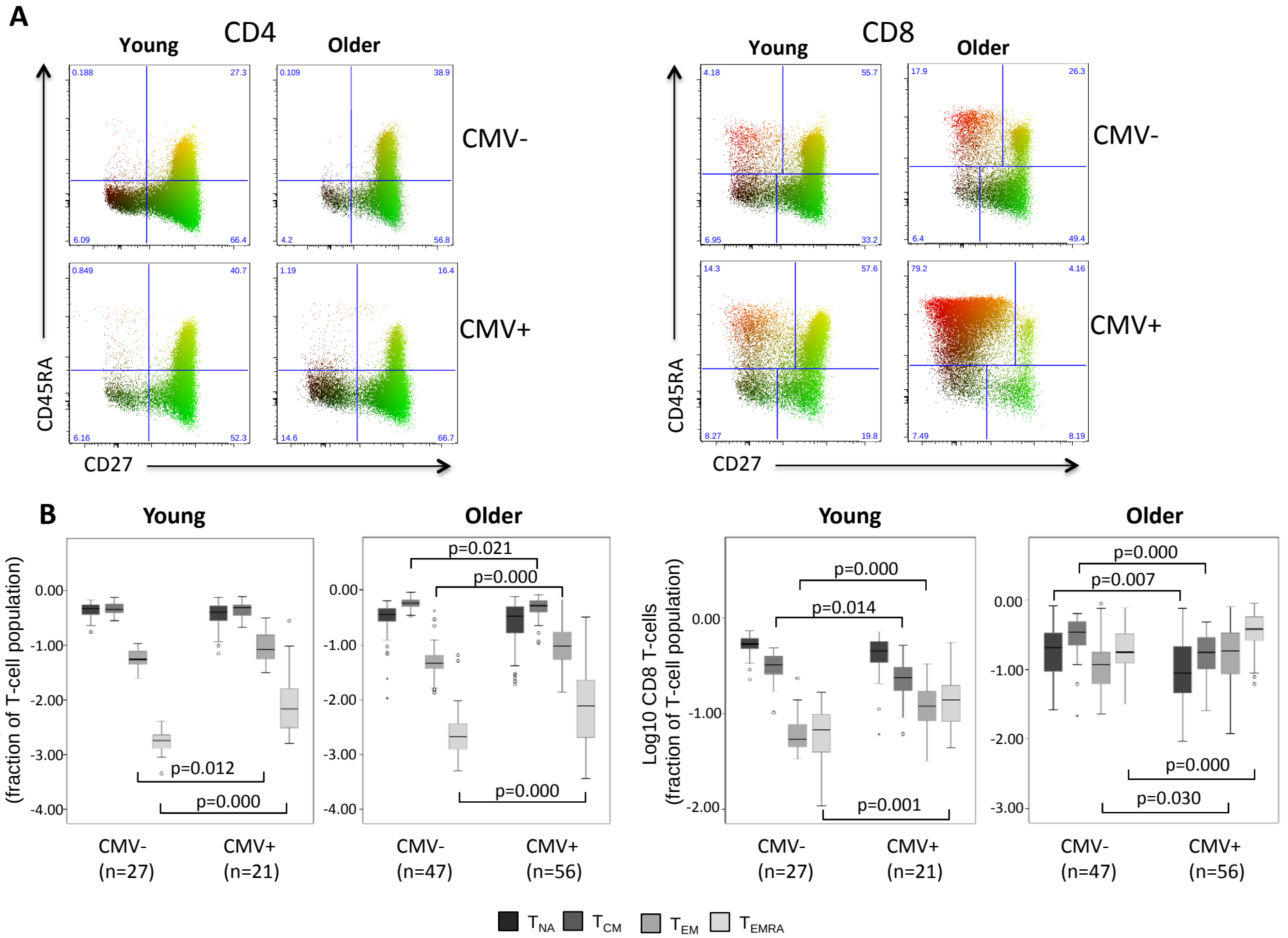

A
CD4 T cells
CD8 T cells
Fraction of participants
1.00
0.80
0.60
0.40
0.20
0.00
UL83
UL55
US24/UL36
UL86
UL32
US3
UL48
UL123
UL28
UL151/UL82
UL153
UL122
UL94/US29
UL103/US32
UL99
B
Log10 T-cells
(fraction of T-cell population)
0
-1
-2
-3
-4
CMV protein

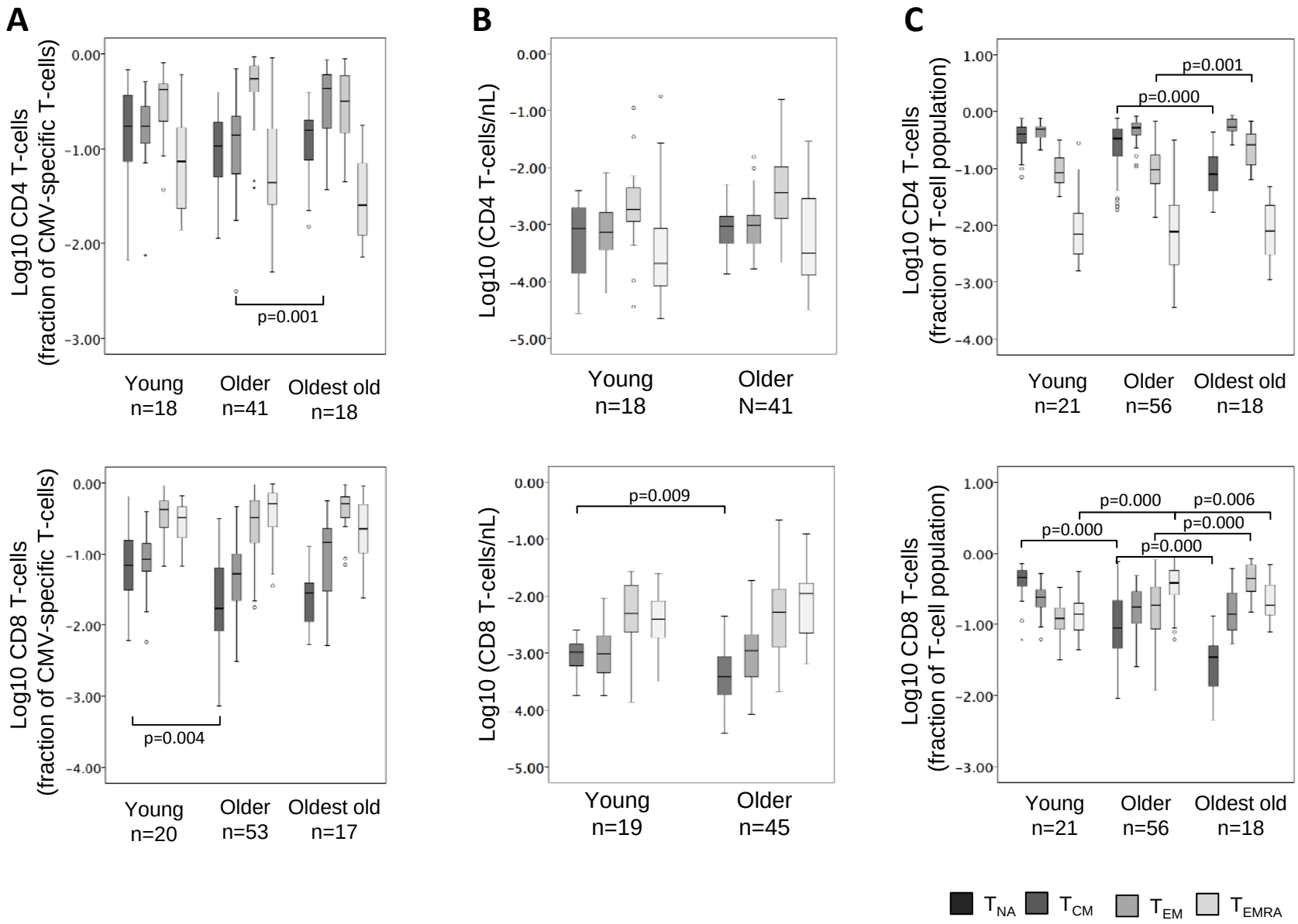

A
CD4 T cells
CD8 T cells
Fraction of participants
1.00
0.80
0.60
0.40
0.20
0.00
Young
Older
Oldest Old
UL83
UL86
UL55
US24/UL36
UL32
US3
UL28
UL48
UL123
UL82/UL151
UL99
UL94/US29
UL153
UL122
UL103/US32
UL83
UL123
UL28
UL32
US3
UL122
UL86
UL48
UL55
US24/UL36
UL94/US29
UL153
UL82/UL151
UL99
UL103/US32
CMV protein
B
No. of recognized target proteins
15
10
5
0
p=0.001
n.s.
p=0.005
n.s.
Young
Older
Oldest old
Oldest Old

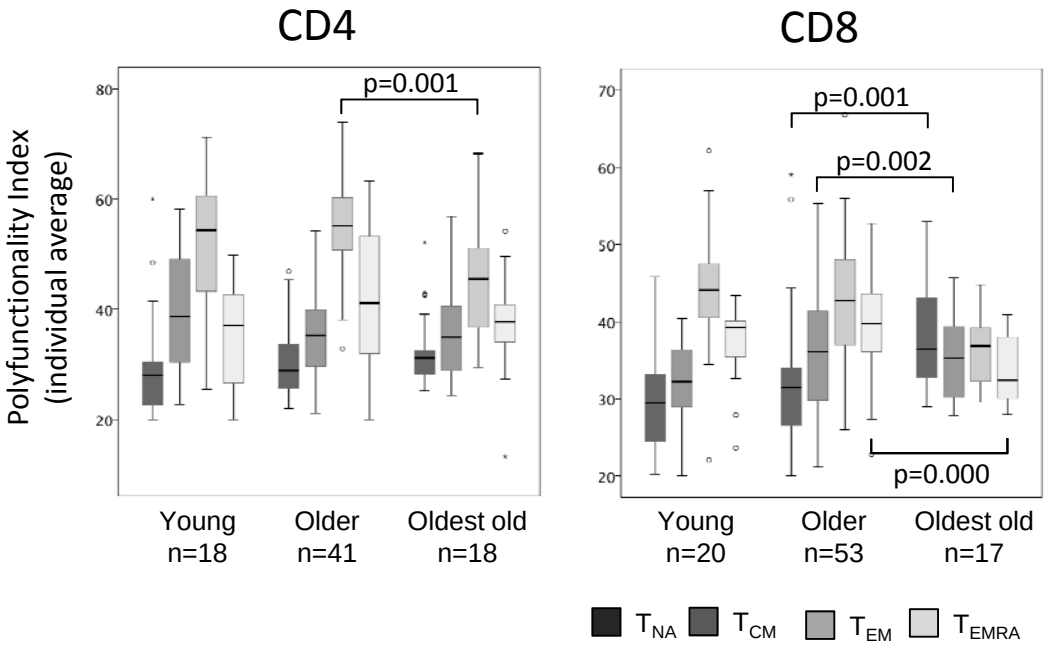

A
all 19 proteins
UL83 (pp65)
UL55 (gB)
Log10 CD4 T-cells
(fraction of T-cell population)
p=0.015
p=0.017
p=0.005
p=0.003
p=0.000
p=0.026
p=0.002
p=0.012
p=0.007
p=0.000
p=0.005
Young
n=17
Older
n=33
Oldest old
n=15
Young
n=10
Older
n=31
Oldest old
n=14
Young
n=6
Older
n=19
Oldest old
n=11
C
Log10 CD4 T-cells
(fraction of T-cell population)
p=0.000
p=0.000
p=0.017
p=0.001
Young
n=18
Older
n=41
Oldest old
n=18
B
all 19 proteins
UL83 (pp65)
UL123 (IE-1)
Log10 CD8 T-cells
(fraction of T-cell population)
p=0.041
p=0.041
p=0.000
p=0.001
p=0.003
p=0.038
p=0.047
p=0.043
p=0.012
p=0.001
Young
n=17
Older
n=42
Oldest old
n=15
Young
n=15
Older
n=45
Oldest old
n=15
Young
n=10
Older
n=41
Oldest old
n=11
D
Log10 CD8 T-cells
(fraction of T-cell population)
p=0.000
p=0.000
p=0.000
p=0.003
Young
n=20
Older
n=53
Oldest old
n=17
at least one marker positive
IFN-γ
TNF
T_NA
T_CM
T_EM
T_EMRA


(1) lymphoc.
SSC-A
FSC-A
(2) singlets
FSC-H
FSC-A
(3) Live dead
FSC-H
viability
(12) CD45RA/CD27
CD45RA
CD27
Combine with (7) – (11)
(4) CD3_8
CD8
CD3
(5) CD3_4
CD4
CD3
Boolean Combination:
'(4) OR (5)'
CD8
(6) CD3 (all)
CD4
(7) CD8/IL-2
IL-2
(8) CD8/IFN-γ
IFN-g
(9) CD8/CD107
CD107
(10) CD8/TNF
TNF
(11) CD8/CD154
CD154
The strategy for CD4 T-cells is analogous.
Example for Assay Controls (TNF):
Neg. control
Pos. control
TNF


A
all 19 proteins
UL83 (pp65)
UL55 (gB)
Log10 (CD4 T-cells/nL)
n.s.
n.s.
p=0.046
p=0.014
p=0.027
Young n=13
Older N=32
Young n=9
Older n=30
Young n=5
Older n=18
C
Log10 (CD4 T-cells/nL)
n.s.
Young n=16
Older n=59
B
all 19 proteins
UL83 (pp65)
UL123 (IE-1)
Log10 (CD8 T-cells/nL)
n.s.
Young n=13
Older n=41
Young n=13
Older n=44
Young N=9
Older n=40
D
Log10 (CD8 T-cells/nL)
p=0.022
Young n=16
Older n=59
at least one marker positive
IFN-γ
TNF


A
CD4
Young
Older
CMV-
CMV+
CD45RA
CD27
0.188
27.3
6.09
66.4
0.109
38.9
4.2
56.8
0.849
40.7
6.16
52.3
1.19
16.4
14.6
66.7
CD8
Young
Older
CMV-
CMV+
CD45RA
CD27
4.18
55.7
6.95
33.2
17.9
26.3
6.4
49.4
14.3
57.6
8.27
19.8
79.2
4.16
7.49
8.19
B
Young
Older
Log10 CD4 T-cells
(fraction of T-cell population)
0.00
-1.00
-2.00
-3.00
-4.00
p=0.012
p=0.000
p=0.021
p=0.000
p=0.000
CMV-
(n=27)
CMV+
(n=21)
CMV-
(n=47)
CMV+
(n=56)
Log10 CD8 T-cells
(fraction of T-cell population)
0.00
-1.00
-2.00
-3.00
p=0.000
p=0.014
p=0.001
p=0.000
p=0.007
p=0.000
p=0.030
CMV-
(n=27)
CMV+
(n=21)
CMV-
(n=47)
CMV+
(n=56)
T_NA
T_CM
T_EM
T_EMRA


A
B
C
Log10 CD4 T-cells (fraction of CMV-specific T-cells)
Log10 (CD4 T-cells/nL)
Log10 CD4 T-cells (fraction of T-cell population)
Log10 CD8 T-cells (fraction of CMV-specific T-cells)
Log10 (CD8 T-cells/nL)
Log10 CD8 T-cells (fraction of T-cell population)
p=0.001
p=0.000
p=0.004
p=0.009
p=0.006
Young n=18
Older n=41
Oldest old n=18
Young n=18
Older N=41
Young n=21
Older n=56
Oldest old n=18
Young n=20
Older n=53
Oldest old n=17
Young n=19
Older n=45
Young n=21
Older n=56
Oldest old n=18
T_{NA} T_{CM} T_{EM} T_{EMRA}



Supplementary Figure S5

Supplementary Materials

CMV-specific T-cell responses at older ages: broad responses with a large central memory component may be key to long-term survival

Short title: Ageing, CMV-specific T-cells, and long-term survival

Martha Bajwa[1,*], Serena Vita[4,*], Rosanna Vescovini[5], Martin Larsen[6,7], Paolo Sansoni[5], Nadia Terrazzini[8], Stefano Caserta[2], David Thomas[1], Kevin A. Davies[1], Helen Smith[3], and Florian Kern[1]

[1]Department of Clinical and Experimental Medicine, [2]Department of Global Health and Infection, [3]Department of Primary Care and Public Health, Brighton and Sussex Medical School, Brighton, United Kingdom;

[4]Institute Pasteur, Cenci-Bolognetti Foundation, Department of Public Health and Infectious Diseases, University Sapienza of Rome, Rome, Italy.

[5]Dipartimento di Clinica Sperimentale, Università di Parma, Parma, Italy.

[6]Inserm UMR-S1135, Centre d'Immunologie et des Maladies Infectieuses (CIMI-Paris), Paris, France.

[7]AP-HP, Groupement Hospitalier Pitié-Salpêtrière, Département d'Immunologie, Paris, France.

[8]School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton, United Kingdom;

Materials and Methods

Participants

Inclusion criteria for UK donors: age 20-35 or 60-85 years; exclusion criteria were known immunodeficiency (including HIV-infection), organ transplantation, use of immunosuppressive or immune-modulating drugs within the last year (excluding acetylsalicylic acid ≤ 100mg/day), cancer or treatment for cancer within the previous 5 years, insulin dependent diabetes, moderate or advanced renal failure, liver disease, endocrine disorders (except corrected thyroid dysfunction), autoimmune disease, dementia/mental incompetence, alcohol/other drug abuse, acute infection or illness in the last 4 weeks, raised body temperature (>37.5C).

Inclusion criteria for Italian volunteers: minimum age 85 years, known CMV responsiveness; exclusion criteria were evidence of endocrine (except thyroid dysfunction), autoimmune and neoplastic diseases, acute infections or illness in the last 2 months, renal or liver failure, and use of immune-modulatory medications (including steroids, non-steroidal anti-inflammatory agents, acetylsalicylic acid >100mg/day, or immunosuppressive drugs).

Peripheral blood mononuclear cell (PBMC) Isolation and activation

Twenty-five µg per peptide of CMV peptide-pools (“PepMix”, JPT Peptide Technologies, Berlin, Germany) was dissolved in 100 µL of dimethyl-sulfoxide (DMSO, Sigma-Aldrich, Gillingham, UK). Two µL of peptide solution, 1.5 µL of anti CD107a (BD) and 0.5 µL of

Monensin (BD) were added to 46 µL of complete media and placed in 4.5 mL polystyrene tubes (BD). After the addition of 200 µL of PBMC suspension the tubes were incubated at 37°C in a standard incubator with a humidified 5% CO_2 atmosphere. After 2 hours, 1 µL of Brefeldin A (5 µg/ml; Sigma) was carefully added in 249 µL of complete media and samples were incubated for a further 14 hours. Final concentrations of peptide were 1 µg/mL per peptide for each pool. Staphylococcus enteroxin B (SEB) (Sigma) was dissolved in DMSO and used at 1 µg/ml (final concentration) as positive stimulation control, 2 µL DMSO alone was added as a negative control.

Antibodies and cell staining

At the end of PBMC stimulation 100 µL EDTA buffer (20 mM in wash buffer containing PBS with 0.5% bovine serum albumin, 0.1% sodium azide, Sigma) was added to each tube. Tubes were vortexed and then incubated for 10 min at 37°C. After spinning at 400g for 8 min at 4°C, cells were washed with wash buffer. Pellets were carefully resuspended before staining antibodies were added and tubes incubated (30 minutes at 4°C). Cells were then washed, lysed with FACS Lysing solution (BD) and permeabilized with BD Permeabilizing 2 solution (BD) according to the manufacturer's instructions. Cells were stained intracellularly, following the same steps as for surface staining. Following a final wash cells were resuspended and fixed in PBS containing 0.5% paraformaldehyde (Sigma) prior to acquisition on an LSRII flow cytometer using FACSdiva 6.1 software (BD).

Absolute T-cell counts

In order to obtain absolute T-cell counts, 100 µl of fresh whole blood (EDTA-anti-coagulated) was stained with CD45 PerCP and CD3 Qdot605 (all from BioLegend) for 30 min at 4°C, prior to adding 1 ml of FACS lysing solution (BD) and incubating for 10 min according to the manufacturer's instructions. Then 3 ml of wash buffer were added, samples were centrifuged, and aquired on an LSR II flow-cytometer (BD).

White blood cells were selected according to CD45 expression on a side scatter versus CD45 plot. The percentage of CD3 T-cells among white blood cells was determined on a side scatter versus CD3 plot. The absolute CD3 T-cell count was determined by multiplying this percentage with the absolute white blood cell count (cells/nL). In order to determine absolute CD4 and CD8 T-cell counts the absolute CD3 T-cell count (cells/nl) was multiplied with CD4 and CD8 T-cell percentages.

T-cell Polyfunctionality

The polyfunctionality index (PI) algorithm was obtained from 'FunkyCells ToolBox' version 0.1.0 beta (www.FunkyCells.com). To calculate the PI, each subset defined by a given number of displayed functions has a weight assigned which is then multiplied with the subset frequency. The PI is the sum of these products (PI = $\sum_{i=0}^{n} F_i \cdot \left(\frac{i}{n}\right)^q$ where *Fi* is the frequency of cells performing *i* simultaneous functions, *q* is the polyfunctionality parameter determining the weight of the subsets, *n* is the number of possible functions). The polyfunctionality parameter *q* was set to 1 as previously described [15]. Samples containing less than 0.1% activated events were not included in correlations of PI and other parameters.

Supplementary Tables

Supplementary Table S1. CMV- participant demographics

Parameter	'young'	'older'
Total number	29	62
Age range (mean ± STD)	20 – 34 (25.5±4.7)	60 – 85 (72.2±8.2)
Females	18 (62 %)	28 (45 %)
Males	11 (38 %)	34 (55 %)
White (British or other European[a])	29 (100%)	62 (100 %)

[a] Other European: 1 young adult from Greece and 1 young adult from Switzerland.

Supplementary Figure Legends

Supplementary Fig. S1: Gating strategy for T-cell activation markers.

(1) Lymphocytes were gated on an FSC-A versus SSC-A plot. (2) Single cells were gated on an FSC-H versus FSC-A plot. (3) Dead cells were excluded using a viability dye in the violet 3 channel. (4) T-cells were first selected on a CD3 versus CD8 plot, allowing for some CD3 down-regulation on activated CD8+ events ('CD3_8'). (5) CD3 T-cells were also gated alternatively on a CD3 versus CD4 plot, this time allowing for some CD3 down-regulation on activated CD4+ events ('CD3_4'). (6) Both CD3 gates were then combined (logical 'OR'), so that the final CD3 T-cell gate included a maximum of activated CD4 and CD8 T cells. (7)-(11) Subsequently, activated CD8 T-cells were gated with respect to each functional parameter (one by one). The same process was repeated for activated CD4 T-cells. (12) Phenotypic subsets based on the expression of CD45RA and CD27 were gated on all CD4 or all CD8 T-cells (including activated and non-activated) and then combined (logical 'AND') with the respective activation marker gates (or gates derived from these). The numbers/frequencies of activated CD4 or CD8 T-cells for each combination of phenotypic and functional subsets were computed subsequently. The positive assay control (SEB) was used to ascertain if the assay had worked (even if individuals were not responding to CMV-antigens), whereas the negative assay control (unstimulated) was used to estimate (and subtract) 'background noise' for each functional subset (subset by subset).

Supplementary Fig. S2: Differences between young and older people in terms of absolute numbers of CMV-specific CD4 and CD8 T-cells are smaller than in terms of relative numbers. PBMC from CMV+ participants were stimulated over night with 19 CMV protein-derived overlapping peptide-pools. Activated T cells were identified by flow-cytometry. The presented data is limited to the UK cohort. **(A, B)** Diagrams show absolute counts/nL of CD4 and CD8 T-cells displaying at least one activation marker (combined read out), IFN-γ, or TNF. Responses are shown to the 19 proteins combined (left panels) and the most frequently recognized CMV proteins in the UK cohort **(A)** for CD4 T-cells with respect to UL83 (middle) and UL55 (right), **(B)** for CD8 T-cells with respect to UL83 (middle) ('pp65') and UL123 (right) ('IE-1'). Significant differences at the $p \leq 0.05$ level are indicated. In addition, 'n.s.' (not significant) is indicated for those differences that were significant using relative T-cell counts (frequencies, compare Fig. 3A-B). Note that in order to determine if there is a general increase in CMV-specific T-cell response size between young and older people, the main end-point was the combined functional read-out ('at least one marker positive') in connection with all 19 tested proteins. The significance level was not adjusted for multiple end-points in (A) or (B). Absolute counts (in cells/nL of blood) of CD4 **(C)** and CD8 T-cells **(D)** seem to diminish in older people. The effect was not significant for CD4 but highly significant for CD8 T-cells. As a result, in particular for CD8 T-cells, fewer differences between the age groups were significant compared to when subset sizes were expressed as a fraction of CD4 or CD8 T-cells (compare Fig. 3A-B). Boxplots show minimum, maximum, median, interquartile range, and outliers (o).

Supplementary Fig. S3: CMV-infection significantly affects memory subset distributions in the young and older groups. The unstimulated control tube for each participant was used for the analysis of CD4 and CD8 T-cell distributions across the canonical memory compartments defined by the expression of CD27 and CD45RA (CD45RA+/CD27+ = 'naïve' or T_{NA}; CD45RA-/CD27+ = 'central memory' or T_{CM}; CD45RA-/CD27- = 'effector memory' or T_{EM}; CD45RA+/CD27- = 'revertant' or T_{EMRA}). Data for CMV+ and CMV- individuals are shown. **(A)** CMV infection *per se* has a major impact on memory subset distribution in both young and older people as previously shown by us and others [27]. Dot-plots show the T_{NA} (upper right quadrant), T_{CM} (lower right quadrant), T_{EM} (lower left quadrant), and T_{EMRA} (upper left quadrant) compartments. **(B)** The effect of CMV-infection on the naïve T-cell pool is only significant in older people. Interestingly, the effect of CMV infection on the T_{EMRA} (CD27-/CD45RA+) compartment seems to be stronger in CD4 than CD8 T cells. Boxplots show minimum, maximum, median, interquartile range, and outliers (o).

Supplementary Fig. S4: Proportional memory subset distributions of CMV-specific T-cells are reflected by distributions in absolute counts but differ from those of all T-cells. (A) In analogy with Fig. 3C-D, the distribution of T-cells across the memory compartments, T_{NA}, T_{CM}, T_{EM}, and T_{EMRA} is shown for each age group. However, instead of frequencies of CD4 or CD8 T-cells, the diagram shows the proportions that each subset contributes to the whole response (normalized). **(B)** In analogy to Fig. 3C-D, the memory subset distributions of CMV-specific CD4 and CD8 T-

cell were analyzed in terms of absolute T-cell counts (limited to the young and older groups). In each individual and with respect to each CMV-peptide pool, the percentages of CD4 and CD8 T-cells in the T_{NA}, T_{CM}, T_{EM}, and T_{EMRA} memory compartments were added up to provide a total response for each memory compartment. These percentages were multiplied with the absolute CD4 and CD8 T-cell counts in cells/nL. Differences between the age groups by and large reflect the distributions observed in terms of fractions of CD4 and CD8 T-cells. **(C)** The unstimulated control tube was used for the analysis of memory subsets for all T-cells (CMV-specific and non-CMV-specific) in CMV+ people across all three age groups. Boxplots show minimum, maximum, median, interquartile range, outliers (o), and extreme values (*).

Supplementary Fig. S5: Polyfunctionality varies between CMV-specific T-cell memory subsets and is generally highest in T_{EM}. The polyfunctionality index (PI) captures functional subset distributions by weighting the number of functions as well as subset size. For the shown analysis a linear relationship between the number of functions and the relative weight of a subset was selected (e.g. subsets with two functions were assigned twice the weight of subsets with one function, subsets with three functions were assigned three times the weight of subsets with one function, etc.). Polyfunctionality is highest in effector memory T-cells, overall similar in young and older but reduced in the oldest old, where, however, it appears to be increased in naïve CD8 T-cells. Boxplots show minimum, maximum, median, interquartile range, outliers (“o”), and extreme values (“*”).

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