Perturbed CD8+ T cell immunity across universal influenza epitopes in the elderly

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Running title: Influenza-specific T cell responses in the elderly

Key words: T cell receptor repertoire, transcription factors, ZAP-70, antigen-specific CD8+ T cells, aging

One-sentence summary: Aging is associated with reduced numbers of influenza-specific CD8+ T-cells across human universal epitopes and large non-canonical clonal TCR expansions.

Character count: 28,783
6 figures; 6 colour figures; 68 references; 2 tables; 250 words in Abstract; 20 words in summary sentence

Abbreviations: adult donor (AD); elderly adult donor (ED); HLA-A*02:01-restricted $M1_{58-66}$ GILGFVFTL epitope (A2/$M1_{58}$); T cell receptor (TCR)

Abstract

Influenza epidemics lead to severe illness, life-threatening complications and deaths, especially in the elderly. As $CD8^+$ T-cells are associated with rapid recovery from influenza, we investigated the effects of aging on antigen-specific $CD8^+$ T-cells across the universal influenza epitopes in humans. We show that aging is characterised by altered frequencies in T-cell subsets, with naïve T-cells being partially replaced by activated effector/memory populations. Although we observed no striking differences in T-cell receptor (TCR) signalling capacity, T-cells in the elderly had increased expression of transcription factors Eomes and T-bet, and such changes were most apparent in $CD8^+$ T-cells. Strikingly, the numbers of antigen-specific $CD8^+$ T-cells across universal influenza epitopes were reduced in the elderly, although their effector/memory phenotypes remained stable. To understand whether diminished numbers of influenza-specific $CD8^+$ T-cells in the elderly resulted from alteration in TCR clonotypes, we dissected TCRαβ repertoire specific for the prominent HLA-A*02:01-restricted-$M1_{58\text{-}66}$ (A2/$M1_{58}$) influenza epitope. We provide the first *ex vivo* data on paired antigen-specific TCRαβ clonotypes in the elderly, showing that influenza-specific A2/$M1_{58}^+$ TCRαβ repertoires in the elderly adults varied from those in younger adults, with the main features being a reduction in the frequency of the public TRAV27-TRBV19 TCRαβ clonotype, increased proportion of private TCRαβ signatures, broader usage of TRAV and TRBV gene segments, and large clonal expansion of private TCRαβ clonotypes with longer CDR3 loops. Our study supports the development of T-cell-targeted influenza vaccines that would boost the T-cell compartment during life, and maintain the numbers and optimal TCRαβ signatures in the elderly.

Introduction

Circulating influenza viruses are an enormous global health burden, with an annual estimate of 3 to 5 million cases of severe illness and 250,000 to 500,000 deaths worldwide (WHO website, updated November 2016), despite annual updates of the influenza vaccine components. Most people experience mild to moderate influenza illness only once or twice in their lifetime [1], with symptoms ranging from fever, cough, sore throat, body aches and fatigue. However, the risk of severe influenza illness, often requiring hospitalisation and can result in death, is more pronounced in the elderly, with increased occurrence, higher hospitalisation rates and increased morbidity and mortality compared to younger adults [2-4]. This is exemplified by the data obtained from 17 Australian major hospitals during the 2015 April-October influenza season, with the elderly (≥65 years) accounting for 46% of all hospitalised influenza-infected patients out of 2,070 laboratory-confirmed cases [3]. Elderly individuals, particularly males, also preferentially succumbed to severe avian H7N9 influenza disease [2, 5, 6]. Such increased susceptibility and morbidity/mortality of influenza infections in the elderly might be partially due to co-existing co-morbidities (i.e. diabetes, chronic respiratory, cardiac or renal disease) in this age group. Concomitantly, aging is also associated with decreased or impaired function of the immune system, named as immunosenescence [7].

As established T cell immunity directed at conserved viral regions can provide protection against influenza viruses and lead to more rapid host recovery [2, 8-10], prolonged influenza disease in the elderly indicates an impairement within the T cell compartment. Indeed, ageing can have multifactorial effects on T cell immunity, arising from decreased thymic export of naïve precursors due to thymic involution [11, 12], perturbed recruitment of naïve $CD8^+$ T cell precursors [13, 14], replicative senescence of memory T cells [15-18], and decreased cytolytic function, as measured by granzyme B production [19, 20]. Aging can also

affect T cell receptor (TCR) composition of naïve and immune T cells in mice, with large antigen-independent clonal expansion [21]. Naïve T cell attrition has also been inferred from observed reductions in the diversity of antigen-specific TCR repertoires in aged mice [22-24] and humans [25].

Our group recently established that human pre-existing memory $CD8^+$ T cells can recognise universally conserved epitopes derived from the internal influenza proteins, matrix protein (M1) and nucleoprotein (NP), that were present across different influenza A virus strains circulating during the past century [26], including the novel avian A/H7N9 influenza virus [6, 27, 28]. Indeed, these universal influenza $CD8^+$ T cell epitopes are restricted to common HLA class I types, HLA-A*02:01, HLA-A*03:01, HLA-B*08:01, HLA-B*18:01, HLA-B*27:05 and HLA-B*57:01 [26], that are prevalent across many different ethnicities. Although recently van de Sandt *et al*. [29] demonstrated elegantly the persistence and longevity of influenza-specific $CD8^+$ T cells over 13 years (1999-2012) in 9 healthy donors aged between 18-64, it is still unclear whether the numbers of influenza-specific $CD8^+$ T cells, especially those directed at the universal influenza epitopes, persist or decline in individuals older than 65 years of age. To understand T cell responses attributed to the declining immunity, we dissected influenza-specific $CD8^+$ T cell responses and TCR$\alpha\beta$ clonal composition in elderly adults, as compared to younger or middle-aged adults. We analysed the effects of aging on both the total (not antigen-specific) $CD8^+$ and $CD4^+$ T cell compartments, their frequencies, effector/memory phenotypes, expression of transcription factors as well as antigen-specific $CD8^+$ T cell immunity directed at universal influenza epitopes, together with their phenotypes and TCR$\alpha\beta$ repertoires. We found that while bulk T cell compartments are associated with increased frequencies of effector/memory T cells and altered transcription factor levels, the numbers of influenza-specific $CD8^+$ T cells are lower in elderly individuals, and display large non-canonical clonal expansions and reduced usage of

the preferred TCRαβ signatures such as TRBV19, TRAV27 and public TRBV19-TRAV27 clonotype. Our study indicates the need for new vaccination strategies to preserve the numbers and optimal TCRαβ features of influenza-specific $CD8^+$ T cell immunity in elderly individuals.

MATERIALS AND METHODS

Human blood samples

Human experimental work was conducted according to Declaration of Helsinki principles and approved by the Human Ethics Committee (University of Melbourne, Parkville, Victoria; ethics ID #0931311.5 and #1443389.3). Peripheral blood was obtained from healthy younger adults recruited at the University of Melbourne (aged 25-58 years) or elderly adult donors recruited at Deepdene Medical Clinic (aged 60+ years). The vaccination status of the donors, when known, is listed in Table 1. The Australian National Immunisation Program for the seasonal influenza is available around April each year. Influenza immunisation is freely available from GP surgeries and other immunization providers for people who are at high risk from influenza and its complications, including people aged 65 and over and pregnant woman.

Buffy packs from healthy donors (aged 22-70 years) were obtained from the Australian Red Cross Blood Service (ARCBS) (Victoria, Australia). Umbilical cord blood was obtained from the Mercy Hospital for Women (Heidelberg, Australia). All donors provided informed written consent. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation and cryopreserved at -196ºC until required. HLA class I molecular typing was performed as

previously described [30] by the Victorian Transplantation and Immunogenetics Service (ARCBS, West Melbourne, Victoria, Australia).

Peptides and tetramers

Influenza A peptides derived from M1 and NP proteins (**Table 2**) were purchased from Genscript (Piscataway, NJ, USA). Monomers were generated in-house by refolding each peptide with their restricted HLA (**Table 2**) α-heavy chain-BirA and β2-microglobulin [31], before 8:1 molar conjugation with streptavidin-phycoerythrin or streptavidin-allophycocyanin (BD Biosciences, San Jose, California, USA) to form tetramers.

Flow cytometry

PBMCs were thawed and cell-surface stained in MACs buffer (PBS containing 0.5% BSA/2mM EDTA) with human anti-CD3 PE-CF594 (#562280, BD Biosciences, San Jose, California, USA), anti-CD4 BV650 (#563875, BD Biosciences), anti-CD8 PerCP-Cy5.5 (#565310, BD Biosciences) or anti-CD8 BV605 (#301039, Biolegend, San Diego, CA, USA), anti-CD14 APC-H7 (#560180, BD Biosciences), or anti-CD14 BV570 (#301831, Biolegend), anti-CD19 APC-H7 (#560177, BD Biosciences) or anti-CD19 BV570 (#302235, Biolegend), anti-CD45RA FITC (#555488, BD Biosciences) or anti-CD45RA APC-H7 (#560674 BD Biosciences), anti-CD27 AF700, (#56027942, eBioscience, San Diego, CA, USA) or anti-CD27 BV711 (#563167 BD Biosciences) and LIVE/DEAD® Fixable Near-IR (Molecular Probes, Eugene, OR, USA); before fixation with 1% paraformaldehyde. For transcription factor staining, cells were fixed and intracellularly stained with anti-Eomes PE [3-4877-42, eBiosciences] and anti-T-bet PE-Cy7 [32-5825-82, eBiosciences] using the FoxP3 transcription factor staining kit (#00-5523-00, eBioscience). Cells were resuspended

in MACs buffer and then acquired on a LSR Fortessa II (BD Biosciences) and analysed by FlowJo software (Treestar, Inc., Ashland, OR, USA).

Flow cytometry based assay for ZAP-70 phosphorylation

Kinetics of TCR stimulation via phosphorylation of ZAP-70 was measured using the BD Phosflow™ Whole Blood Starter Kit (BD Biosciences). Firstly, whole blood was incubated with anti-CD3 antibody (OKT3 clone, Walter and Eliza Hall Institute, Parkville, Victoria, Australia) for 10 mins on ice, washed, then incubated with goat anti-mouse F(ab')2 for 5 mins on ice. TCR signalling was initiated by incubating the samples in a 37ºC water bath before adding Lyse/Fix solution to each time-point tube at 0 sec, 30 secs, 60 secs, 90 secs, 120 secs, 150 secs, 4 mins and 7 mins to stop the reaction. Samples were then incubated with anti-ZAP-70/Syk PE (#557881, BD Phosflow™, BD Biosciences) and cell-surface mAbs in Perm Wash buffer on ice, as previously described in [33]. Cells were acquired by flow cytometry.

PMA and Ionomycin stimulation

Adult and elderly PBMCs ($1x10^6$) were stimulated *ex vivo* with PMA (10ng/ml) and Ionomycin (1uM) for 6 hrs in the presence of protein transport inhibitors Brefeldin A (#555029, BD Biosciences) and Monensin (#554724, BD Biosciences). Samples were subsequently stained with live/dead discrimination marker and cell surface MAbs, anti-CD3, anti-CD4, and anti-CD8, as above. Cells were then fixed with BD Cytofix/Cytoperm kit (#554722, BD Biosciences) prior to intracellular staining with anti-IFNγ (#560371, BD Biosciences) and anti-TNF (#557647, BD Biosciences), and samples acquired by flow cytometery, as described above.

Tetramer-associated magnetic enrichment (TAME) of influenza-specific CD8$^+$ T cells

Cryopreserved PBMCs (4-35x10^6) were thawed and incubated with anti-human FcR block (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 mins on ice. Cells were then stained with PE- and/or APC-SA conjugated tetramer for 1 hour at room temperature, washed once, then incubated with anti-PE and/or anti-APC microbeads (Miltenyi Biotec) before passing through a LS column (Miltenyi Biotec) to enrich for tetramer$^+$ cells as previously described [30, 34-36]. Cells were then cell-surface stained and either fixed with 1% paraformaldehyde for acquisition, or resuspended in MACs buffer for single-cell sorting.

T cell proliferation assay

HLA-A2$^+$ responder PBMCs (~1x10^7) were pre-incubated with cell trace violet (Violet Proliferation Dye 450, BD Horizon™, BD Biosciences) before generating A2/M1$_{58}$-specific CD8$^+$ T cell lines as previously described [26, 37]. Briefly, one-third of unlabelled PBMCs were pulsed with 10 μM A2/M1$_{58-66}$ peptide for 90 mins at 37°C, washed twice, and then incubated with the remaining two-thirds of cell trace violet-labelled autologous PBMCs for 10 days (37°C, 5% CO_2) in complete RF10 medium containing 10% heat-inactivated FCS, 2mM L-glutamine, 1mM MEM sodium pyruvate, 100μM MEM non-essential amino acids, 5mM HEPES buffer solution, 55μM 2-mercaptoethanol and 100U/ml penicillin/100μg/ml streptomycin in RPMI-1640 media, plus 10U/mL recombinant IL-2 (Roche Diagnostics, Mannheim, Germany). Media reagents were from Gibco (Thermo Fisher Scientific, Scoresby, Victoria, Australia). Cells were assessed on days 0, 3 or 4, 5, 6, 7 and 10 by tetramer staining and cell-surface mAbs staining, followed by flow cytometry.

Single-cell RT-PCR and sequencing

PBMCs were stained with tetramer for 1 hour at room temperature, washed twice, then cell-surface stained with mAbs. Live $CD3^+CD8^+tetramer^+CD19^-/CD14^-$ cells were individually sorted into 96-well Twin.tec PCR plates (Eppendorf, Hamburg, Germany) using a BD FACSAria III (BD Biosciences). Analysis of paired CDR3α and CDR3β regions were performed by multiplex-nested reverse transcriptase PCR before sequencing of TCRα and TCRβ products, essentially as described [35, 36, 38, 39]. Sequences were analysed according to the IMGT/V-QUEST web-based tool [40, 41]. Circos plots were generated using the Circos software package [42].

Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (San Diego, CA, USA). Spearman rank analyses were used to derive age-based correlations. Mann-Whitney and Student-t test were used where appropriate for comparison between two groups as indicated. Statistical significance was described as *=$p<0.05$, **=$p<0.01$, and ***=$p<0.001$, and r values stated where relevant.

RESULTS

Distinct age-related profiles within the $CD8^+$ and $CD4^+$ T cell compartments.

$CD8^+$ and $CD4^+$ T cell compartments change throughout a human lifespan. At birth, T cells are predominantly immunologically naïve, and thereafter gain the effector/effector memory phenotype with age, consequent to encounters with foreign pathogens. We used two surface phenotypic markers, CD45RA and CD27 to delineate naïve ($CD45RA^+CD27^+$), effector/memory ($CD45RA^-CD27^{+/-}$) and terminally differentiated effector ($CD45RA^+CD27^-$) T cell subsets [34, 43] in cord blood, younger adults and elderly adults (**Fig. 1A**), across

different ages (a range of 0-88 years; **Table 1**). At birth, both $CD8^+$ and $CD4^+$ T cells were predominantly of a naïve-like $CD45RA^+CD27^+$ phenotype (~80%, n=7). Expectedly, the naïve $CD8^+$ and $CD4^+$ T cell compartments significantly declined with age ($p<0.0001$; r= -0.8232 and -0.7071, respectively) to a mean(±SD) of 44.6±19.9% in adults (n=16) and 22.8±14.0% in the elderly (n=15) for $CD8^+$ T cells, and to 41.3±18.1% in younger adults and 28.2±14.0% in elderly adults for $CD4^+$ T cells (**Fig. 1Bi)**. On the other side of the spectrum, terminally-differentiated $CD45RA^+CD27^-$ $CD8^+$ T cells [44] significantly increased with age from 3.8±4.1% in cord blood to 16.5±13.0% in younger adults and 33.3±12.9% in elderly adults (**Fig. 1Bii**). Whilst the terminally differentiated $CD45RA^+CD27^-$ effector $CD8^+$ T cell subset was prominent in younger adults and the elderly adults, $CD45RA^+CD27^-$ $CD4^+$ T cells were found only at minimal frequencies (<3%) across the human lifespan. Furthermore, the frequency of the activated effector/memory populations lacking CD45RA expression, and either $CD27^+$ (**Fig. 1Biii**) or $CD27^-$ (**Fig. 1Biv**), significantly increased with age for both $CD8^+$ and $CD4^+$ T cells.

To further explore the transcriptional profiles within activated $CD27^-$ or resting $CD27^+$ T cells during aging, we analysed expression of the key transcription factors for T cell function and differentiation, Eomesoderm (Eomes) and T-bet [45]. Bi-modal distribution of Eomes in $CD27^+$ T cells demonstrated two populations of "naïve-like" $CD27^+Eomes^{lo}T\text{-}bet^{lo}$ T cells, also negative for the cytolytic molecules perforin and granzyme (data not shown), and a mixture of effector/memory $Eomes^{hi}T\text{-}bet^{hi}$ T cells heterogeneously expressing CD27 (**Fig. 1C**). In agreeance with previous findings [43], we observed significantly higher frequencies of $Eomes^{hi}$ expression in both $CD27^+$ (p=0.0317) and $CD27^-$ (p=0.0317) $CD8^+$ T cell populations, and higher frequencies of $T\text{-}bet^{hi}$ expression in $CD27^+CD8^+$ T cells (p=0.0317) in elderly individuals compared to the younger adults (**Fig. 1D**). Although similar trends were observed in $CD4^+$ T cells, these were not significant.

Thus, with aging, naïve T cells are replaced with the activated effector/memory T cell subsets characterised by increasing Eomes/T-bet expression, and such changes are most apparent in the $CD8^+$ T cell compartment.

Elderly $CD8^+$ and $CD4^+$ T cells retain their TCR signalling capacity.

As the composition of T cell compartments and their transcription factor profiles change with age, we next asked whether aging also affects the initial TCR engagement and TCR signalling efficacy. We measured the kinetics of tyrosine phosphorylation of the zeta-chain-associated protein kinase 70 (ZAP-70), which plays a critical role in T cell signal transduction after TCR-dependent activation. TCR stimulation was initiated by cross-linking with anti-CD3 antibody and the kinetic analysis of the peak geometric mean fluorescent intensity (MFI) of ZAP-70 phosphorylation was measured in $CD8^+$ and $CD4^+$ T cells after 0 sec, 30 secs, 60 secs, 90 secs, 120 secs, 150 secs, 4 mins and 7 mins of TCR stimulation (**Fig. 2A**). Evidently, there were no significant differences in ZAP-70 phosphorylation kinetics for either $CD8^+$ or $CD4^+$ T cell populations between elderly and young adult donors, as assessed by phospho-ZAP-70 MFI measured as fold-changes from the baseline (**Fig. 2B)**. ZAP-70 phosphorylation was initiated between 60 and 90 secs for both T cell subsets and peaked at 120 secs for $CD8^+$ T cells, 150 secs for $CD4^+$ T cells, albeit the 30-second delay was not significant. As a negative control, non-T cells ($CD3^-$ lymphocytes), including NK cells, which also show increased pZAP-70/Syk staining when activated, remained inactive during anti-CD3 cross-linking and showed stable expression of ZAP-70 over time (**Fig. 2A**).

Given that the distribution of T cell subsets changes with age, as shown by a decline in "naïve-like" T cells and an increase in effector/memory T cell subsets (**Fig. 1**), we further examined TCR signalling capacity within the phenotypically-distinct T cell subsets of adults and the elderly. In the $CD8^+$ T cell compartment, we observed no significant differences in

the naïve/effector/memory subsets between younger adults and elderly adults (**Fig. 2C**; left panels). However, the CD45RA$^-$CD27$^-$ effector memory subset within CD4$^+$ T cells showed significantly higher ZAP-70 MFI fold-change at 150 secs and 4 mins in the elderly donors (**Fig. 2C**; right panels), suggesting that effector memory CD4$^+$ T cells in the elderly may have a higher and prolonged signalling capacity compared to younger individuals.

To assess the functional capacity of adult and elderly T cells, we assessed cytokine (IFNγ and TNF) production by PBMCs stimulated *ex vivo* with Phorbol myristate acetate (PMA) and Ionomycin. No significant differences (p>0.05) in the single or dual cytokine-producing T cells were observed across the CD8$^+$ and CD4$^+$ T cell compartments within younger (n=7) to elderly adults (n=7) (Fig. 2D).

Overall, although aging is associated with altered frequencies in T cell subsets and transcription profiles, no striking differences in TCR signalling or cytokine producing capacity, especially in CD8$^+$ T cells, were observed.

Aging is associated with reduced numbers of antigen-specific memory CD8$^+$ T cells across universal influenza HLAs.

Following the broad analysis of total CD8$^+$ and CD4$^+$ T cells in the elderly, we subsequently dissected T cells directed at a single epitope level. As influenza virus infections cause significant morbidity and mortality in the elderly, and CD8$^+$ T cells are associated with rapid recovery [2, 8-10], we investigated whether aging affects the numbers of CD8$^+$ T cells across the universal influenza HLAs in humans [26]. To enumerate influenza-specific memory CD8$^+$ T cells in elderly adults compared to younger adults, we used a tetramer-associated magnetic enrichment (TAME) method, which increases detection of tetramer-positive CD8$^+$ T cells by up to 100-fold [30, 36, 46]. Dual enrichment with two tetramers was used when donors had more than one of the universal HLAs (**Table 1**; in red). Tetramer-enriched

influenza-specific $CD8^+$ T cell precursor frequencies were calculated based on the total $CD8^+$ T cell population according to Alanio *et al*. [46] where $1x10^{-4}$ represents a frequency of 1 in 10,000 $CD8^+$ T cells. We assessed enriched memory precursor frequencies for four universal HLA/peptide tetramers (HLA-A*02:01-$M1_{58-66}$ ($A2/M1_{58}$), HLA-B*08:01-$NP_{225-233}$ ($B8/NP_{225}$), HLA-B*27:05-$NP_{383-391}$ ($B27/NP_{383}$), HLA-B*57:01-$NP_{199-207}$ ($B57/NP_{199}$) (**Table 2**; **Fig. 3A**). Our analysis showed significantly reduced numbers of influenza-specific $CD8^+$ T cells across two universal influenza epitopes tested, $A2/M1_{58}$ and $B27/NP_{383}$ (**Fig. 3B**). The mean(±SEM) frequency of $A2/M1_{58}^+CD8^+$ T cells was $2.78x10^{-4}$±$6.21x10^{-5}$ cells (of total $CD8^+$ T cells) in younger adults (range: $1.48x10^{-5}$-$1.04x10^{-3}$) versus $8.07x10^{-5}$±$2.58x10^{-5}$ cells (range: $2.15x10^{-5}$-$5.05x10^{-4}$) in the elderly adults (p=0.0154), whilst for $B27/NP_{383}^+CD8^+$ T cells there were on average $3.73x10^{-5}$±$6.88x10^{-6}$ cells in younger adults (range: $1.62x10^{-5}$-$5.05x10^{-5}$) and $1.41x10^{-5}$±$2.67x10^{-6}$ cells (range: $1.12x10^{-5}$-$1.94x10^{-5}$) in the elderly adults (p=0.0481). Of note, due to the limited number of donors available for $B8/NP_{225}$ and $B57/NP_{199}$ TAME experiments, statistical analysis could not be performed and data points were graphed together (**Fig. 3B**). Further, phenotypic analysis of $A2/M1_{58}^+CD8^+$ T cells revealed comparable activation/memory phenotypes, as defined by CD45RA and CD27 profiling (**Fig. 3C**). Furthermore, as we found no difference (p=0.111) in the vaccination rate between our healthy adult volunteers (46%) and elderly adults (43%), for the donors with known vaccination history, it seems unlikely that the vaccination status of the donors would impact our results.

Taken together, while the numbers of antigen-specific $CD8^+$ T cells across two universal HLAs are reduced in the elderly individuals, the activation/memory phenotype of those influenza-specific $CD8^+$ T cells remain stable.

Robust proliferative capacity of $A2/M1_{58}$-specific $CD8^+$ T cells in the elderly.

Given that the numbers of influenza-specific $CD8^+$ T cells across several specificities were reduced in the elderly individuals, we next asked whether those epitope-specific $CD8^+$ T cells were capable of effective proliferation upon subsequent antigenic stimulation. We performed these experiments for the immunodominant influenza epitope, $A2/M1_{58}$ [36, 47], by *in vitro* $A2/M1_{58}$-peptide stimulation for 10 days. Since *ex vivo* numbers of $A2/M1_{58}$-tetramer$^+$$CD8^+$ T cells detected on day 0 were below the detection limit, we used TAME to measure *ex vivo* d0 precursor frequencies (**Fig. 3**). Proliferation of $A2/M1_{58}$-tetramer$^+$$CD8^+$ T cells was first observed between days 3-4, as shown by the loss of cell trace violet over time (**Fig. 4A**). From undetectable levels on day 0, the total numbers of $A2/M1_{58}$-tetramer$^+$$CD8^+$ T cells expanded similarly to $1.0\text{-}4.8\text{x}10^4$ cells in younger adults and $1.2\text{-}3.5\text{x}10^4$ cells in the elderly adults by d10 (**Fig. 4B**). Furthermore, fold expansions of $A2/M1_{58}$-tetramer$^+$$CD8^+$ T cells from days 3-4 did not differ between elderly adult and younger adult cultures (**Fig. 4C**). Although our sample size was limited for these experiments, both younger and elderly adults had similar trends in the proliferation of $A2/M1_{58}$-tetramer$^+$$CD8^+$ T cells during the 10-day *in vitro* culture as reflected in their fold increase of $A2/M1_{58}$-tetramer$^+$$CD8^+$ T cells at day 7 and 10.

Given that our data suggest similar intrinsic TCR signalling capacity and expansion potential, but reduced numbers of epitope-specific $CD8^+$ T cells in the elderly, we next asked whether such reduced numbers during aging were associated with striking alterations in the TCRαβ repertoires of influenza-specific $CD8^+$ T cells.

Large non-canonical expansions of $A2/M1_{58}^+CD8^+$ TCRαβ clonotypes in the elderly.

To dissect the composition and diversity of epitope-specific TCRαβ repertoires in the elderly, we used a single-cell multiplex RT-PCR capable of amplifying both TCRα and TCRβ chains [35, 36, 38, 39]. Here, we provide the first *ex vivo* data on paired epitope-

specific TCRαβ repertoire in the elderly. Using direct *ex vivo* tetramer staining, we examined TCRαβ clonotypes within A2/M1$_{58}^{+}$CD8^{+} T cells for 3 HLA-A*02:01-expressing elderly adult donors (ED9, ED31 and ED18) (**Fig. 5A**). As compared to our *ex vivo* TCRαβ data for memory CD8^{+} T cells in five young adults [36], we found that TCRαβ repertoire in the elderly adult donors utilised a broader array of TCRβ variable (TRBV) and TCRα variable [48] [48] gene usage (n=3.6 and 9.3, respectively) than in younger adults [36] (n=1 and 5, respectively) (**Fig. 5B** and **E**). This suggests that the strong bias towards prominent TRAV27 and TRBV19 segments (as found in adults) decreases with aging, and is replaced by a broader representation of TRAV and TRBV segments. The frequency of TRBV19 in the elderly was decreased to a mean of 54.9% (range 0.0-83.5%), detected in 2/3 elderly adult donors and was completely absent in another (ED31; **Fig. 5C**), compared to 100% TRBV19 gene usage reported in five younger adult donors (**Fig. 5C**). The prominence of TRAV27 was even more strikingly diminished, with a mean of 14.8% in the elderly (range of 0.0-25.1%), as compared to the younger adults with a mean of 64.9% (range of 48.1-91.0%) (**Fig. 5C**). Similarly, we observed marked differences in the use of complementarity-determining region β and α (CDR3β and CDR3α; **Fig. 5D**). While in younger adults the predominant CDR3β length is 8 amino acids (a.a.; utilised by 87%±9% (mean±SD) clonotypes) and CDR3α length is 7 a.a. (utilised by 48.8%±34.7% clonotypes) [36], these preferred CDR3 length features were reduced with aging (to 26.3%±11.4% for CDR3β length and 25.5%±29.4% for CDR3α) and instead a broad range of both CDR3β and CDR3α lengths were utilised in the elderly adult donors, and these varied greatly across different individuals (**Fig. 5D**).

Further dissection of CDR3αβ clonotypic signatures revealed that the elderly A2/M1$_{58}^{+}$CD8^{+} T cells had a diminished sharing of the pubic TCRαβ clonotype CDR3β-SIRSSYEQ (within TRBV19) and CDR3α-GGSGQGNL (within TRAV27) and was found

in only one elderly donor (ED18) at 6.3% of the total TCRαβ repertoire (clone Y; **Fig. 5E, 6A** and **6B**). Likewise, the well-documented CDR3β, "IRS" motif that provides a structural basis for peg-notch recognition of A2/M1$_{58}$ [49] was present in both younger adult and elderly adult donors (11/35 and 8/42 CDR3β clonotypes, respectively), but occurred less frequently albeit not significantly in the elderly (13.7%±14.1% of CDR3β sequences in elderly versus 32.4%±29.6% of CDR3βs in younger adults [36]). Thus, while public TCRβ signatures were still present, aging was associated with increased TCRβ diversity and oligoclonal expansion of clonotypes with longer CDR3α loops. Conversely, TCRαβ repertoire in the elderly displayed more private clonotypes that were not typically shared across the elderly donors, except for clonotype (clone C) bearing the public TCRβ sequence but a varied TCRα sequence, which was present in two elderly donors (**Fig. 6B** and **C**). Interestingly, this was associated with large clonal expansions found in all the elderly individuals, and these were characterised by longer CDR3α loops (**Fig. 6B**). This is most evident from ED31 characterised by one prominent TRAV13-1-TRBV27 TCRαβ clonotype (clone N), with a 10 a.a. CDR3α constituting 60% of the A2/M1$_{58}^{+}$CD8^{+} TCRαβ repertoire (**Fig. 6B** and **C**). In ED9, a large clonal expansion of a TRAV38-1-TRBV19 TCRαβ clonotype with an 11 a.a. CDR3α loop (clone B; **Fig. 6B**) was observed, further demonstrating that longer TCRα chain sequences can be detected in the elderly donors, in accordance with Gil *et al*. [25].

Overall, the influenza-specific A2/M1$_{58}^{+}$TCRαβ repertoire in the elderly individuals varied strikingly from A2/M1$_{58}^{+}$TCRαβ clonotypes observed in the younger adult donors, with the main features being a reduction in the usage of the public TRAV27-TRBV19 TCRαβ clonotype, higher proportion of non-shared private TCRαβ signatures, broader usage of TRAV and TRBV segments, as well as large clonal expansion of selected private TCRαβ

clonotypes with longer CDR3 loops. Thus, it appears that with aging, changes within epitope-specific TCRαβ repertoires are associated with selective loss of the public TCRαβ and/or random clonal expansion of certain private TCRs.

Discussion

In the absence of neutralising antibodies, one way of minimising the effects of a novel influenza virus is to recall pre-existing cross-strain protective $CD8^+$ T cell memory directed at peptides derived from internal proteins shared by many influenza strains. Influenza-specific $CD8^+$ T cells generated by seasonal influenza infection can promote virus elimination and host recovery, leading to a milder disease after infection with distinct strains [2, 8-10, 50]. Following the 2013 H7N9 avian influenza outbreak in China, we analysed immune responses in patients hospitalised with severe and fatal influenza disease at the Shanghai Public Clinical Centre and found that recovery from severe H7N9 disease was associated with diverse response mechanisms driven predominantly by $CD8^+$ T cells [2]. We also provided evidence that the emergence of different effector mechanisms contributed to H7N9 disease resolution and survival. Patients with most rapid recovery had early robust H7N9-specific $CD8^+$ T cell immunity, while those with prolonged hospital stays displayed late recruitment of $CD8^+$ and $CD4^+$ T cells and antibodies, augmented by NK cells. In contrast, individuals who died from severe H7N9 disease had no detectable influenza-specific immunity and no T cell activation, thus illustrating the importance of pre-existing $CD8^+$ T cell memory for protection against severe influenza disease (and death) caused by novel influenza viruses. However, it is far from clear what determines optimal, long-lasting, influenza-specific $CD8^+$ T cell immunity in humans, and how such robust memory $CD8^+$ T cell responses persist throughout the human lifespan, which is particularly important given our aging population and the fact that human

lifespans are increasing. This reflects the relative paucity of data on human influenza-specific $CD8^+$ T cells, especially on the numbers of influenza-specific $CD8^+$ T cells and the underlying TCRαβ characteristics, despite the great body of knowledge available for mice.

Here, we dissected *ex vivo* influenza-specific $CD8^+$ T cell responses and TCRαβ clonal composition in the elderly individuals and compared them to younger adult donors. Our analyses showed that while bulk T cell compartments display increased frequencies of effector/memory T cells and altered transcription factor levels, we observed stable phenotype of influenza-specific $CD8^+$ T cells across different ages. Nevertheless, the numbers of influenza-specific $CD8^+$ T cells are reduced in the elderly, and they are characterized by large non-canonical clonal expansions and reduced usage of preferred TCRαβ signatures (such as TRBV19 and TRAV27) and the public TRBV19-TRAV27 clonotype. Our findings suggest a need for new vaccination strategies to maintain the numbers and optimal TCRαβ features of influenza-specific $CD8^+$ T cell immunity in the elderly individuals.

Enhanced susceptibility to influenza and exacerbated disease severity can reflect over-activation of the innate immune system (cytokine storm, alveolar oedema and pulmonary complications), impaired humoral and cellular immunity [51], and can be influenced by host genetic factors such as HLA [52] or interferon-induced transmembrane protein 3 (IFITM3) [5]. Understanding the key deficits that contribute to severe disease in high-risk groups is needed for understanding how immune interventions might minimise the incidence of severe influenza acute pneumonia. Our study provides new insights into how dysregulated $CD8^+$ T cell immunity, declining influenza-specific $CD8^+$ T cell numbers and perturbed TCRαβ repertoires may contribute to the higher infection rates and more severe disease observed for the elderly.

Using a human-adapted tetramer enrichment technique, naïve antigen-specific T cell precursor frequencies for HLA-A*02:01-restricted NY-ESO-$1_{1157-165}$, HIV Gag $p1_{777-85}$, HCV $Core_{132-140}$, CMV $pp65_{495-503}$ [46] and WT1 [30] antigens in cancer-free and seronegative individuals range between $0.5x10^{-6}$-$5.0x10^{-6}$. In comparison, frequencies of epitope-specific memory $CD8^+$ T cells are ~100-times higher in the range of 1 in 10^{-3}-10^{-4} cells [30, 36, 46]. In our study, this was also the case for influenza-specific memory $CD8^+$ T cells directed at the universal influenza epitopes in adults (pooled mean $2.6x10^{-4}$; range $1.5x10^{-5}$-$1.0x10^{-3}$), but reduced on average by 2.8-fold in the elderly individuals (pooled mean $9.2x10^{-5}$; range $3.7x10^{-6}$-$7.0x10^{-4}$). Although the population coverage by the universal influenza HLAs varies greatly across different ethnicities (16-57%) [26], the maintenance of robust memory $CD8^+$ T cell pools directed at conserved viral regions is of importance for providing broad, long-lasting protection across different influenza strains, especially for the aging population at high-risk from severe influenza disease. Our previous studies in mice show that (1) early priming of influenza-specific $CD8^+$ T cells preserves both the magnitude and TCR repertoire composition for life of the experimental mouse [24], and (2) sequential priming of the $CD8^+$ T cell compartment in the absence of antibody responses greatly reduces influenza-induced morbidity [53]. However, it is still unknown how frequently influenza-specific $CD8^+$ T cells in humans need to be primed to preserve the memory numbers and the optimal TCR repertoires. Recent evidence [29], however, suggests that memory $CD8^+$ T cells are maintained in healthy donors for up to 12 years. Furthermore, it is thought that humans are exposed to influenza viruses every 5-10 years [54], where natural infection would prime/boost T cell compartments. However, this would differ in vaccinated individuals, in whom neutralizing antibodies might prevent regular T cell boosting. However, despite the existing evidence in mice, there are still not enough data to understand how influenza-specific $CD8^+$ T cell immunity is generated and maintained in children. We still do not know

how many times T cells need to be boosted in children for the maintenance of $CD8^+$ T cell numbers, effector function and optimal TCR repertoire. These are important questions for future studies.

The diversity and composition of the peripheral TCRαβ repertoire have important implications for subsequent immune responses in both animal models and human disease [55]. Links between TCR repertoire diversity, public TCR usage, avidity, immune protection and viral escape are well established [56-59]. In mice, the diverse and high peptide/MHC-I avidity of the D^bPA_{224}-specific TCR repertoire "protects" against viral escape during influenza infection. Conversely, emergence of escape variants occurs within the immunodominant D^bNP_{366} epitope, recognised by $CD8^+$ T cells expressing a restricted TCRβ repertoire [60]. The selection of $CD8^+$ T cell escape mutants is driven by selective pressure from $D^bNP_{366}{}^+CD8^+$ T cells as the NP_{366} variants revert to the wild-type NP_{366} sequence in the absence of immune pressure in MHC-mismatched mice [59]. Pre-emptive priming against the influenza viral mutants, however, can expand T cells expressing additional distinct TCRs that are capable of mounting robust recall responses against the viral variants [59]. Published evidence also points to the importance of TCR selection for viral control and disease-associated morbidity in human HIV, HCV, CMV and influenza [61-63]. Although HCMV seropositivity can be one of the factors affecting immunosenescence and T cell responses to co-infecting viruses [64, 65], HCMV status of our donors is unknown. It is most likely as a diverse TCR repertoire provides a greater range of clonotypes with scope for the preferential selection of high avidity TCRs into the immune response. This is particularly advantageous for disease control when diverse TCR repertoires with high peptide/MHC-I avidity TCR clonotypes are capable of promoting superior $CD8^+$ T cell function and recognising escape mutants.

In the current study, we provide the first *ex vivo* data on paired epitope-specific TCRαβ clonotypes in the elderly, showing that the influenza-specific A2/M1$_{58}$$^{+}$TCRαβ repertoires in the elderly adults varies from that in younger adults, with the main features being a reduction in the frequency of the public TRBV19-TRAV27 TCRαβ clonotype, increased proportion of non-shared private TCRαβ signatures, broader usage of TRAV and TRBV segments, and large clonal expansion of private TCRαβ clonotypes with longer CDR3α loops. These are striking alterations within the A2/M1$_{58}$ TCRαβ repertoire, especially with respect to the lack of the prominent public TCRαβ clonotype, which has previously been shown to have a characteristic peg-and-notch mode of A2/M1$_{58}$ peptide recognition [49] and is capable of recognizing both the wild-type peptide as well as the mutated versions of the A2/M1$_{58}$ peptide found in circulating influenza viruses [36]. It is possible that with aging there may be a shift or skewing in the predominant structural basis of A2/M1$_{58}$ recognition by the TCRαβ repertoire, which may have implications for the protective capacity of the A2/M1$_{58}$-specific CD8^{+} T cell response. Selection of public clones (exactly the same TCRs shared between unrelated individuals) is also associated with superior CD8^{+} T cell immunity during HIV-1 infection, while lacking the public clonotypes leads to suboptimal immunity [61]. Thus, large random clonal expansions, rather than preservation of the key public TCRαβ clonotypes, appears to be a feature of the aging TCRαβ repertoire, in accordance with previous studies in mice and humans [25, 66].

One caveat of our study was our inability to directly assess the TCR signalling capacity of epitope-specific CD8^{+} T cells in the younger adults compared to elderly adults. Due to the low frequencies of tetramer-specific CD8^{+} T cells in 100μl of blood, our *ex vivo* ZAP-70 signalling assay could not be modified to determine TCR-mediated ZAP-70 phosphorylation efficacy in influenza-specific CD8^{+} T cells. Although, based on our previous studies in mice [67, 68], we speculate that the M1$_{58}$-specific TCR repertoires lacking the

"best-fit" immunodominant public clonotypes in the elderly donors, might have less of an effector function as compared to younger adult $M1_{58}$-specific TCR repertoires predominantly encompassing the public clonotypes.

The future design of T cell-based vaccination strategies should provide effective long-lasting protection across the lifespan of an individual. The changes to the composition and protective abilities of T cell memory subsets across the human lifespan require future studies to understand what constitutes optimal T cell memory. The potential complicating factors in the maintenance of life-long immunity are the changes at the cellular and molecular levels, which occur during aging and have been associated with impaired immune responsiveness in the elderly. However, studies to date suggest that age-related defects in T cell immunity predominantly affect the generation and maintenance of T cell memory in old age. This indicates that counteracting the increased immune susceptibility in the elderly may require either the development of vaccines to be administered early in life to generate long-lasting T cell memory, even for pathogens likely to be encountered later life, or the development of strategies to prevent age-related perturbations in T cell immunity. Either approach might potentially boost antigen-specific $CD8^+$ T cell numbers and maintain optimal TCR$\alpha\beta$ repertoires for life.

Authorship

T.H.O.N., S.S., S.G., J.C., L.L., and K.K. designed research; T.H.O.N., S.S., N.L.B., E.J.G., E.B.C., M.K., S.G., and L.L. performed research; T.H.O.N., S.S., N.L.B., S.A.V., L.L., and K.K. analysed data; A.J. provided ZAP-70 assay and expertise; M.L. and J.C. provided crucial donor samples; T.H.O.N., S.S., L.L., and K.K. wrote the paper. All authors read and edited the manuscript.

Acknowledgments

Thank you to Thakshila Amarasena and Sheilajen Alcantara for their technical assistance. We thank all donors for donating blood for this study and to Bernie McCudden for collecting blood. Thank you to the clinical research midwives Genevieve Christophers, Gabrielle Pell, and Rachel Murdoch for cord blood sample collection; and the Obstetrics and Midwifery staff of the Mercy Hospital for Women for their co-operation. We thank Matthew Caverley and Paul Thomas for their technical expertise in TCR sequence analysis. S.S. is a recipient of the Victoria India Doctoral Scholarship and Melbourne International Fee Remission Scholarship (MIFRS), University of Melbourne. E.J.G. is an Australian National Health and Medical Research Council (NHMRC) CJ Martin Fellowship. E.B.C. is an NHMRC Peter Doherty Fellow. M.K. is supported by a Melbourne International Research Scholarship (MIRS) and MIFRS. M.L. is supported by an NHMRC Career Development Fellowship (ID 1047025). K.K. is supported by the NHMRC Program Grant (ID 1071916) and the NHMRC Senior Research Fellowship Level B.

Conflict of Interest Disclosure

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Changes in $CD8^+$ and $CD4^+$ T cell compartments with aging. $CD8^+$ and $CD4^+$ T cell frequencies and phenotypes were compared between human cord blood, younger adults and elderly adults. (A) Representative FACS plots show four subsets of $CD8^+$ and $CD4^+$ T cell differentiation in a cord blood (CB), young adult (AD) and elderly adult donor (ED) based on CD45RA versus CD27 expression. (B) Frequency of (i) $CD45RA^+CD27^+$, (ii) $CD45RA^+CD27^-$, (iii) $CD45RA^-CD27^+$ and (iv) $CD45RA^-CD27^-$ subsets plotted against age for $CD8^+$ (black circles) and $CD4^+$ T cells (red squares). *P*-values in black and red are shown for $CD8^+$ and $CD4^+$ T cells, respectively (Spearman rank correlation). (C) Histograms of (i) Eomes and (ii) T-bet expression on $CD8^+$ (top panels) and $CD4^+$ T cells (bottom panels) in the presence or absence of CD27 co-expression. (D) Box-and-whisker plots of (i) $Eomes^{hi}$ and (ii) $T\text{-}bet^{hi}$ populations in $CD27^+$ and $CD27^-$ subpopulations of $CD8^+$ and $CD4^+$ T cells in AD (red, n=4) versus ED donors (blue, n=5). Rectangles represent the second and third quartiles separated by the median horizontal line. Lower and upper quartiles are shown as vertical lines either side of the rectangle (*=$p<0.05$, Mann-Whitney).

Figure 2. Phosphoflow measurement of CD3-dependent ZAP-70 in T cell subsets. (A) Representative histograms are shown for ZAP-70 expression in T cell subsets and non-T cells following CD3 cross-linking in an AD and ED donor at indicated times. Right panel depicts representative FACS plots of $CD3^-$ non-T cells and $CD3^+$ T cells gated on viable lymphocytes. (B) Fold change in ZAP-70 geometric MFI from baseline (0 sec) at indicated times in $CD8^+$ and $CD4^+$ T cells for ED (blue lines, n=17) and AD (red lines, n=4) donors, and (C) within $CD8^+$ and $CD4^+$ T cell differentiation subsets based on CD45RA and CD27 expression. Of note, ZAP-70 measurement was not included for the effector ($CD45RA^+CD27^-$) $CD4^+$ T cell population, as this subset was minimally expressed in $CD4^+$ T cells. (D) IFNγ and TNF cytokine expression in $CD8^+$ and $CD4^+$ T cells after stimulation of

younger adult (red, n=7) and elderly adult (blue, n=7) PBMCs for 6hrs *ex vivo* with PMA and Ionomycin. Error bars represent standard error of the mean. Rectangles represent the second and third quartiles separated by the median horizontal line. Lower and upper quartiles are shown as vertical lines either side of the rectangle. Abbreviations: s, seconds; min, minutes.

Figure 3. Lower frequencies of elderly influenza-specific CD8$^+$ T cells across universal influenza HLAs. PBMCs from elderly adults (ED) and younger adults (AD) were enriched for antigen-specific CD8$^+$ T cells using TAME. (A) Representative FACS plots of enriched fractions of epitope-specific CD8$^+$ T cells in AD and ED donors. Cells were gated as viable CD14$^-$CD19$^-$CD3$^+$CD8$^+$tetramer$^+$ cells. (B) Precursor frequencies of epitope-specific CD8$^+$ T cells of total CD8$^+$ T cells in younger adult (closed symbols) and elderly adult donors (open symbols) for A2/M1$_{58}^+$CD8$^+$ T cells (AD: n=27; ED: n=20), B27/NP$_{383}^+$CD8$^+$ (AD: n=5; ED: n=3), and combined B8/NP$_{225}^+$CD8$^+$ (AD: n=1; ED: n=3) and B57/NP$_{199}^+$CD8$^+$ (AD: n=2; ED: n=2) frequencies (due to a limited number of donors), where 1x10^{-4} represents a frequency of 1 in 10,000 CD8$^+$ T cells. Mean and *p*-value symbols (*=p<0.05; Student-t test) are shown. **(C) (i)** Representative FACS plots of CD27 and CD45RA profiling of TAME-enriched A2/M1$_{58}$-specific CD8$^+$ T cells (in red) superimposed onto total CD8$^+$ T cells (in gray) in AD and ED donor. **(ii)** CD45RA$^-$CD27$^{+/-}$ memory profiles of TAME-enriched A2/M1$_{58}$-specific CD8$^+$ T cells in AD and ED donors are comparable.

Figure 4. *In vitro* proliferation of A2/M1$_{58}$-specific CD8$^+$ T cells. (A) Representative FACS plots of a young adult (AD48) and elderly adult (ED25) donor showing proliferation of expanded A2/M1$_{58}$-tetramer$^+$CD8$^+$ T cells by the loss of cell trace violet over time. Cells were gated on total CD8$^+$ T cells and the values indicate percentages. (B) Total numbers and (C)

fold increase of A2/M1$_{58}$-tetramer^{+}CD8^{+} T cells from first detectable tetramer^{+} time-point (day 3 or 4), following *in vitro* expansion in AD (red lines) and ED donors (blue lines).

Figure 5. Large expansions of private A2/M1$_{58}^{+}$CD8^{+} TCRαβ clonotypes in the elderly. Viable CD19^{-}CD14^{-}CD3^{+}CD8^{+}A2/M1$_{58}$-tetramer^{+} T cells from three ED donors were enriched by TAME and then single-cell sorted for TCRαβ analysis. (A) FACS plots of enriched fraction of A2/M1$_{58}$-tetramer^{+}CD8^{+} T cells gated on CD3^{+} T cells. Percentages in red are based on gated CD8^{+} T cell population. (B) Pie charts of TRBV and TRAV gene usage. (C) Frequency of TRBV19 and TRAV27 gene usage amongst AD (numbers in red indicate age) [36] and ED donors. (D) Distribution of CDR3α and CDR3β amino acid (a.a.) lengths in AD and ED donors. CDR3 lengths of AD donors were taken from [36]. (E) Complete repertoire of paired TRBV-TRBJ/TRAV-TRAJ clonotype frequencies are shown. Clonotypes in bold represent the dominant clonotypes observed for each ED donor. Clonotype shaded in blue (clone Y) represent the public A2/M1$_{58}^{+}$TCRαβ clonotype. Long CDR3α a.a. lengths in red are not commonly detected in younger adult donors [36] (ND, not determined).

Figure 6. Dominant private TCRαβ clonotypes abundant in elderly. (A) Frequency of public A2/M1$_{58}^{+}$TCRαβ clonotype is predominantly observed in younger adults [36], but not the elderly. (B) Paired TRBV-TRBJ/TRAV-TRAJ frequencies of dominant TCRαβ clones observed for each ED donor are shown. (C) Circos plots of paired TRAV-TRAJ (Vα-Jα; black labels) and TRBV-TRBJ (Vβ-Jβ; blue labels) TCR clonotype analysis for each ED donor. Band width is proportional to the frequency and each band represents a single clonotype. Red bands represent dominant private A2/M1$_{58}^{+}$TCRαβ clones from (B). Blue

bands represent shared TCR clone between DMC9 and DMC18 (clone C). Grey bands represent other remaining clonotypes listed in Fig. 5D.

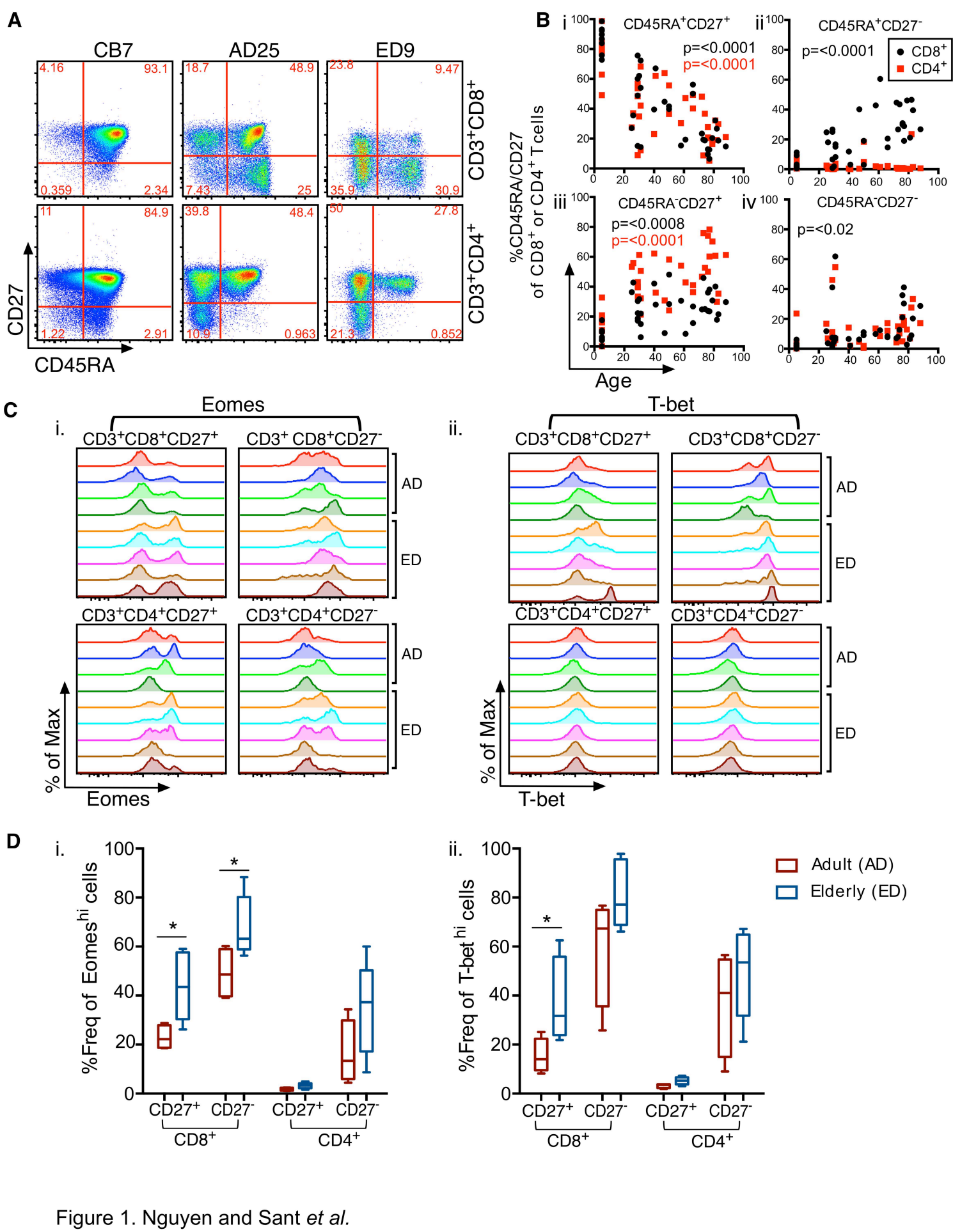


Figure 1. Nguyen and Sant *et al.*

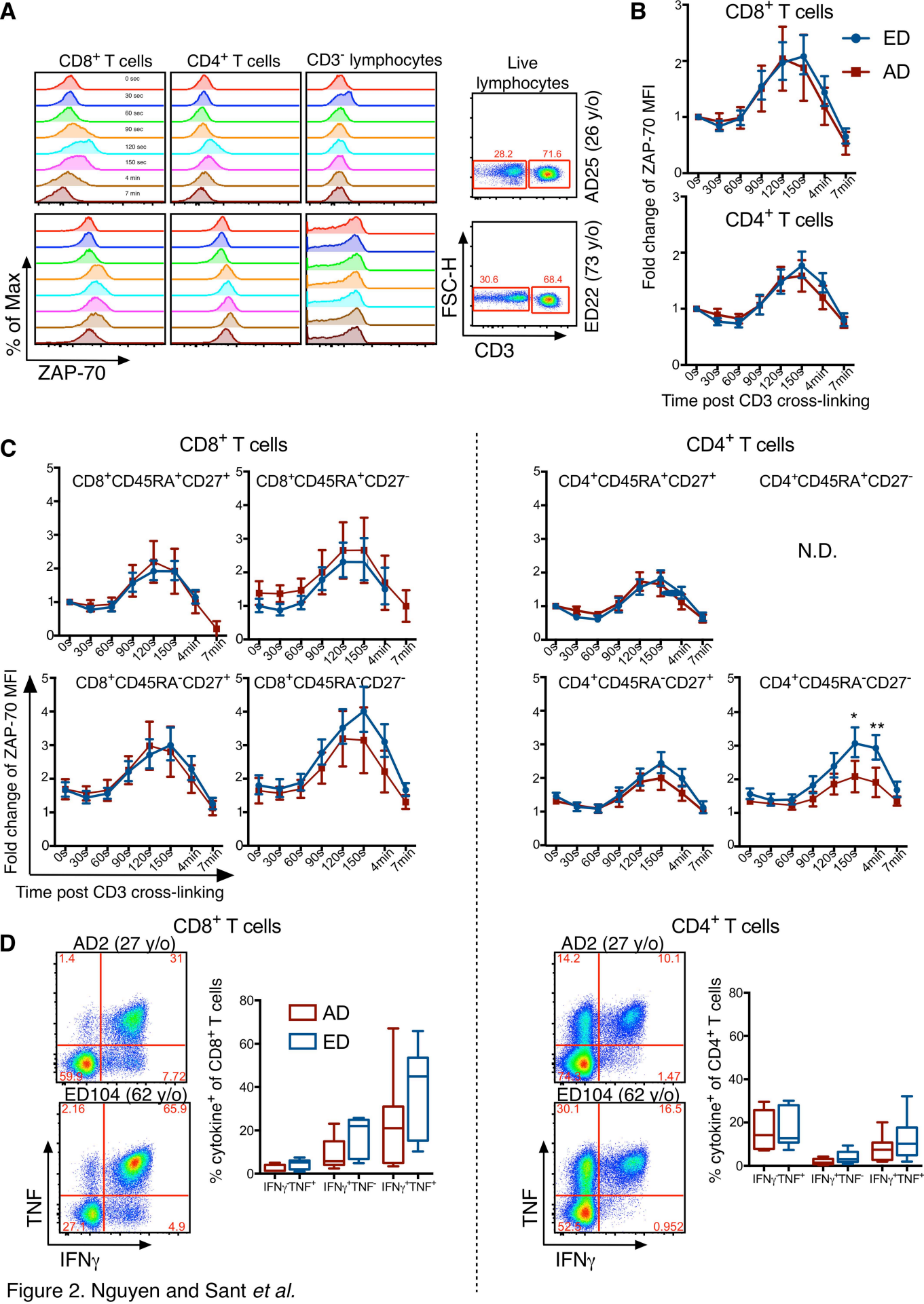


Figure 2. Nguyen and Sant *et al.*

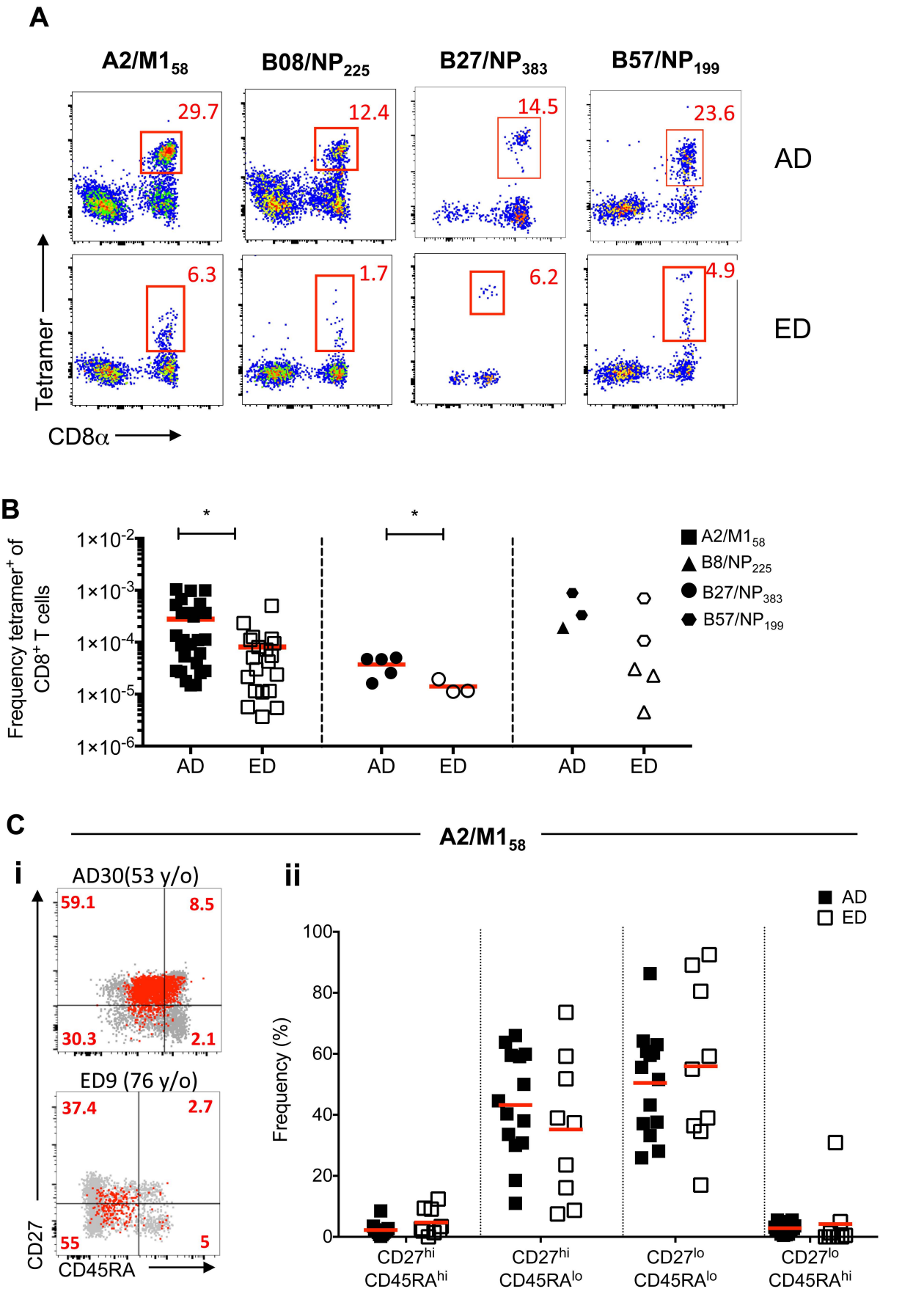


Figure 3 Nguyen and Sant *et al.*

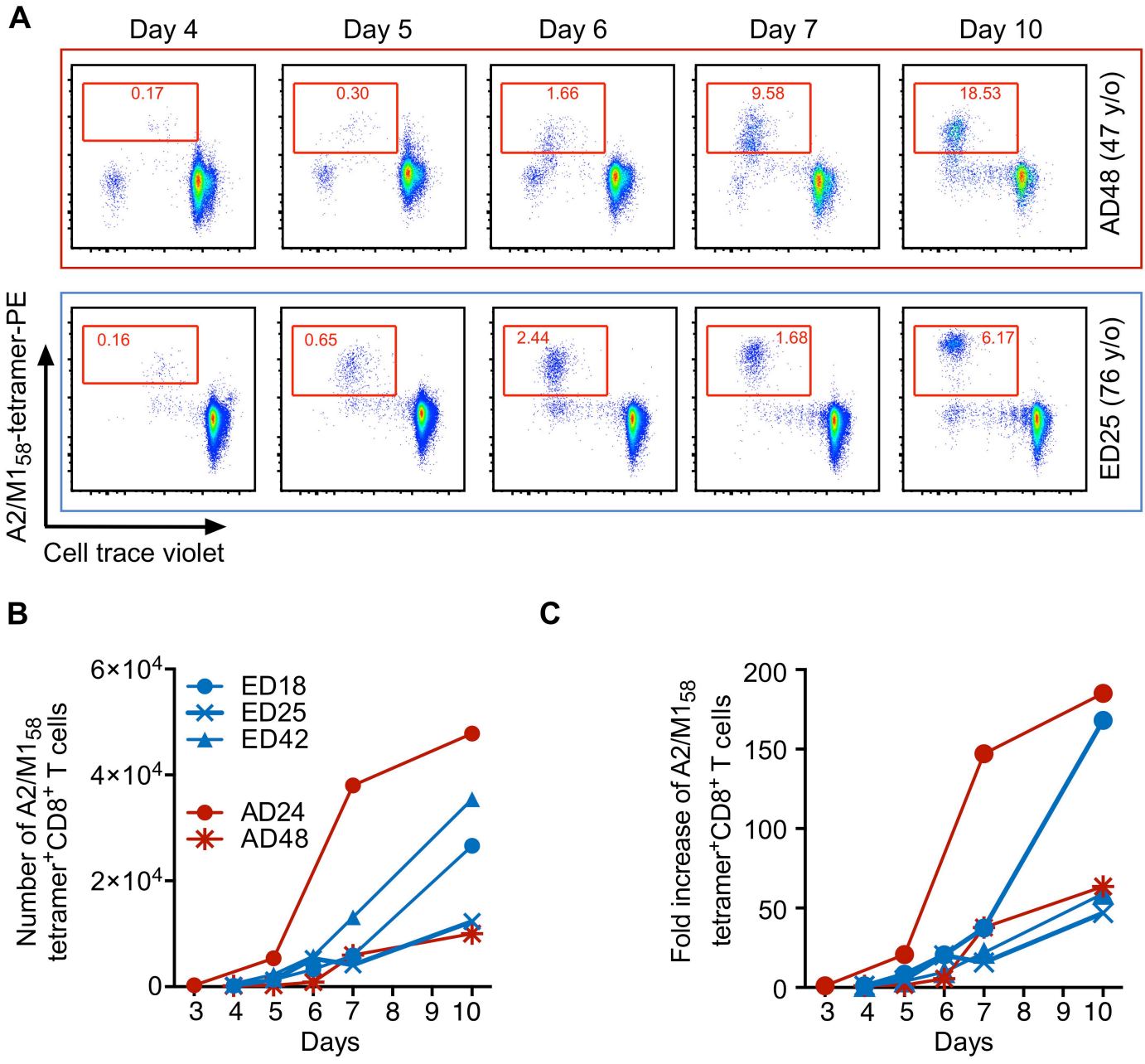
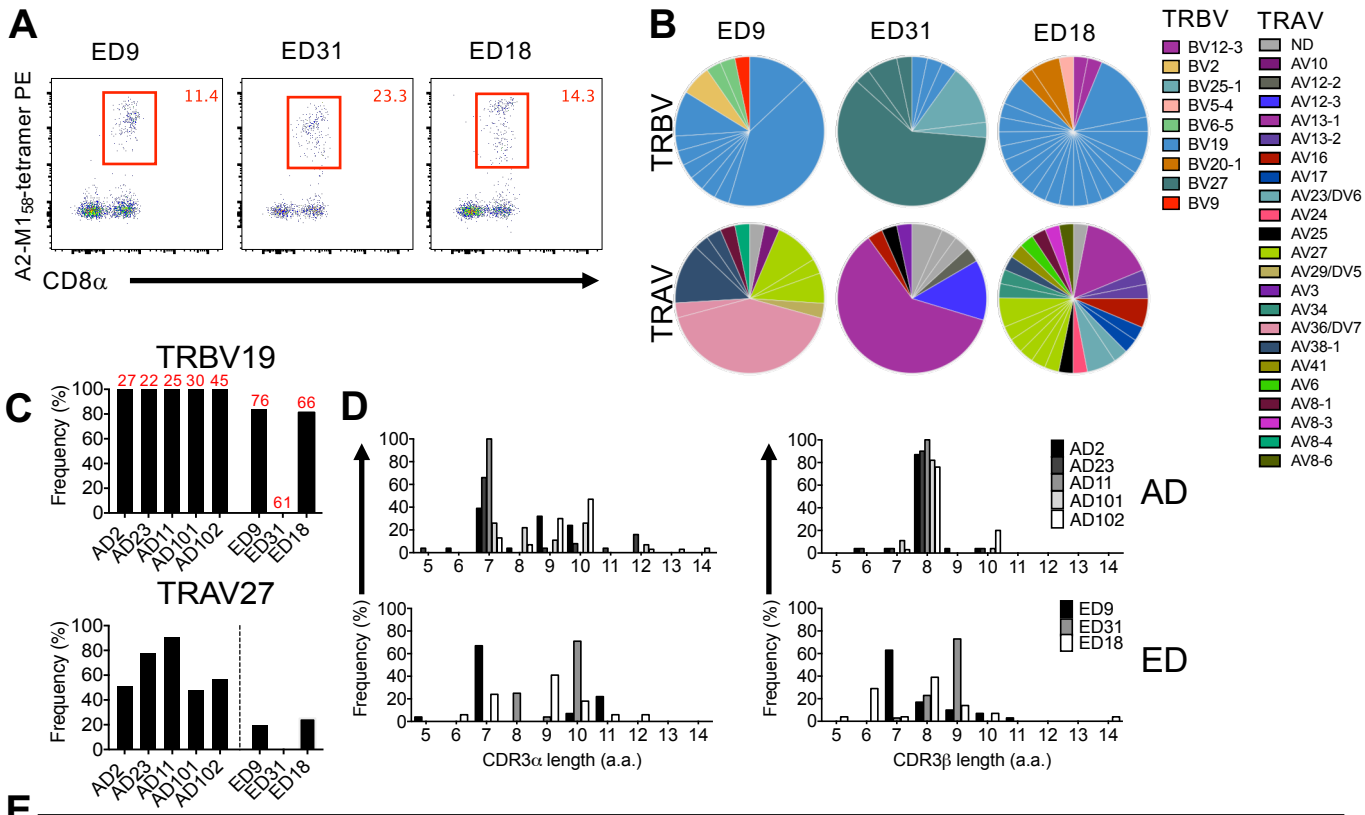


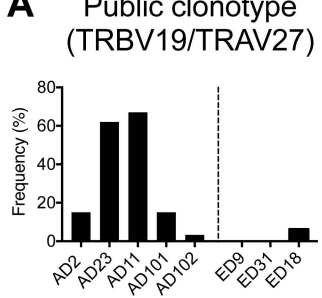
Figure 4. Nguyen and Sant et al.



E

Clone ID	TRBV	TRBJ	CDR3β	Length	TRAV	TRAJ	CDR3α	Length	ED9	ED31	ED18
A	**19**	**1-2**	**SIGVAGY**	**7**	**36/DV7**	**49**	**EDGTGNQ**	**7**	**41.6**		
B	**19**	**1-2**	**SIGVAGY**	**7**	**38-1**	**52**	**KDAGGTSYGKL**	**11**	**12.9**		
C	**19**	**2-7**	**SIRSSYEQ**	**8**	**27**	**42**	**AGSQGNL**	**7**	**9.7**		**6.3**
D	**2**	**1-1**	**SEAPGLTNTE**	**10**	**27**	**38**	**ND**		**6.5**		
E	6-5	1-2	SYLGVDGY	8	8-4	49	NTGNQ	5	3.2		
F	19	2-7	SIRSAYEQ	8	27	37	WLGSSTAGKL	10	3.2		
G	9	2-3	SVEGGGLTDTQ	11	29/DV5	32	SQGGGATNKL	10	3.2		
H	6-5	2-7	ND		10	10	ND		3.2		
I	19	1-2	SIGVAGY	7	ND		ND		3.2		
J	19	1-2	SIGVAGY	7	38-1	52	RGAGGTSYGKL	11	3.2		
K	19	1-2	SIVGTGYGY	9	8-1	45	GGGADGL	7	3.2		
L	19	1-2	SIGVAGPTP	9	38-1	52	KDAGGTSYGKL	11	3.2		
M	19	1-2	SIVGTGYGY	9	36/DV7	32	GGATNKL	7	3.2		
N	**27**	**2-4**	**SPLAGWDIQ**	**9**	**13-1**	**54**	**SMIIQGAQKL**	**10**		**60.0**	
O	**25-1**	**2-3**	**ARDRDNTQ**	**8**	**12-3**	**26**	**SAGNYGQN**	**8**		**13.4**	
P	**27**	**2-4**	**SPLAGWDIQ**	**9**	**ND**		**ND**			**6.6**	
Q	27	2-1	RTLDSYNE	8	3	9	RDKGGGKT	8		3.3	
R	27	2-7	SGQGLEQ	7	ND		ND			3.3	
S	19	2-3	SRGSTDTQ	8	16	9	SEGRRHCNP	9		3.3	
T	19	2-1	SIGGVSYNE	9	25	27	LEANAGKS	8		3.3	
U	25-1	2-3	ARDRDNTQ	8	ND		ND			3.3	
V	19	2-7	SIGTTAEQ	8	12-2	42	KEGSQGNL	8		3.3	
W	**19**	**2-3**	**SSTDTQ**	**6**	**25**	**42**	**NYGGSQGNL**	**9**			**15.6**
X	**20-1**	**1-1**	**ND**		**41**	**58**	**GEEETSGSRL**	**10**			**6.3**
Y	**19**	**2-7**	**SIRSSYEQ**	**8**	**27**	**42**	**GGSQGNL**	**7**			**6.3**
Z	19	2-1	SIGPGE	6	6	12	ND				3.1
AA	5-4	2-3	SDGTSGRGDGTDTQ	14	13-1	33	SLSGYQLI	8			3.1
AB	20-1	1-5	ND		16	29	ND				3.1
AC	19	2-3	SSRSTDTQ	8	ND		ND				3.1
AD	19	1-5	SGRSNQPQ	8	13-2	53	ND				3.1
AE	19	1-6	ND		23/DV6	48	SMREGNEKL	9			3.1
AF	19	1-2	SGGGY	5	17	42	ND				3.1
AG	19	2-1	SPLAGGHNE	9	24	49	QTSNQ	6			3.1
AH	19	2-7	SIRSSYEQ	8	27	42	GGSQRNL	7			3.1
AI	19	1-2	SAGSYGY	7	38-1	52	TNAGGTSYGKL	11			3.1
AJ	19	2-7	SIRSAYEQ	8	8-6	42	SGSQGNL	7			3.1
AK	19	2-7	SSSYEQ	8	27	42	SAGGSQGNL	9			3.1
AL	19	2-1	SMSSGSYNE	9	8-1	20	ND				3.1
AM	19	2-7	SIRSAYEQ	8	27	37	GRTSKAGKL	9			3.1
AN	19	2-7	AIRSSYEQ	8	17	24	DWTDSWGKL	9			3.1
AO	12-3	2-2	SPLLAGGTGE	10	34	37	ASV#GSSNTGKL	12			3.1
AP	19	2-7	SSSYEQ	8	8-3	30	ND				3.1
AQ	19	2-7	SIRSSYEQ	8	27	37	AIGSSNTGKL	10			3.1
AR	19	1-5	SIHAGNQPQ	9	23/DV6	7	ND				3.1
AS	19	2-1	SIAGTTYNE	9	13-2	52	NTGANAGGTSYGKL	14			3.1
AT	12-3	2-2	SPLLAGGTGE	10	34	37	DQCGALATQGKL	12			3.1
							Number of αβ pairs		**31**	**30**	**32**

Figure 5 Nguyen and Sant *et al.*



B

Clone ID	TRBV	TRBJ	CDR3β	Length	TRAV	TRAJ	CDR3α	Length	ED9	ED31	ED18
A	19	1-2	SIGVAGY	7	36/DV7	49	EDGTGNQ	7	41.6		
B	19	1-2	SIGVAGY	7	38-1	52	KDAGGTSYGKL	11	12.9		
C	19	2-7	SIRSSYEQ	8	27	42	AGSQGNL	7	9.7		6.3
D	2	1-1	SEAPGLTNTE	10	27	38	ND		6.5		
N	27	2-4	SPLAGWDIQ	9	13-1	54	SMIIQGAQKL	10		60.0	
O	25-1	2-3	ARDRDNTQ	8	12-3	26	SAGNYGQN	8		13.4	
P	27	2-4	SPLAGWDIQ	9	ND		ND			6.6	
W	19	2-3	SSTDTQ	6	25	42	NYGGSQGNL	9			15.6
X	20-1	1-1	ND		41	58	GEEETSGSRL	10			6.3
Y	19	2-7	SIRSSYEQ	8	27	42	GGSQGNL	7			6.3

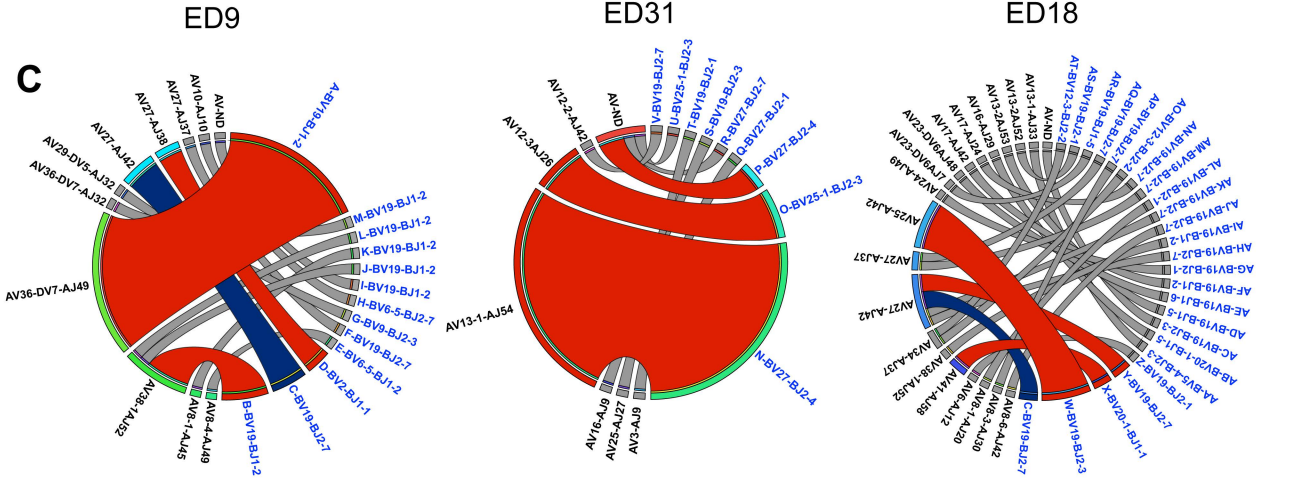


Figure 6 Nguyen and Sant *et al.*

TABLE 1. Cohort demographics of all donors used in the study

Donor code	Age	HLA-A or -A*	HLA-B or -B*	Figure	Vaccination status**	Year of vaccination (if known)
Cord blood						
CB1	0			Fig. 1A and B	NA	-
CB2	0			Fig. 1B	NA	-
CB3	0			Fig. 1B	NA	-
CB4	0			Fig. 1B	NA	-
CB5	0			Fig. 1B	NA	-
CB6	0	02:01, 31:01	27:05, 38:01	Fig. 1B	NA	-
CB7	0			Fig. 1A and B	NA	-
CB8	0			Fig. 1B	NA	-
Adult healthy volunteers						
AD2	27	02:01, 11:01	35:01, 39:01	Fig. 3B, 5B, C, D and 6A	yes	2016,
AD3	41	01:01, 68:01	35:03, 37:01	Fig. 1A and B	yes	2017
AD4	31	02:01, 11:01	40:01, 44:02	Fig. 3B, 5C i and ii	yes	2014
AD5	31	01:01, 26:01	08:01, 44:03	Fig. 1A and B	yes	2014
AD6	40	01:01, 03:01	08:01, 15:01	Fig. 1A and B	yes	
AD11	25	02:01, 11:01	3501, 39:01	Fig. 5B, C, D and 6A	yes	2014
AD15	31	01:01, 11:01	35:01, 52:01	Fig. 1A and B	yes	2014, 2015
AD16	46	02:01, 11:01	35:03, 44:02	Fig. 3B	yes	
AD17	29	32:01	07:02, 44:02	Fig. 1C and D	yes	2010
AD18	42	02:01, 03:02	18:01, 35:08	Fig. 3B	yes	2015
AD19	28	01:01	08:01	Fig. 3A and B	yes	2014
AD21#	29	26:01, 33:03	40:02, 52:01	Fig. 1B	yes	2014, 2015, 2016
AD21#	31	26:01, 33:03	40:02, 52:01	Fig. 1B, 2B and C	yes	2014, 2015, 2016
AD25#	26			Fig. 1A and B	yes	2015, 2016
AD25#	29			Fig. 1B, 2B and C	yes	2015, 2016
AD26	25	11:01, 26:01	14:01, 27:05	Fig. 1B, 2B and C	yes	2015, 2016
AD27	29	02:01	15:01, 44:02	Fig. 1B	yes, prior to 2009 pH1N1	2008
AD28	30	02:07, 11:01	15:02, 57:01	Fig. 1B, 2B and C	yes	2014, 2015, 2016, 2017
AD40	29	03:01, 33:03	35:01, 44:03	Fig. 1B	yes	2014, 2015, 2016
AD51	31	02:01, 33:03	07:02, 27:02	Fig. 3B	yes	2015, 2016
AD52	47	02:01, 03:01	07:02, 55:02	Fig. 1A and B	yes	2015
AD69	58	2, 3	14, 27	Fig. 1B and 3B	yes	2011, 2012, 2013, 2014, 2015, 2016, 2017
AD101	30	02:01, 34:01	13:01, 40:01	Fig. 5C, D and 6A	ND	-
AD102	45	02:01, 34:01	13:01, 56:01	Fig. 5C, D and 6A	ND	-
Adult buffy packs						
AD13	50	02:01, 68:01	27:05, 15:18	Fig. 3B	ND	-
AD23	22	02:01, 68:01	44:02, 51:01	Fig. 5B, C, D and 6A	ND	-
AD24	48	01:02, 02:01	08:01, 44:02	Fig. 4B and C	ND	-
AD30	53	02:01, 30:04	15:03, 57:01	Fig. 3A, B, 5C i and ii	ND	-
AD29	54	03:01, 26:01	07:02, 27:05	Fig. 3B, 5C i and ii	ND	-
AD31	50	02:01, 26:01	07:02, 27:05	Fig. 3A and B	ND	-
AD34	30	02:01, 03:01	40:01 (60), 56:01	Fig. 3B, 5C i and ii	ND	-
AD36	25	02:01, 03:01	07:02, 40:01	Fig. 3B, 5C i and ii	ND	-
AD48	47	02:01, 29:02	15:01, 35:01	Fig. 4A, B and C	ND	-
AD60	54	02:01, 03:01	35:01, 57:01	Fig. 3B, 5C i and ii	ND	-
AD71	56	02:01	07:02, 57:01	Fig. 3A and B	ND	-
AD73	45			Fig. 1D	ND	-
AD76	31	02:01, 02:05	44:02, 50:01	Fig. 3B, 5C i and ii	ND	-
AD78	50			Fig. 1D	ND	-
AD86	51	02:01	07:02, 39:01	Fig. 3B, 5C i and ii	ND	-
AD87	58	02:01, 32:01	40:01, 44:02	Fig. 3B, 5C i and ii	ND	-
AD88	22	02:01, 11:01	15:02, 54:01	Fig. 3B, 5C i and ii	ND	-
AD94	25	02:01, 24:02	27:05, 35:01	Fig. 3B, 5C i and ii	ND	-
AD95	43	02:01	07:02, 44:02	Fig. 3B, 5C i and ii	ND	-

AD97	36	02:01, 02:05	07:02, 44:01	Fig. 3B, 5C i and ii	ND	-
AD100	45	02:01	07:02, 44:02	Fig. 3B, 5C i and ii	ND	-
EM6	<60	2	18	Fig. 3B	ND	-
EM4	<60	02:01, 2501	07:02, 18:01	Fig. 3B	ND	-
EM5	<60	2, 24	7, 40	Fig. 3B	ND	-
Elderly adults						
ED1	87	02:01, 31:01	40:01, 44:02	Fig. 3B	yes	2009,p2009, 2010, 2011, 2012, 2013, 2014, 2015
ED3	73	02:01	07:02, 44:02	Fig. 2B, C, 3B, 5C i and ii	yes	2007,2009,p2009,2010,2011,2012,2013,2014
ED4	81	01:01, 24:02	08:01, 57:01	Fig. 2B, C, 3A and B	yes	2007,2009,2010,2011,2013,2014,2015
ED5	84	02:01, 11:01	13:02, 44:02	Fig. 3B	ND	
ED6	75	01:01, 31:01	07:02, 44:03	Fig. 2B and C	ND	
ED7	81			Fig. 1D	ND	
ED8	78			Fig. 2B and C	ND	
ED9	76	02:01, 03:01	37:01, 44:02	Fig. 1A, B, 5A, B, C i, C ii, D, E, 6A, B and C	ND	
ED11	78			Fig. 2B and C	ND	
ED12	65	02:01, 24:02	27:05, 40:01	Fig. 3B	ND	
ED15	66	01:01, 02:01	08:01, 27:05	Fig. 3B	ND	
ED16	72	01:01, 29:02	08:01, 44:03	Fig. 1B, 2B and C	ND	
ED17	81	11:01, 23:01	27:05, 44:03	Fig. 2B, C, 3A and B	ND	
ED18	66	02:01	15:01, 44:02	Fig. 4B, C, 5A, B, C, D, E, 6A, B and C	ND	
ED19	83	01:01, 02:01	08:01, 13:02	Fig. 2B, C, 3A and B	yes	2009,p2009,2010,2011,2012,2013
ED20	82	02:01, 03:01	27:05, 44:02	Fig. 3B	yes	2009,p2009,2010,2011,2012,2013
ED21	79	01:01	37:01, 57:01	Fig. 1B, 2B and C	yes	2008,2009,p2009,2010,2011,2012,2013
ED22	73	03:01, 33:03	15:01, 58:01	Fig. 1B, 2B and C	yes	2008,2009,p2009,2010,2011,2012,2013
ED23	74	01:01, 68:01	08:01, 44:02	Fig. 1B, 2B and C	yes	2007,2008,2009,p2009,2010,2011,2012,2013
ED24	77	02:01	35:01, 40:01	Fig. 2B, C, 3B, 4A, B, 5C i and ii	yes	2008,2009,p2009,2010,2011,2012,2013
ED25	76	02:01, 03:01	07:02, 39:06	Fig. 2B, C, 3B, 4A, B, C, 5C i and ii	yes	2009,2010,2011,2012,2013
ED26	77	02:05, 26:01	38:01, 49:01	Fig. 1B, 2B and C	yes	2007,2009,p2009,2010,2011,2012,2013
ED27	88	03:01, 33:01	14:02, 15:01	Fig. 1B, 2B and C	yes	2007,2009,2010,2011,2012,2013
ED28	80	02:01, 11:01	27:05, 55:01	Fig. 2B, C and 3B	yes	2005, 2007, 2009,2010,2011,2012,2013, p2009
ED31	61	02:01, 03:01	07:02, 50:01	Fig. 3A, B, 5A, B, C, D, E, 6A, B and C	yes	
ED33	82	03:01, 30:02	18:01, 35:01	Fig. 1D	yes	2015
ED36	66	01:01, 03:01	08:01, 40:02	Fig. 1A and B	yes	2013,2015
ED37	82	02:01, 11:01	08:01, 41:02	Fig. 3B	yes	2007,2009,p2009,2010,2012,2013,2015
ED38	68	02:01	15:01	Fig. 1D, 2B, C, 3B, 5C i and ii	yes	2012,2013,2014,2015
ED39	84	01:01	08:01, 57:01	Fig. 3B, 5C i and ii	yes	2010,2011,2012,2013,2014,2015
Elderly adult buffy packs						
ED102^	70			Fig. 1C and D	ND	-
ED103^	68			Fig. 1D	ND	-
ED104	62			Fig. 2D	ND	-
ED41^	69	02:01, 32:01	07:02, 44:03	Fig. 3B, 5C i and ii	ND	-
ED42^	62	02:01	07:02, 44:02	Fig. 3B, 5C i and ii	ND	-
ED43^	67	01:01, 02:01	13:02, 44:32	Fig. 3B, 5C i and ii	ND	-

* Molecular resolution of HLA class I antigens (4-digit) are shown where available.

** Vaccinated, post 2009 pandemic H1N1.

Of note, AD21 and AD25 were bled twice.

NA: Not applicable; ND: Not determined.

^ Elderly adult donors obtained from buffy packs, therefore vaccination status ND.

HLA typing in red indicates donor and their HLA used for TAME (Fig. 3).

TABLE 2. Highly conserved “universal” influenza epitopes used in this study

HLA restriction	Peptide	Sequence	Epitope acronym
HLA-A*02:01	$M1_{58-66}$	GILGFVFTL	$A2/M1_{58}$
HLA-B*08:01	$NP_{225-233}$	ILKGKFQTA	$B8/NP_{225}$
HLA-B*27:05	$NP_{383-391}$	SRYWAIRTR	$B27/NP_{383}$
HLA-B*57:01	$NP_{199-207}$	RGINDRNFW	$B57/NP_{199}$

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Title:
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Date:
2018-02-01

Citation:
Nguyen, THO; Sant, S; Bird, NL; Grant, EJ; Clemens, EB; Koutsakos, M; Valkenburg, SA; Gras, S; Lappas, M; Jaworowski, A; Crowe, J; Loh, L; Kedzierska, K, Perturbed CD8(+) T cell immunity across universal influenza epitopes in the elderly, JOURNAL OF LEUKOCYTE BIOLOGY, 2018, 103 (2), pp. 321 - 339

Persistent Link:
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File Description:
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