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Early T-cell differentiation with well-maintained function across the adult lifecourse in sub-Saharan Africa

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Abstract

Immune senescence is a significant contributor to health problems in the developed world and may be accelerated by chronic viral infections. To date, there have been few studies of immune function in healthy older people in sub-Saharan Africa. We assessed T-cell and B-cell phenotypes and immune responses to CMV, EBV and influenza virus in Malawians aged 20-69 years. Notably, the proportion of naïve ($CCR7^{+}CD45RA^{+}$) CD4 and CD8 T-cells was only 14% of the lymphoid repertoire even in donors aged under 30 years, but did not decrease further with age. A small increase in the late differentiated ($CD27^{-}CD28^{-}$) CD8 T-cell subpopulation was observed in older donors but the CD4:CD8 T-cell ratio remained stable in all age groups. Interestingly, the regulatory ($CD25^{hi}FoxP3^{hi}$) T-cell subpopulation was small in all age groups and we observed no age-associated accumulation of cells expressing the senescence- and exhaustion-associated markers CD57 and PD-1. We assessed functional T-cell responses to mitogenic and viral antigenic stimulation by expression of CD154, IFNγ, TNFα, IL-2 and IL-17 and proliferation. All responses were robust across the lifecourse although we observed an age-associated shift from IFNγ to TNFα in the response to EBV. In summary we found the naïve T-cell subpopulation of young adult Malawians was smaller than in their contemporaries in high-income settings but remains stable thereafter, and that lymphocyte function is retained across the lifecourse. These observations indicate that studies of the genetic and environmental factors influencing immune function in different environments may be provide insights into minimizing immune ageing.

Introduction

Infection remains a major cause of morbidity and mortality in older people and there is ongoing interest in the mechanisms and clinical importance of immune senescence. Many studies have investigated phenotypic and functional variation in the human immune system throughout the life course (1–6) and have identified important determinants that can help to

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guide interventions such as vaccination protocols. However, such studies have been undertaken almost entirely within high-income populations and as longevity is now increasing dramatically in low-income countries (7), there is a need to extend these analyses to these settings.

To date, research on immune function in low-income settings has focused largely on infancy and on the response to specific infections. Several studies have shown qualitative differences between immune development in the first two years of life in infants in sub-Saharan Africa compared to high-income countries (8–11). However, there is very little information on phenotypic and functional features of immune function through the adult life course in low-income settings.

Malawi is one of the world's poorest countries (https://data.worldbank.org/indicator/NY.GDP.PCAP.CD?locations=MW) with an average life expectancy at birth of only 58 years, driven by high levels of mortality early in life and related primarily to childhood infectious diseases and HIV infection in young adulthood (12). Poor nutrition is also common and impairment of growth potential is seen in up to 50% of the population. Nonetheless, healthy individuals who survive into adulthood can reasonably expect to live to at least 70 years (13).

The herpesviruses cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are very common and persistent infections and each has a profound impact on many immune parameters. This is driven partly by the phenomenon of memory inflation: the accumulation of CMV-specific T-cells through the life course (6) and has been associated with the development of an ‘immune risk phenotype’ in older people with features such as an inverted CD4:8 ratio and accumulation of CD8 effector T-cells associated with early mortality (14–16). Most African infants are infected with both viruses by four years of age (9, 17, 18) and as CMV infection induces a substantial T-cell response within weeks of primary infection (8, 19) it was of particular interest to assess how the immune response was maintained within adults in the sub-Saharan setting. Likewise, influenza virus is also endemic throughout Africa (20), but little is known regarding influenza-specific immune responses within adults in this setting.

We therefore investigated major cell phenotypic and functional immune parameters within adults in Malawi between the age of 20 and 70 years to assess how these contrast with findings from individuals from higher income settings. In particular, we compared the number and ratio of major lymphoid subsets, and the functional responses to mitogenic stimulation and to selected acute and chronic viral infections. We show that lymphoid subsets and function are remarkably stable across the life course in this African setting with no evidence of immune senescence. These findings indicate that immune function remains robust in older people within sub-Saharan Africa and suggest that genetic and environmental factors within higher income populations deserve further investigation as potential mediators of immune senescence.

Materials and Methods

Study design and recruitment

We recruited healthy volunteers from Blantyre, Malawi. Individuals aged 20-29, 30-39, 40-49, 50-59 and 60-69 years were identified and asked to attend the recruitment clinic at Queen Elizabeth Hospital, Blantyre. Exclusion criteria were: pregnancy; body mass index (BMI) <18.5 (underweight) or >30 (obese); any long-term medication; history of treatment for tuberculosis; hospital admission in preceding twelve months; any illness within the preceding four weeks and seropositivity for HIV or malaria. The study was approved by the University of Malawi College of Medicine Research Ethics Committee (P.11/11/1150) and endorsed by the University of Birmingham's Research Ethics Committee.

Sample collection and preparation

Blood was collected into EDTA and heparinised vacuum tubes (Becton Dickinson). Differential counts were carried out on EDTA blood using an HmX analyser (Beckman-Coulter). Lymphocyte subsets were quantified using 150μl aliquots of heparinised whole blood, prepared as described below. Plasma was collected from the remaining sample. The pelleted cells were diluted to 80ml in pre-warmed PBS (Fisher) and PBMCs were collected by gradient centrifugation on lymphoprep (Axis-Shield) and counted in a haemocytometer following trypan blue (Sigma) staining.

T-cell and B-cell subsets

Whole blood samples were stained with either the T-cell antibody panel, B-cell panel or the regulatory panel excluding the anti-FoxP3 antibody (Table SI) for 15min at 26°C in the dark. Cells were vortexed in 3ml FACSlyse (BD Biosciences) and incubated for a further 15min. They were centrifuged at 450g for 5min, the FACSlyse was poured off and the cells for T-cell and B-cell analysis were resuspended in 200μl FACSfix (2% v/v formalin [Sigma] in PBS). They were then stored at 4°C in the dark and Accucheck counting beads (Invitrogen) were added immediately before acquisition on a 9-colour CyAn ADP (Beckman Coulter).

T-regs were identified by resuspending the surface stained cells in 3ml PBS-BSA (2% w/v bovine serum albumin [Sigma] in PBS) followed by centrifuging at 450g for 5min. Intranuclear staining for FoxP3 was carried out using FoxP3/transcription factor fixation/permeabilization solution and anti-FoxP3 antibody (eBioscience) according to manufacturers' instructions. Cells were stored in 200μl FACSfix at 4°C in the dark until they were acquired.

Following acquisition, subsets were identified as described below and, if the Accucheck bead acquisition passed validation according to manufacturers' instructions, the subsets were quantified.

Antigens

CMV-infected dermal fibroblast lysate and uninfected dermal fibroblast lysate were prepared using a standard protocol using strain AD169. EBV-infected B-cell lysate and uninfected B-cell lysate were obtained commercially (Virusys). Infected cell lysates were diluted to the

same protein concentration as the respective uninfected cell lysate in PBS (Fisher) before they were used.

Responses to the CMV pp65 and IE1 proteins were assessed using mixes of immunogenic peptides (Table SII) (21–26). Responses to influenza were assessed using a complete peptide pool spanning the haemagglutinin (HA) protein of the A/California/07/2009 (H1N1) strain, consisting of 139 14-mers and 15-mers overlapping by 11 amino acids (BEI Resources). All peptides were diluted in dimethyl sulphoxide (Sigma).

Preparation of samples for short-term T-cell response assay

PBMCs were resuspended at 10^6 cells/ml in R10F (90% v/v RPMI [Sigma], 10% v/v foetal bovine serum [PAA Labs], 100U/ml penicillin [Sigma], 100μg/ml streptomycin [Sigma], 2mM L-glutamine [Sigma]) and aliquoted to a 48-well microplate at 1ml for each treatment.

Antigenic stimuli included CMV-infected dermal fibroblast lysate (20μg/ml) with uninfected dermal fibroblast lysate as a negative control, EBV-infected B-cell lysate (10μg/ml) with uninfected B-cell lysate as a negative control, pp65 and IE1 peptide mixes (0.6μg/ml) and HA peptide pool (25μg/ml). Mitogenic stimulation with PMA (1.5ng/ml; Sigma) and calcium ionophore (250ng/ml; Sigma) was added from a stock diluted in DMSO. A negative control of 1μl DMSO was used for the peptide pools and polyclonal stimulus. All treatments were incubated for 18h at 37°C in a 5% carbon dioxide atmosphere with 10μg/ml brefeldin A added after 2h. Dead cells were identified by staining with live/dead fixable aqua (Invitrogen), then stained for CD3, CD4 and CD8 (Table SI). Cells were permeabilised using Cytofix/Cytoperm (BD Biosciences) and stained for IFNγ, IL-2, IL-17, CD154 and TNFα (Table SI). After staining, cells were stored in 250μl FACSfix at 4°C in the dark.

Proliferation

PBMCs were stained with 0.25μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen) according to a standard protocol (27). After staining, they were resuspended at 2×10^6 PBMCs ml^{-1} in R10AB (90% v/v RPMI, 10% v/v human AB serum [Sigma], 100U ml^{-1} penicillin, 100μg ml^{-1} streptomycin, 2mM L-glutamine) and aliquoted to a 48-well microplate at 0.5ml for each treatment. Treatments used were: 10μg ml^{-1} CMV-infected dermal fibroblast lysate, with 10μg ml^{-1} uninfected dermal fibroblast lysate as a negative control. 10μg ml^{-1} EBV-infected B-cell lysate with 10μg ml^{-1} uninfected B-cell lysate as a negative control. 10μg ml^{-1} of the HA peptide pool, with 0.2μl DMSO as a negative control. As a positive control, a polyclonal stimulus of 1.5ng ml^{-1} PMA (Sigma) and 250ng ml^{-1} calcium ionophore (Sigma) was added from a stock diluted in DMSO, also with the DMSO treatment as a negative control.

The PBMCs were incubated for 8d at 37°C in 5% carbon dioxide in the dark. They were then stained with live/dead fixable aqua and stained for CD19, CD3, CD4 and CD8 to complete the proliferation panel (Table SI) as described above. PBMCs were stored in 250μl FACSfix at 4°C in the dark until acquisition.

Flow cytometry and validation criteria

For every acquisition, a set of compensation samples was acquired using cells prepared in the same way as the experimental cells and either surface stained for CD3, CD4 or CD8 using the same fluorochromes as in the panel or stained with CFSE or live/dead fixable aqua. Analysis was carried out using FlowJo (Treestar). The early stages of the analysis strategy were the same for all samples (Fig S1A-C), after which it was adapted as appropriate. For the T-cell and B-cell subsets, a concentration of cells could only be calculated if at least 2,000 beads were successfully acquired and the passed the validation criteria specified by the certificate of analysis (lot 58060A).

For the short-term and proliferation assays, validation and limit of detection (LoD) for B-cells and for CD4 and CD8 T-cells were calculated separately. A result was classed as valid if at least 2,000 live cells were acquired in both the treatment and control samples. Invalid samples were excluded from analysis. LoD was achieved if the number of responding live cells in the treatment exceeded the number of responding live cells in the negative control by at least 0.015% and also by at least 30 cells. Responding cells were calculated as the sum of live cells expressing CD154, IFNγ, IL-2, IL-17 or TNFα.

B-cell memory assay

B-cell memory to influenza was measured by polyclonal stimulation followed by B-cell ELISpot (28). Briefly, cells were cultured at 2×10^6 ml^{-1} in B-cell medium (90% v/v RPMI, 10% v/v human AB serum, 100U ml^{-1} penicillin, 100μg ml^{-1} streptomycin, 10mM HEPES [Sigma], 2mM L-glutamine) in a 48-well plate. The stimulant was 1.7μg ml^{-1} CpG (InvivoGen), 8.3μg ml^{-1} pokeweed mitogen (Sigma) and 0.02% v/v pansorbin cell suspension (Calbiochem). One well was left unstimulated as a negative control. The PBMCs were incubated for 6d at 37°C, 5% carbon dioxide in the dark. After 5d, an ELISpot plate (96-wells, based with polyvinylidene difluoride [Millipore]) was prepared by coating each well with 50μg ml^{-1} haemagglutinin protein from influenza virus A/California/04/09 (H1N1) (BEI Resources) in 50μl PBS. Positive control wells were prepared with 10μg ml^{-1} unconjugated goat anti-human IgG (Cambridge Bioscience). The plate was incubated at 24°C and the wells were blocked with 2% w/v bovine serum albumin (Sigma) in PBS and washed with PBS. The cells were washed three times by centrifuging at 450g for 5min in 10ml PBS, then resuspended at 2×10^6 ml^{-1} in B-cell medium, based on the concentration of PBMCs when the culture was begun. Treatments were prepared in triplicate 100μl of the suspension and incubated for 20h at 37°C in a 5% CO2 atmosphere. Anti-haemagglutinin IgG was identified with goat anti-human IgG conjugated to alkaline phosphatase (Sigma) and developed with AP developing solution (Bio-Rad) according to manufacturer's instructions. Spots that indicated a B-cell producing antibodies against haemagglutinin were identified with an ELISpot reader running version 4.0 software (AID-Diagnostika). The number of HA-specific memory cells is expressed as the mean number of spots produced by stimulated cells in HA-coated wells after the mean response of unstimulated cells in HA-coated wells was subtracted, expressed per 10^6 circulating cells.

Confirmation of CMV and EBV infection

All volunteers were confirmed seropositive for CMV using ELISA to detect IgG against CMV strain AD169. All volunteers were confirmed seropositive for EBV using immunofluorescence technique to detect IgG against the VCA protein.

Antibody response to influenza

Functional antibody response to influenza was assessed by inhibition of the activity of the haemagglutinin protein using a standard protocol (29). Three different haemagglutinin proteins were used based on strains circulating at the time of recruitment according to WHO surveillance: B/Brisbane/60/2008, A/California/7/2009 H1N1 and antigen A/Victoria/210/2009 (NIBSC) H3N2.

Statistical analysis

Data were managed using Microsoft Access and analysed using MINITAB 14.1 (MINITAB Inc).

Results

Low proportion of naïve T-cells in younger Malawian adults

68 individuals aged 20-68 years were recruited between May and November 2012. Analysis of blood counts revealed that the total white cell and lymphocyte counts did not vary with the age of the donor. In addition, no differences were seen in the absolute or relative CD4 T-cell, CD8 T-cell or B-cell populations according to age (Table I). As the CD4 and CD8 T-cell counts were similar across the lifecourse, the CD4:8 T-cell ratio also remained stable with median values of 1.95 and 2.03 in the young and oldest cohorts respectively.

More detailed phenotyping of the T-cell subpopulations was carried out by flow cytometry (Fig S1).

Relative proportions of naive, central memory, effector and effector memory RA cells within the CD4 and CD8 populations was assessed by co-expression of CD45RA and CCR7 (30). A striking finding was that the naïve ($CCR7^{+}CD45RA^{+}$) T-cell subpopulation accounted for only 14% of both the CD4 and CD8 T-cells in donors aged 20-29 years (Table II), which is considerably less than has been measured in high-income settings among healthy adults of equivalent age (5, 31, 32). Moreover, the naïve subpopulations were similarly low in all age groups.

The relative size of the central memory ($CCR7^{+}CD45RA^{-}$) CD4 T-cell subpopulation rose between the 30-39 and 40-49 age groups, with a corresponding drop in the effector ($CCR7^{-}CD45RA^{-}$) and effector memory RA ($CCR7^{-}CD45RA^{+}$) subpopulations (Fig 1).

Among the CD8 T-cells, the relative size of the central memory ($CCR7^{+}CD45RA^{-}$) did not significantly differ across the different age groups although the effector memory RA ($CCR7^{-}CD45RA^{+}$) subpopulation was lower in donors aged over 40 years.

We next assessed age-associated differentiation of T-cells based on the pattern of CD27 and CD28 co-expression, which revealed that the differentiation profile of CD4 T-cells was stable within all age groups.

However, we did observe a modest increase in the relative size of the late differentiated ($CD27^-CD28^-$) CD8 T-cell subpopopulation, from 28% among 20-29-year-olds to 41% among 60-69-year-olds which corresponded with a decrease in the early differentiated ($CD28^+CD27^+$) subpopulation (Table II).

Interestingly, regulatory T-cells ($CD25^{hi}FoxP3^{hi}$) (33) comprised less than 1% of the CD4 T-cell pool in all but two individuals. These values were particularly low in younger people and increased only marginally in those over the age of 40 years (Table II, Fig 2).

We further assessed the expression of PD-1, which is expressed by T-cells undergoing sustained activation and may indicate exhaustion in some settings (34, 35). PD-1 expression was rare among both CD4 and CD8 T-cells, and the only age-associated difference was that the 20-29-year-olds had a higher median percentage of CD4 T-cells that expressed PD-1 than any of the older age groups (Table II).

CD57 is a marker of late differentiation on T-cells (36) but again, relatively few $CD57^+$ T-cells were observed within peripheral blood and no differences were seen in relation to age.

The pattern of CD10, CD21 and CD27 expression was also used to define a range of B-cell subpopulations (Fig S2) (37, 38) but no consistent trends in subset distribution were observed in relation to age (Table II).

Functional immune responses are maintained across the life course

We next examined the functional activity of CD4 and CD8 T-cells within the different age groups. PBMCs were stimulated with a range of antigens including CMV and EBV viral lysates, and peptide pools from cytomegalovirus pp65 and IE1 and hemagglutinin (HA) peptides from influenza. The mitogenic combination of PMA and ionomycin was used as a positive control.

Functional responses were measured by expression of CD154 (CD40L) or production of IFNγ, IL-2, IL-17 or TNFα (Fig S1C), and the percentage of cells that were positive for at least one of these five parameters was determined. No age-related differences were observed between the proportion of donors with a detectable response to the different antigenic stimuli (Table III). Similarly, no age-associated differences were seen in the magnitude of the detectable responses. An assessment of T-cell proliferation using CFSE-dilution also failed to reveal any age-related variation in either the frequency or magnitude of proliferative responses.

As no differences were observed between the age groups, we next combined the results from all donors in response to each antigen to compare the overall response to each antigenic stimulus. We observed stronger CD4 and CD8 T-cell responses against CMV and EBV compared to influenza haemagglutinin (Fig 3A).

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We next compared the magnitude of the proliferative response to each stimulus using paired comparisons. Again, CD4 and CD8 T-cell responses to CMV or EBV were greater than those against the HA peptide pool (Fig 3B). A similar trend was apparent in both the functional and proliferative responses of B-cells although proliferation in response to CMV lysate was particularly low (Fig 3B).

The polyfunctional response to mitogenic stimulation increases with age

We next assessed the polyfunctional capacity of CD4 and CD8 T-cell responses through comparison of the percentage of samples that expressed each combination of CD154 expression or IFNγ, IL-2, IL-17 or TNFα production. Of the 32 (2^5) potential profiles within the CD4 and CD8 subsets, we selected those observed in at least 10% of individuals for further analysis. The percentage of individuals that expressed each of those profiles was then compared between the different age groups.

In response to mitogenic stimulation, all donors had both CD4 and CD8 T-cells that expressed both one and two of the measured mediators. Many donors also had cells producing three or four mediators simultaneously, and such multifunctional cells became progressively more common through early adulthood, peaking among individuals in their forties and then falling in the later age groups (Fig 4A). As we observed very few fivefold responses, those fourfold responses were the most multifunctional that we were able to observe.

The peaks in those responses were reflected in many of the specific combinations of responses, although the age-associated differences were not large enough to be statistically significant in most cases. One notable exception was the CD4 T-cell response that included expression of both IL-17 and CD154. Another was the CD8 T-cell response that involved CD154, IL-2 and TNFα, with or without IFNγ. Both of those response types were more commonly observed in the 40-49 and 50-59 age groups (Fig 4B).

The profile of the CMV-specific immune response is maintained across the lifecourse whilst the cytokine response to EBV is dependent on donor age

We next went on to determine and contrast the T-cell response profiles following stimulation with the different viral antigens. CD4 T-cell responses to CMV lysate were dominated by the production of IFNγ which was observed either in isolation or in combination with CD154 and/or TNFα. By contrast, the CD4 response to peptide pools of CMV proteins pp65 and IE1 comprised populations that produced either IFNγ or TNFα in isolation, although some dual subpopulations were observed in response to pp65. Interestingly the majority of CD4 T-cell responses to EBV lysate consisted of cells that expressed CD154, IFNγ or TNFα alone, although some individuals had subpopulations that co-expressed TNFα with either IFNγ or CD154 (Fig 5).

CD8 T-cell responses to all antigenic stimuli were far less diverse than CD4 T-cell responses (Fig 6). Stimulation with viral lysate induces poor CD8 T-cell responses as viral proteins require antigen processing and cross-presentation which is relatively inefficient within PBMC (39). Indeed the only CD8 T-cell response to CMV lysate involved cells that produced TNFα and this was observed in relatively few individuals. Whilst EBV lysate

induced a more diverse response, this was again restricted to monofunctional production of CD154, IFNγ or TNFα. IE1 and pp65 peptide pools induced IFNγ and TNFα responses within CD8 T-cells although dual responses were again only observed following pp65 stimulation. T-cell responses to stimulation with HA were also weak although monofunctional CD8 T-cells producing TNFα were observed in some donors.

Very few age-associated differences were observed in the functional responses of CD4 or CD8 T-cells. In particular, responses to CMV stimulation were stable across the life course. However, we did observe a significant age-related decline in the percentage of individuals whose CD8 T-cells produced IFNγ in response to EBV lysate, associated with a corresponding but non-significant rise in the percentage of individuals responding with TNFα (Fig 6). A similar increase in TNFα production with age was also observed in CD4 T-cells in response to EBV lysate.

Humoral responses against influenza are observed in virtually all donors and are comparable across age groups

A haemagglutinin-specific antibody response against at least one of three influenza strains was observed in 94% of the 68 donors and 65% had humoral responses against all three viruses. More individuals demonstrated protective immunity against the influenza A California H1N1 (90%) and Victoria H3N2 (91%) strains compared to the influenza B Brisbane strain (66%). Again we found no age-associated differences in either the proportion of individuals with detectable HA-specific antibody responses (Chisquare test) or the magnitude of the haemagglutinin-specific response (Kruskal-Wallis test).

Although most individuals had adequate protective levels of antibody against both of the influenza A strains, the Victoria H3N2-specific antibody titre was substantially greater than that against California H1N1. Moreover, both were substantially higher than the level of humoral immunity to the influenza B (Fig S2).

Consistent with the findings from the functional assays, the responses detected by the B-cell memory ELISpot were low with only two individuals have more than ten spots (50 HA-specific memory B-cells per 10^6 PBMCs). Also consistent with results from the proliferation assay, we found no age-associated effect on the frequency of memory B-cells.

Discussion

Life expectancy is increasing throughout the world and there is a need to understand the parameters that determine healthy ageing. Relatively little attention has been directed toward the study of ageing in sub-Saharan Africa despite the recent dramatic increase in life expectancy from middle age in the region. We found marked differences between the immune responses of Malawian adults and those reported from adults studied in high-income settings (2, 3, 40–44). Factors such as host and pathogen genetics, environmental factors, infectious disease burden and nutritional status are all likely to impact on immune function and differ significantly according to income and geographic region (45). These observations therefore have a range of important implications.

Perhaps the most striking observation in our study was the low proportion of naïve CD4 and CD8 T cells that was observed in adults within all age groups. In particular, only 14% of CD4 and CD8 T-cells expressed a naïve ($CD45RA^{+}CCR7^{+}$) phenotype in Malawians under 30 years. These values are considerably lower than has been reported from studies in high-income settings where a median value of 50% naive cells has been reported in the CD4 repertoire within a US population (31). However, it was noteworthy that although the naïve pool is relatively small in early adulthood, we did not observe any further decline during ageing which differs from the pattern reported from high-income settings (4, 31, 43).

Our findings indicate that the relative balance of the naïve and antigen-experienced (memory and effector) T-cell pools differs substantially between low- and high-income settings by early adulthood. This may well reflect more frequent, and perhaps repeated, exposure to environmental pathogens that drive expansion of the memory T-cell pool. In support of this, the proportion of effector/memory T-cells was already increased at an early age and did not increase further across the life course which is in contrast to studies from more affluent areas (43, 46).

African children do exhibit substantial expansion of differentiated T-cell subpopulations in the first year of life (8, 19, 27, 47–49), due at least partly to primary CMV infection which drives the accumulation of late differentiated T cells (1, 50). However, this does not involve a concurrent loss of naïve T-cells, but rather an increase in the total number of circulating T-cells (19).

The only phenotypic evidence of age-associated differentiation we observed was a relatively large subpopulation of highly differentiated ($CD27^{-}CD28^{-}$) CD8 T-cells among 60-69-year-olds and a correspondingly small early-differentiated ($CD27^{+}CD28^{+}$) subpopulation. $CD27^{-}CD28^{-}$ T-cells are often associated with co-expression of senescence markers such as CD57, PD1 and KLRG1, which represents an interesting area for future study. Importantly, the ratio of CD4 to CD8 T-cells, which acts as a strong correlate of the CMV-associated ‘immune risk profile’ in several previous studies (4), appeared stable across the lifecourse.

It was also striking that expression of CD57, a marker of senescence (36), was low across the cohort and in a similar range to that seen in CMV-seronegative Western Europeans (3, 40, 44). We have previously identified significant subpopulations of $CD57^{+}$ and $PD\text{-}1^{+}$ T-cells in infants from the same population (27) which suggests that these subpopulations have contracted by adulthood (51).

Malawian adults had very low levels of regulatory T-cells, with median values below 0.3% of the CD4 repertoire in all ages although the relative subpopulation size was slightly larger in individuals over 30 than among 20-29-year-olds. Comparisons between regulatory T-cell subpopulations reported by different studies must be interpreted with caution as there is no defined standard of FoxP3 expression for calibration. However, our value is within a similar range to that reported from a Kenyan cohort (52) and suggests that the proportion of regulatory T-cells is some 5 to 10-fold lower in these settings in comparison to higher income environments (53, 54).

It is of interest that the age-associated differences between T-cell subpopulations that we observed were below twofold in magnitude in most cases, implying that the changes we observed over five decades of adulthood were less dramatic than those that typically occur within the first year of childhood (8, 19, 47). Furthermore, the stable naïve pool and CD4:CD8 ratio suggest that the influence of immune senescence may be relatively muted in this setting. As such it became important to consider the functional activity of T cells and here again, we observed a very stable pattern in all age groups. Malawians of all ages were able to mount robust T-cell responses to both mitogenic stimulation and viral antigens, whether measured by short-term expression of CD154 and cytokines or by proliferation.

As expected, mitogenic stimulation revealed a broader spectrum of responses from the CD4 than the CD8 T-cells and this profile was consistent across all age groups. Small subsets of CD4 T-cells producing IL-17 were observed, which increased in size from the 20-29 to the 50-59-year-old age groups where they peaked, and then decreased in the 60-69-year-old age group. IL-17 was not detected in virus-specific responses, which is in keeping with previous observations (55). Interestingly, the number of individuals whose CD4 T-cells expressed IL-17, which was always co-expressed with CD154, was at its highest among the 40-59-year-old Malawians.

The main age-associated variation was in the frequency of multifunctional responses to mitogenic stimulation, which peaked in the 30-39 and 40-49 age groups.

In response to CMV and EBV, most individuals mounted a robust overnight response. The only significant age-associated difference was in the response to EBV lysate by CD8 T-cells: there was an age-associated trend toward less individuals having cells produing IFNγ only. We also observed a corresponding but non-significant trend toward cells producing TNFα only. Taken together, the two trends imply an age-associated shift away from IFNγ and toward TNFα production.

CMV-specific T-cell responses were both more diverse and more likely to be polyfunctional than EBV-specific responses, which is in keeping with findings from high-income settings (6, 56, 57).

Responses to influenza, which causes repeated acute infections, were similar to those reported from high-income settings. Influenza-specific antibodies were present in all donors and were similar in specificity to those found in the USA (58) and UK (59). It is unlikely that any donors had a recent influenza infection as donors who reported any sign of illness within four weeks were not recruited and assessment took place outside the January-April rainy season when influenza transmission is highest (60). As influenza-specific antibodies decline rapidly, with a half-life of six months (61), it is unsurprising that the anti-influenza antibody titres that we recorded were relatively low. Cellular immune responses to influenza were detected in less than half of the individuals tested, which is again consistent with findings from a high-income setting (62).

It is interesting to speculate on some of the factors that might determine these observations on lymphoid repertoire and function across the life course in a sub-Saharan setting. The timing of herpesvirus infections, most notably CMV, may be one such factor. Primary CMV

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infection in Africa occurs almost universally in infancy, whilst the immune system is still developing (9, 48, 63), whereas most infection now occurs during adulthood in high income countries (64). CMV infection has been virtually ubiquitous during human evolution and it is possible, indeed likely, that we are ‘hardwired’ to anticipate infection during the first year of life. Of note, CMV has been associated with beneficial effects on immune function including moderation of EBV-associated immune impairment (65, 66), enhanced T-cell responses to polyclonal stimulus (19) and enhanced antibody responses to influenza vaccine (67). These may indicate that CMV and humans have co-evolved into a partial mutualism such that CMV has become an valuable component of the human 'virobiota' (68) or 'metagenome' (69). Infection during adulthood is therefore likely to be a recent evolutionary anomaly that may disrupt the natural symbiosis between humans and CMV.

Our conclusions are limited by a number of factors. Individuals who survive until adulthood might have been selected for more robust immune function during childhood. Estimates for under-5 mortality in Malawi in 1965, when our 50-59-year-old age group was born, stood at 35% but had dropped to 23% by 1992, the year of birth of our youngest donor (http://data.worldbank.org/indicator/SH.DYN.MORT?locations=MW). Such high childhood mortality might have acted to select against individuals with poor immune function and as such, it will be important to repeat these studies on future cohorts now that childhood mortality rates are significantly reduced.

Another limitation was the difficulty in recruiting elderly people in an urban setting, which dictated the upper limit of the age of donor recruitment at 69 years, and as such the number of donors over the age of 50 years was modest. Studies of immune senescence often recruit subjects over 80 years of age (1, 2, 15, 40) and this population should be targeted in future work. Finally, despite this encouraging observation on immune function, around 30% of Malawians show severe physical and functional limitation by the age of 65 years (13) so the contribution of immune dysfunction to this decline needs be clarified.

In conclusion, our data demonstrate that the profile and stability of lymphoid repertoire and function varies considerably between sub-Saharan Africa and higher income settings. The relative contribution of genetic and environmental factors should now be studied, potentially including an assessment of the influence of migration on immune function into older age, and these data could provide information that could help to guide novel approaches to support immune function in older people.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Key points

- Only 14% of CD4 or CD8 T-cells of young adult Malawians were naïve (CCR7+CD45RA+).
- Little age-associated accumulation of differentiated or senescent T-cells.
- Robust T-cell responses to viral antigens and mitogens from early 20s to late 60s.

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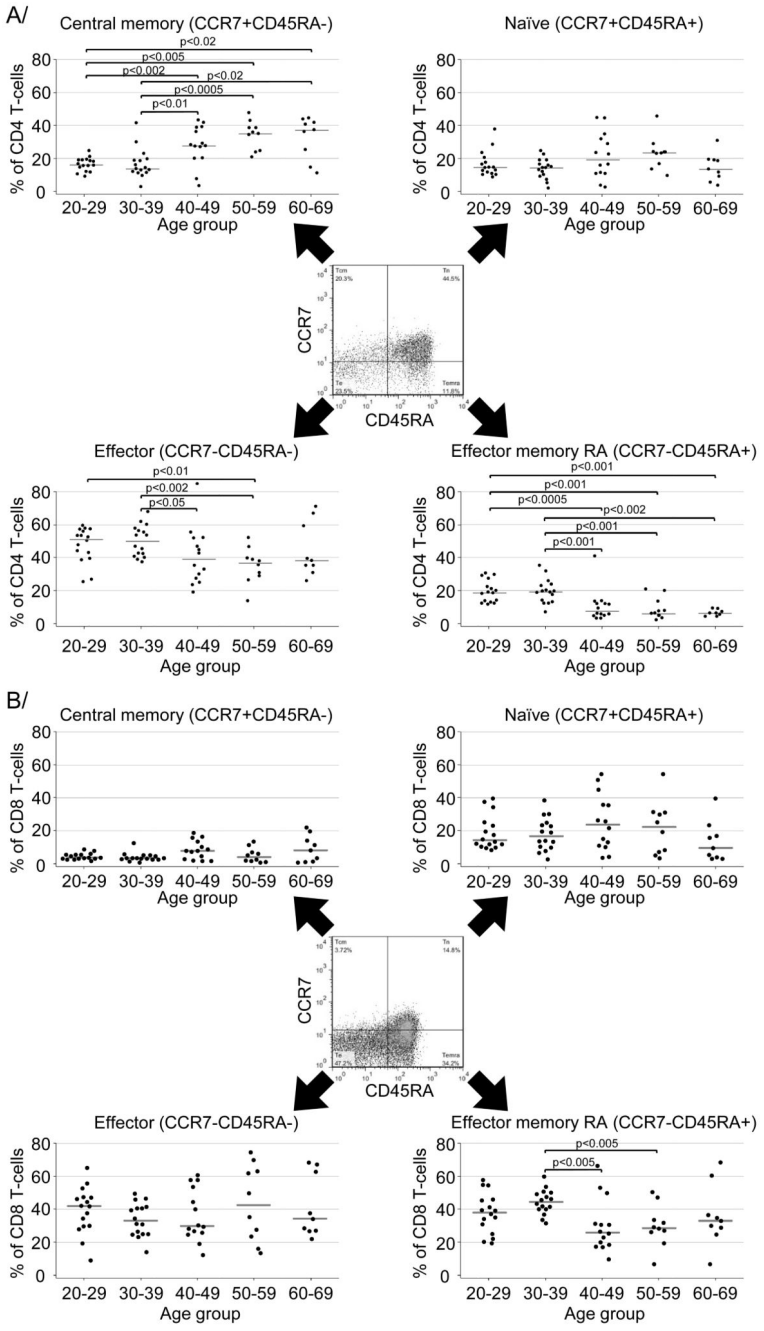

Fig 1.

T-cell subpopulations classified by CCR7 and CD45RA. A/ CD4 T-cells show an age-associated trend toward larger central memory subsets at the expense of effector and effector memory RA cells. B/ CD8 T-cells show no clear age-associated trends in subpopulation sizes. Bars indicate medians. Gating is illustrated using the individuals with the median central memory subpopulations (50-year-old male for CD4 T-cells, 51-year-old male for CD8 T-cells). Comparisons by Kruskal-Wallis test, multiple comparisons by Mann-Whitney U-test.

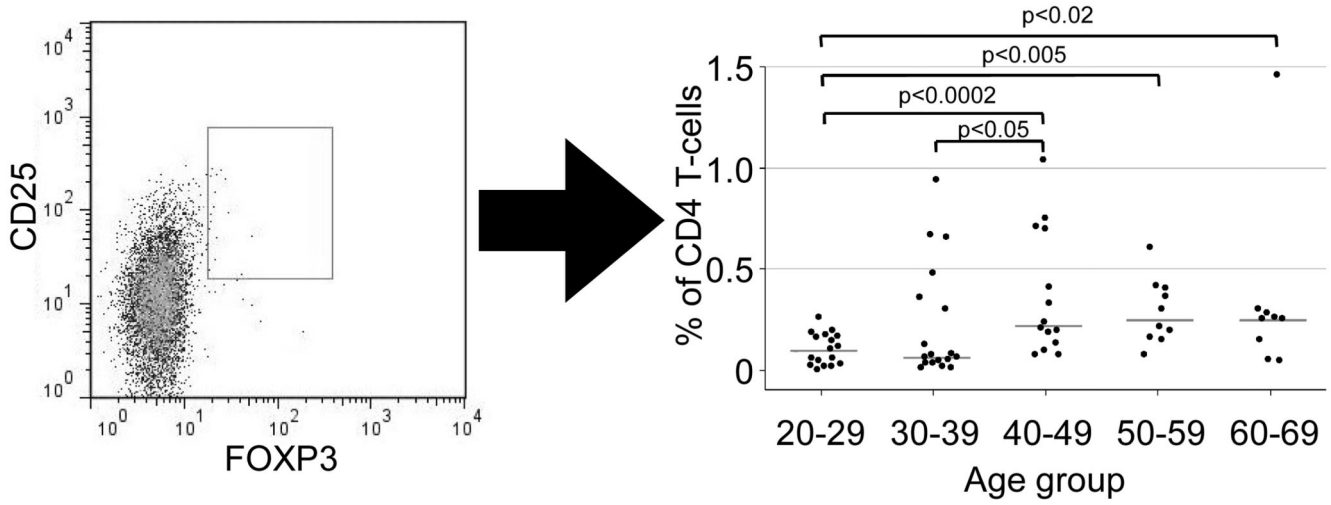

Fig 2.

Larger subsets of regulatory T-cells in individuals over 40 years old. Bars indicate medians. Gating strategy is based on identifying the regulatory T-cells as $CD25^{hi}FoxP3^{hi}$, illustrated using the individual with the median percentage of regulatory T-cells (21-year-old female). Comparisons by Kruskal-Wallis test, multiple comparisons by Mann-Whitney U-test.

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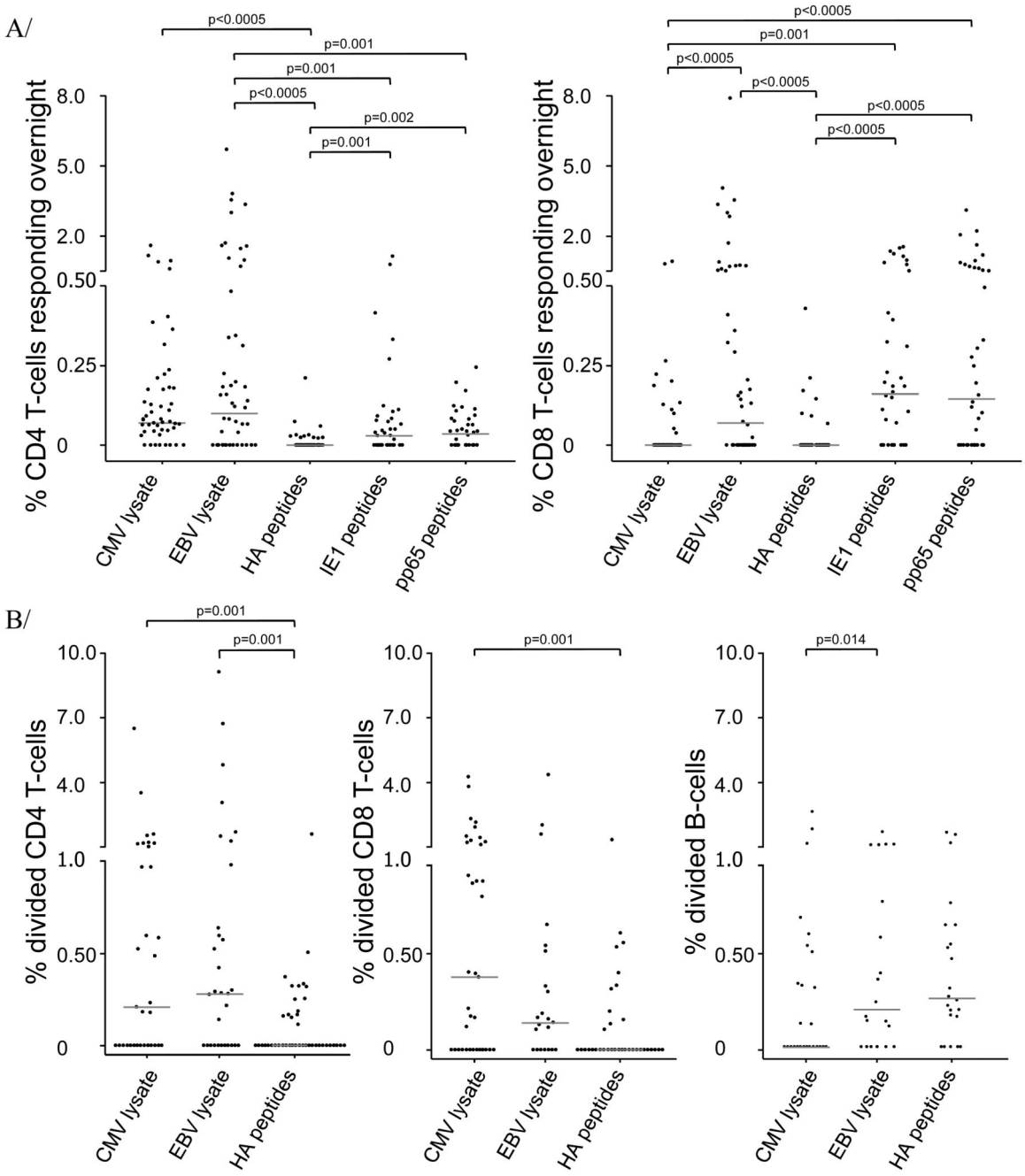

Fig 3.

Magnitude of responses to the antigenic stimulants. Plots show the percentage of CD4 and CD8 T-cells A/ producing either IFNγ, IL-2, IL-17, CD154 or TNFα in response to short-term stimulation or B/ having undergone at least one division after 8d of stimulation. Only individuals with a detectable response to at least one stimulant were included. Bars indicate medians. Comparisons by 1-sample Wilcoxon tests.

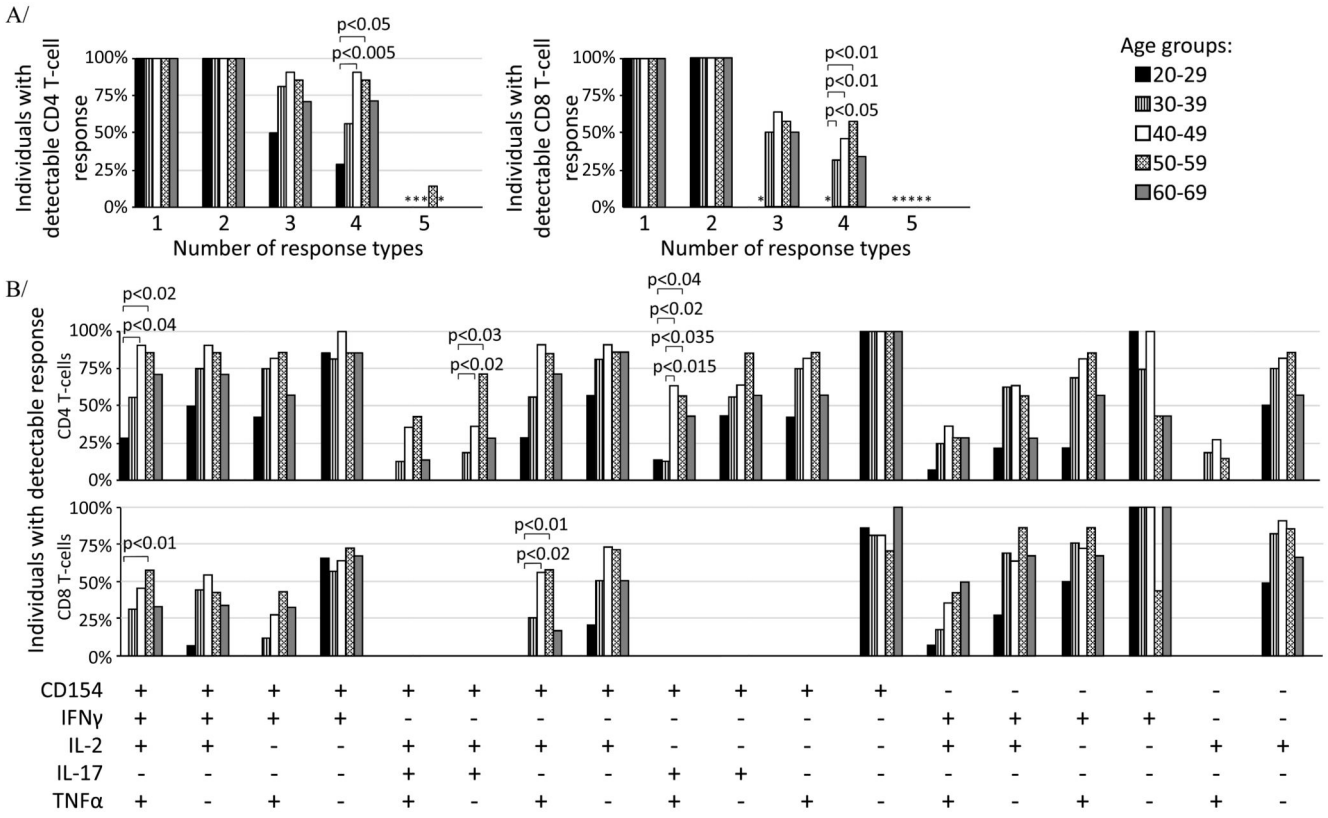

Fig 4.

Expression profile of CD154, IFNγ, IL-2, IL-17 and TNFα amongst CD4 and CD8 T-cells after short-term stimulation with PMA and ionomycin. A/ Percentage of individuals in whom 1, 2, 3, 4 and 5-fold responses were observed, irrespective of response type. B/ Percentage of individuals in which each possible combination of CD154, IFNγ, IL-2, IL-17 and TNFα were observed among CD4 (top panel) and CD8 (bottom panel) T-cells. Only combinations that were observed in at least 10% of individuals are plotted. Comparisons by Chisquare test with multiple comparisons by Fisher's exact probability test.

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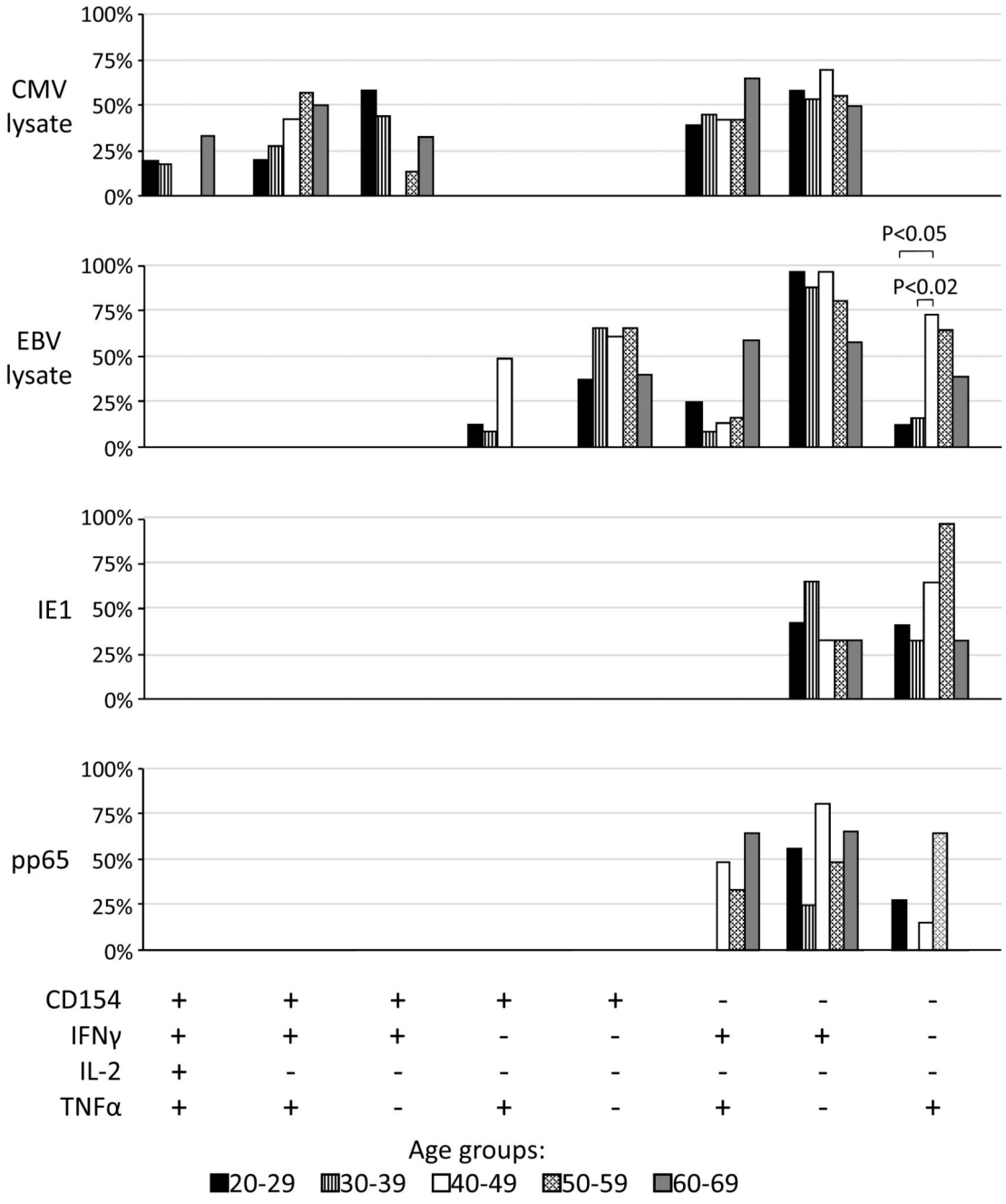

Fig 5.

Short-term response profile of CD4 T-cells. Bar chart shows the percentage of individuals in which each response type was detectable, split by age group which is indicated by the pattern of the bars. Comparisons by Chisquare test with multiple comparisons by Fisher's exact probability test.

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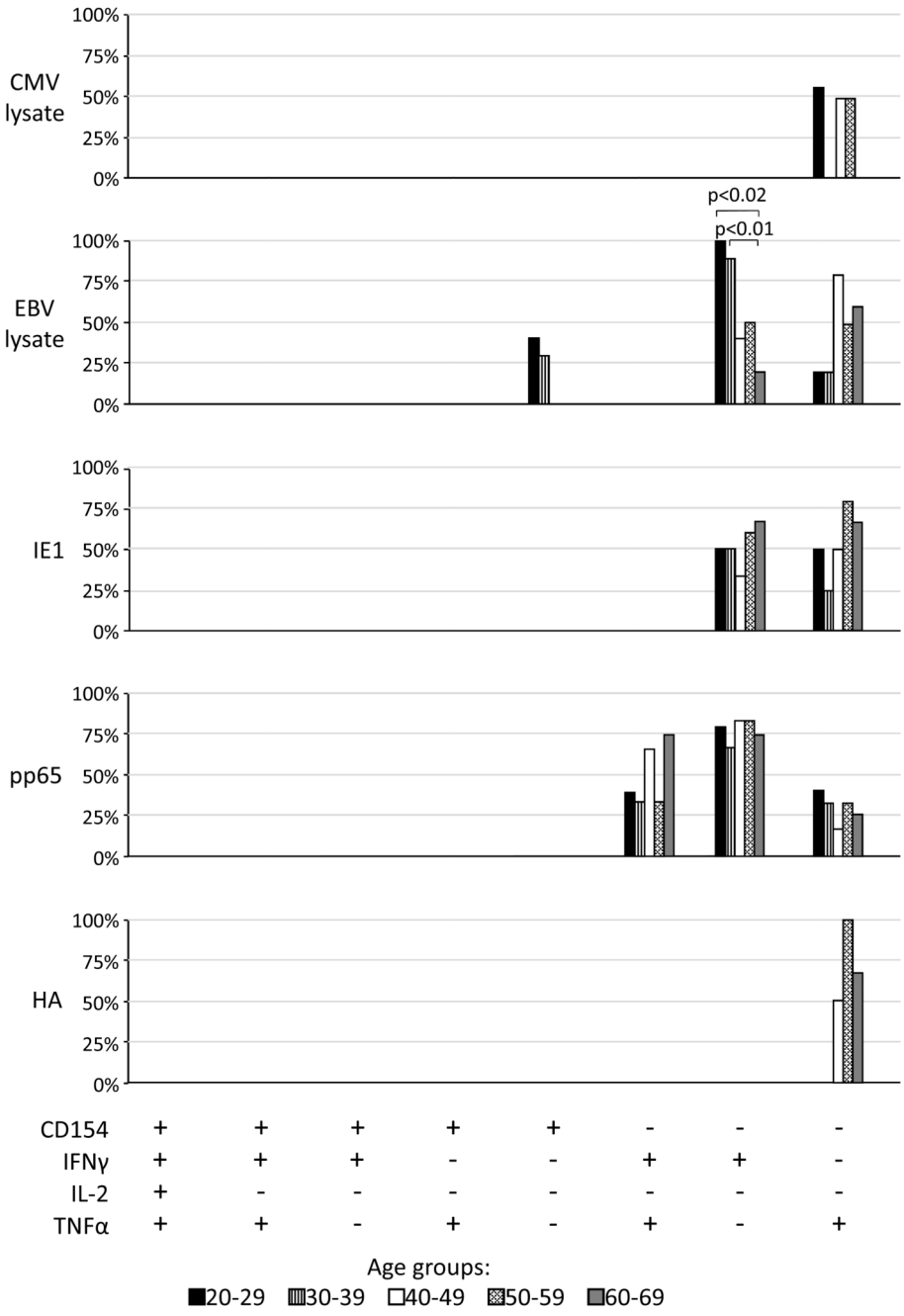

Fig 6.

Short-term response profile of CD8 T-cells. Bar chart shows the percentage of individuals in which each response type was detectable, split by age group which is indicated by the pattern of the bars. Comparisons by Chisquare test with multiple comparisons by Fisher's exact probability test.

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Table I

Characteristics of individuals recruited into the cohort.

Characteristics of individuals recruited into the cohort. There was no significant effect of age group on any parameter listed. Comparisons by Kruskal-Wallis test.

Age group:	**20-29**			**30-39**			**40-49**			**50-59**			**60-69**		
	Median	**IQR**	**n**	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**
Number recruited			17			18			14			10			9
Number of women			7			9			4			5			7
Body mass index	22	20-23	17	22	20-26	18	21	20-23	14	24	20-24	10	26	23-27	9
White cell count (10^6 cells ml^{-1})	5.30	4.35-5.85	17	5.85	4.93-6.75	18	5.30	4.20-6.05	13	5.25	4.35-6.48	10	4.60	4.30-6.45	9
Lymphocyte count (10^6 cells ml^{-1})	1.80	1.45-2.20	17	2.15	2.00-2.75	18	1.90	1.63-2.53	13	2.00	1.65-2.85	10	2.10	1.30-2.60	9
B-cell count (10^3 cells ml^{-1})	339	192-628	10	446	218-1,051	9	524	383-744	14	566	248-643	9	480	340-808	8
CD4 T-cell count (10^3 cells ml^{-1})	999	866-1,242	11	958	710-1,093	16	912	714-1,788	13	1164	750-1,714	9	907	436-1,357	8
CD8 T-cell count (10^3 cells ml^{-1})	521	339-597	11	458	344-805	16	554	234-1,151	13	502	232-1,008	9	411	312-621	8
CD4/CD8 ratio	1.95	1.76-2.80	11	1.85	1.391-2.49	16	2.47	1.46-3.07	13	2.17	1.62-2.75	9	2.03	1.29-2.64	8

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Table II

Major lymphocyte subsets in relation to donor age.

Major lymphocyte subsets by age group. Each subset is expressed as the percentage of cells within the CD4 T-cell, CD8 T-cell or B-cell population. Superscript letters indicate significance groups (at $p<0.05$) such that, where any difference was found in a row, two age groups that are not significantly different are shown to share a letter in common whilst if they do differ they are not shared. Comparisons by Kruskal-Wallis test, multiple comparisons by Mann-Whitney U-test.

Age group:		20-29		30-39		40-49		50-59		60-69	
		Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
CD4 T-cells (CD3+CD4+CD8-)											
Central memory	CCR7+CD45RA-	16^{a}	13-19	14^{a}	11-19	28^{b}	20-39	35^{b}	24-40	37^{b}	20-43
Effector	CCR7-CD45RA-	51^{ac}	40-57	50^{c}	41-57	39^{ab}	27-52	37^{b}	28-42	38^{abc}	33-63
Effector memory RA	CCR7-CD45RA+	19^{a}	13-26	19^{a}	13-24	7.5^{b}	4.8-12	6.0^{b}	4.3-9.1	6.4^{b}	4.8-9.1
Naïve	CCR7+CD45RA+	14	11-20	14	9.4-18	19	11-32	23	16-25	13	7.2-19
Early differentiated	CD28+CD27+	75	70-81	72	63-80	67	57-74	70	66-73	60	56-75
Intermediate	CD28+CD27-	17	11-20	20	15-25	27	19-29	23	17-25	23	17-29
Late differentiated	CD28-CD27-	2.4	1.8-8.0	3.4	2.0-5.9	2.7	1.9-11.13	5.75	1.82-13	7.03	5.12-14.57
Senescent	CD57+	0.20	0.042-0.63	0.14	0.049-0.35	0.22	0.17-0.42	0.24	0.066-0.59	0.17	0.16-0.36
Sustained activated	PD-1+	0.29^{a}	0.19-0.92	0.18^{ab}	0.15-0.33	0.14^{b}	0.093-0.29	0.33^{ab}	0.11-0.54	0.18^{b}	0.034-0.27
Regulatory	$CD25^{hi}FoxP3+$	0.10^{a}	0.024-0.17	0.069^{ac}	0.033-0.39	0.22^{bc}	0.12-0.70	0.2544^{b}	0.16-0.41	0.25^{bc}	0.10-0.29
CD8 T-cells (CD3+CD4-CD8+)											
Central memory	CCR7+CD45RA-	3.6	2.94-5.4	3.3	2.6-4.9	7.8	2.5-14	3.8	1.5-7.9	8.1	1.1-17
Effector	CCR7-CD45RA-	42	30-47	33	25-41	30	26-55	42	22-65	34	27-65
Effector memory RA	CCR7-CD45RA+	38^{ab}	26-46	45^{a}	40-50	26^{b}	18-36	29^{b}	24-37	33^{ab}	27-49
Naïve	CCR7+CD45RA+	14	11-25	17	10-25	24	11-38	22	6.7-31	10	3.6-20
Early differentiated	CD28+CD27+	41^{ac}	30-52	35^{ab}	27-39	47^{c}	33-55	41^{abc}	27-44	28^{b}	20-35
Intermediate CD27+	CD28-CD27+	10^{a}	7.9-18	18^{a}	9-23	14^{a}	7.7-16	6.4^{b}	4.5-11	7.8^{ab}	5.7-17
Intermediate CD28+	CD28+CD27-	16^{a}	10-20	16^{a}	9.7-22	15^{a}	11-17	17^{ab}	12-24	19^{b}	11-23
Late differentiated	CD28-CD27-	28^{a}	16-37	33^{a}	27-35	23^{a}	19-44	36^{ab}	26-48	41^{b}	35-50

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	Age group:	20-29		30-39		40-49		50-59		60-69	
		Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Senescent	CD57+	1.5	0.8-2.9	1.3	0.50-2.1	1.4	0.62-2.0	1.6	0.43-3.7	1.9	1.6-5.6
Sustained activated	PD-1+	0.15	0.076-0.52	0.11	0.048-0.36	0.18	0.09-0.36	0.36	0.18-1.1	0.11	0.036-0.35
B-cells (CD19+)											
Naïve	CD20+CD27-CD10-CD21+	55	41-68	64	50-72	53	45-68	58	51-64	66	62-67
Immature	CD20+CD27-CD10+CD5+CD21+	9.3	3.1-19	10	4.8-22	13	9.3-24	15	5.5-30	12	6.6-22
Memory	CD20+CD27+CD10-CD21+	16	11-18	11	8.8-16	16	12-27	18	8.8-24	14	11-18
Activated mature	CD20+CD27+CD10-CD21-	3.1[ac]	2.5-5.6	1.8[b]	1.3-3.0	2.9[ac]	2.2-4.6	3.0[abc]	1.6-4.8	1.8[b]	1.3-2.2
Exhausted memory	CD20+CD27-CD10-CD21+	9.3	3.1-19	10	4.8-22	13	9.3-24	15	5.5-30	12	6.6-22
Plasmablasts	CD20-CD27+CD38+	0.62	0.22-1.1	0.61	0.16-1.3	0.59	0.36-0.82	0.26	0.18-0.78	0.84	0.31-1.2

Table III

Individuals with detectable CD4 T-cell, CD8 T-cell or B-cell responses in overnight and proliferation assays.

Total short-term and proliferative responses. Number of individuals with detectable response to each stimulation is given over total number of samples that returned a valid result. Percentages are given in brackets. Validity was assessed separately for CD4 T-cells, CD8 T-cells and B-cells within each sample. No significant differences were detected between age groups, so results from all age groups were pooled to compare the total number of individuals who responded to each antigen. Within the 'Total' column, superscript letters indicate significance groups (at $p<0.05$) such that, where any difference was found in a row, two age groups that are not significantly different are shown to share a letter in common whilst if they do differ they are not shared. Comparisons by Chisquare test, multiple comparisons with Fisher's exact probability test.

	Age group					
	20-29	**30-39**	**40-49**	**50-59**	**60-69**	**Total**
CD4 T-cell response in overnight assay						
CMV lysate	12/17 (71%)	12/18 (67%)	9/13 (69%)	7/9 (78%)	7/8 (88%)	47/65 (72%)[a]
EBV lysate	8/17 (47%)	12/18 (67%)	8/12 (67%)	6/8 (75%)	5/7(71%)	39/62(63%)[a]
pp65 peptides	7/12 (58%)	4/12 (33%)	6/10 (60%)	6/7 (86%)	3/4 (75%)	26/45 (58%)[a]
IE1 peptides	7/12 (58%)	3/12 (25%)	6/10 (60%)	3/7 (43%)	3/4 (75%)	22/45 (49%)[a]
HA peptides	4/17 (24%)	2/18 (11%)	2/13 (15%)	2/9 (22%)	3/8 (38%)	13/65 (20%)[b]
PMA / I	17/17 (100%)	18/18 (100%)	13/13 (100%)	8/8 (100%)	8/8 (100%)	64/64 (100%)[c]
CD4 T-cell response in proliferation assay						
CMV lysate	5/14 (36%)	5/9 (56%)	3/4 (75%)	3/4 (75%)	3/6 (50%)	19/37 (51%)[a]
EBV lysate	6/13 (46%)	6/11 (55%)	4/5 (80%)	1/3 (33%)	3/4 (75%)	20/36 (56%)[ac]
HA peptides	3/16 (19%)	7/17 (41%)	2/7 (29%)	2/5 (40%)	1/6 (17%)	15/51 (29%)[b]
PMA / I	11/16 (69%)	11/17 (65%)	6/7 (86%)	4/5 (80%)	5/6 (83%)	37/51 (73%)[c]
CD8 T-cell response in overnight assay						
CMV lysate	7/17 (41%)	1/18 (5.6%)	4/13 (31%)	2/9 (22%)	0/8 (0%)	14/65 (22%)[a]
EBV lysate	6/17 (35%)	12/18 (67%)	7/12 (58%)	4/8 (50%)	5/7 (71%)	34/62 (55%)[b]
pp65 peptides	5/10 (50%)	3/10 (30%)	6/8 (75%)	6/6 (100%)	4/4 (100%)	24/38 (63%)[bc]
IE1 peptides	8/12 (67%)	4/12 (33%)	6/10 (60%)	5/7 (71%)	3/4 (75%)	26/45 (58%)[b]
HA peptides	4/17 (24%)	0/18 (0%)	2/13 (15%)	1/9 (11%)	3/8 (38%)	10/65 (15%)[ac]
PMA / I	17/17 (100%)	17/18 (94%)	12/12 (100%)	8/8 (100%)	7/8 (88%)	61/63 (97%)[d]
CD4 T-cell response in proliferation assay						
CMV lysate	5/14 (36%)	6/12 (50%)	5/6 (83%)	3/5 (60%)	4/6 (67%)	23/43 (53%)[a]
EBV lysate	6/10 (60%)	2/7 (29%)	3/4 (75%)	1/2 (50%)	3/4 (75%)	15/27 (56%)[a]
HA peptides	3/16 (19%)	5/14 (36%)	3/5 (60%)	0/3 (0%)	0/6 (0%)	11/44 (25%)[b]
PMA / I	11/16 (69%)	10/14 (71%)	5/5 (100%)	2/3 (67%)	4/6 (67%)	32/44 (73%)[a]

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	Age group					
	20-29	**30-39**	**40-49**	**50-59**	**60-69**	**Total**
	B-cell response in proliferation assay					
CMV lysate	5/12 (42%)	2/9 (22%)	2/4 (50%)	1/2 (50%)	2/4 (50%)	12/31 (39%)[ab]
EBV lysate	6/10 (60%)	4/9 (44%)	2/4 (50%)	1/3 (33%)	1/3 (33%)	14/29 (48%)[a]
HA peptides	4/15 (27%)	7/16 (44%)	4/6 (67%)	1/3 (33%)	1/6 (17%)	17/46 (37%)[ab]
PMA/I	5/15 (33%)	2/16 (13%)	1/6 (17%)	0/3 (0%)	0/6 (0%)	8/46 (17%)[b]