ChAdOx1 nCoV-19 vaccination generates spike-specific CD8+ T cells in aged mice.

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Abstract

Effective vaccines have reduced SARS-CoV-2 morbidity and mortality; however, the elderly remain the most at risk. Understanding how vaccines generate protective immunity, and how these mechanisms change with age is key for informing future vaccine design. Cytotoxic CD8+ T cells are important for killing virally infected cells, and vaccines that induce antigen specific CD8+ T cells in addition to humoral immunity provide an extra layer of immune protection. This is particularly important in cases where antibody titres are sub-optimal, as can occur in older individuals. Here, we show that in aged mice, spike-epitope specific CD8+ T cells are generated in comparable numbers to younger animals after ChAdOx1 nCoV-19 vaccination, although phenotypic differences exist. This demonstrates that ChAdOx1 nCoV-19 elicits a good CD8+ T cell response in older bodies, but that typical age-associated features are evident on these vaccine reactive T cells.

Introduction

Since the beginning of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) pandemic in March 2020, multiple effective vaccines have been produced. This includes the ChAdOx1 nCoV-19 vaccine (AZD1222) which has been supplied in billions of doses worldwide. The ChAdOx1 nCoV-19 vaccine is an adenovirus vectored vaccine, which can stimulate CD8+ T cell responses as well as inducing neutralising antibodies [1, 2]. ChAdOx1 nCoV-19 can prevent symptomatic infections, limit viral transmissibility, reduce infection severity, and prevent hospitalisations and death caused by variants of concern [3-6]. Vaccine efficacy is associated with the effective generation of anti-spike protein antibodies [7]. While generation of protective humoral immunity is a key function of vaccines, adenovirus-vector vaccine-induced memory CD8+ T cells also provide cellular immunity [8, 9]. Murine experiments using protein-based vaccines and challenge with SARS-CoV-2 variants showed that, in the absence of viral-neutralising antibodies, CD8+ T cells provide protection, with depletion of CD8+ T cells leading to an increase in viral load [10]. Furthermore, CD8+ T cells can provide cross protective

immunity to variants of concern, across multiple vaccine platforms, providing protection as the virus continues to evolve [11].

Generally, ageing negatively impacts the function of immune system, and for COVID-19 infections, likely contributes to the strong correlation between advancing age and risk of hospitalisation or death [12]. Moreover, vaccination becomes less effective with increased age, as older individuals have lower serum neutralisation and IgG/A titres after a single vaccination with Pfizer's BNT162b2 mRNA vaccine [13]. Furthermore, older patients have responses which wane more quickly, meaning increased risk over time [14]. Despite this, the phase 3 trial of ChAdOx1 nCoV-19 found that vaccine efficacy was maintained in participants over 65 years old, despite lower humoral immunity [15]. During ageing, the CD8+ T cell compartment changes; thymic involution occurs in early adulthood and reduces the supply of new naïve T cells, and later, an accumulation of differentiated cells with a central memory (CD44+ CD62L+) phenotype occurs, although these cells may include antigen-independent differentiation of virtual memory T cells [16]. Age-associated effector subset expansion is more pronounced in CD8+ T cells as compared to CD4+ T cells, with CD8+ T cells exhibiting lesser homeostatic stability [17]; naïve CD8+ T cell frequency declines more drastically over time , as clones are either lost or differentiate into central/virtual memory cells [18, 19].

Here we aimed to characterise the production of antigen specific CD8+ T cells following a single ChAdOx1 nCoV-19 vaccination, in order to understand how their number and phenotype changes in ageing. We report that while aged mice have decreased humoral immunity following a single ChAdOx1 nCoV-19 vaccination, they successfully generate comparable numbers of spike specific CD8+ T cells to younger adult animals, albeit with an altered phenotype.

Results

Aged mice have reduced serum humoral immunity after ChAdOx1 nCoV-19 vaccination

Ageing is known to impact humoral immunity after vaccination. In order to test whether the CD8+ T cell response is intact in ageing despite humoral immunity being reduced, we first determined the total and neutralising antibody titre after ChAdOx1 nCoV-19 vaccination. Indeed, 42 days after intramuscular vaccination, 22-month-old mice had lower serum SARS-CoV-2 pseudo-virus neutralising capacity, as compared to 3-month-old mice (Figure 1A). Consistent with this, 22-month-old mice also had a reduction in both RBD (Figure 1B) and Spike (Figure 1C) binding IgG in the serum. We also correlated these two metrics of vaccine protection, showing that mice with higher neutralising capacity had higher serum spike specific antibody (Figure 1D), indicating that titre rather than quality of the antibody response is impaired with advanced age. These results confirm previous findings; that ageing negatively affects the generation of antibody following prime vaccination for SARS-CoV-2 [2, 13, 20].

Aged mice generate comparable numbers of spike-specific CD8+ T cells after ChAdOx1 nCoV-19 vaccination

To track spike-epitope specific CD8+ T cells directly ex vivo after vaccination we used a peptide Class I tetramer (SARS-CoV-2 S 539-546 – VNFNFNGL) to detect binding CD8+ T cells in the draining medial iliac lymph node (mILN) in both 3-month-old and 22-month-old mice (Figure 2A). We found that the 22-month-old mice had an increased frequency of spike-specific CD8+ T cells at both 14- and 42-days post vaccination, which corresponded to a greater number of spike-specific CD8+ T cells in the mILN at day 14 (Figure 2B). Typically, after vaccination, the draining lymph node has a higher frequency of

antigen specific B or CD4+ T cells, as compared to the spleen [2]. However, we found that the spleen has a greater frequency of antigen specific CD8+ T cells following ChAdOx1 nCoV-19 vaccination compared to the draining mILN, and is therefore the primary reservoir of these cells (Figure 2C). In the spleen, there was no difference in either the frequency, or the total number of spike-specific CD8+ T cells between 3-month-old and 22-month-old mice at either 14- or 42-days post vaccination. Thus, despite the various age-associated defects in lymphocyte biology following vaccination, expansion of spike-specific CD8+ T cells occurs effectively in 22-month old mice in response to ChAdOx1 nCoV-19 and these cells are maintained for at least 42 days.

Spike protein specific CD8+ T cells from aged mice have an altered phenotype.

Following the observation that old mice generated spike-specific CD8+ T cells effectively, we next explored their phenotype. Given that the spike-specific CD8+ T cells in the spleen outnumbered those of the mILN by a factor of around 100, we first analysed the spleen. We sought to contextualise our observations by performing a side by side comparison of the spike-specific CD8+ T cell population alongside total CD8+ T cells in the vaccinated mice. The majority of spike+ CD8+ T cells, were of CD44+ CD62L- T-effector (Teff) phenotype that marks both effector and effector memory T cells, and 22-month-old mice had a near ubiquitous Teff phenotype within their spike+ CD8+ T cells (Figure 3A). For the total CD8 cell population, 22-month-old mice had a higher frequency of CD8+ T cells with Teff phenotype, at both timepoints after vaccination (Figure 3B). PD-1 can be a marker of T cell activation, and consistent with this spike-specific CD8+ T cells from mice of both age groups had a high frequency of PD-1 expression 14-days post-vaccination, which dropped after 42 days, with spike-specific CD8+ T cells from 22-month-old mice retaining higher PD-1 expression at this later time point compared to 3-month-old mice (Figure 3C). This trend was also seen across the entire CD8+ T cell pool, in which 22-month old mice had more PD1+ cells than young mice. Expression of T-BET and CXCR3 in antigen-specific CD8+ T cells is associated with an activated effector phenotype [21, 22]. We quantified expression of these markers in our study and found that spike+ CD8+ T cells for both 3-month-old and 22-month-old mice were >95% positive for these markers, with subtle differences between age groups (Figure 3E). Across all CD8+ T cells, 22-month-old mice had an increased frequency of T-BET+ CXCR3+ dual expressing cells, suggesting an overall skew towards an activated phenotype (Figure 3F).

These splenic observations were largely recapitulated in the mILN, as nearly all spike-specific cells had a Teff phenotype (Figure 4A), while 22-month old mice had an overall increased frequency of Teff phenotype CD8+ T cells (Figure 4B). We also noted that Teff phenotype cells were less frequent in the mILN than in the spleen (Figure 3B, 4B). Similar to the spleen, 22-month-old mice had an increased frequency of PD-1 expression in both spike-specific and total CD8+ cells 14-days post-vaccination, with spike-specific CD8+ T cells largely maintaining their PD-1 expression 42 days after vaccination (Figure 4C, D). Again, spike-specific mILN CD8+ cells were near ubiquitously T-BET and CXCR3 double positive (Figure 4E). Finally, total CD8+ cells from 22-month-old mice had increased frequency of T-BET and CXCR3 co-expression as compared to 3-month-old mice (Figure 4F). Thus, ageing is associated with the increased acquisition of activation markers associated with effector phenotype on CD8+ T cells, with these markers being very frequently expressed by spike-specific CD8+ T cells.

Discussion

In this study we compared the response made by young and old mice following ChAdOx1 nCoV-19 vaccination, showing that while humoral immunity is compromised in ageing, spike-epitope specific CD8+ T cells expand, albeit with an altered phenotype. Six weeks after vaccination, serum from 22-month-old mice had reduced SARS-CoV-2 pseudo-neutralising capacity, as well as lower spike-specific IgG titre. This agrees with our previous findings, as well as findings of many others that humoral

immunity is compromised in advanced age [2, 13, 23]. Despite this reduction in humoral immunity, we found that expansion of spike-specific CD8+ T cells occurs effectively, and is potentially enhanced in aged mice, with the spleen acting as the primary reservoir in which millions of spike-specific CD8+ T cells can be found. In aged mice however, there is a shift of phenotype amongst CD8+ T cells after vaccination, and this is seen to an even greater extent within the recently activated Spike-epitope specific pool of CD8+ T cells evaluated here, with increased expression of PD-1, CD44 and dual expression of T-BET and CXCR3.

A known feature of the ageing immune system is a reduction of T cell receptor repertoire diversity which occurs as a result of thymic involution and expansion of antigen experienced clones [24]. This loss of CD8+ T cell diversity is non-random, being at least in part driven by T cell receptor:pMHC avidity, such that cells specific for epitopes with poor self-pMHC avidity are likely to be lost [25]. This therefore impacts the response to specific antigens; for example, following influenza infection aged mice have a reduced response to the immunodominant nucleoprotein epitope $NP_{366-374}$ [26]. Our results suggest that clones specific for spike epitopes are maintained in aged mice, such that antigen specific CD8+ T cells are successfully generated following ChAdOx1 nCoV-19 vaccination, but produce fewer effector cytokines following spike peptide-pool stimulation, despite their activated phenotype [2].

As ageing occurs, the microenvironment becomes more pro-inflammatory [27]. This increased concentration of inflammatory cytokines and resultant low-level signalling via various pathways, likely contributes to an altered baseline phenotype and leaves CD8+ T cells less capable to respond to stimuli [28]. A continual supply of low-grade activation signals results in differentiation into a virtual memory state, the development of which correlates with the age-related decline seen in primary responses. These virtual memory cells have limited functionality and become senescent with age, and transfer of old CD8+ T cells into young mice does not rescue their responsiveness, potentially due to epigenetic imprinting [29, 30]. Therapeutics aiming to reverse T cell exhaustion by targeting PD-1 are becoming popular, and a recent paper highlighted that cancer patients treated with anti-PD-1 immunotherapy had an increase in proliferation within their PD-1 expressing CD4+ T follicular helper cells [31]. Short term anti-PD-1 treatment has been shown to increase the protection provided by SIV vaccines in macaques, and increases viral clearance and CD8+ memory precursor formation during LCMV infection of mice [32, 33]. Given the increased expression of PD-1 in aged mice shown here, targeting this pathway during SARS-CoV-2 vaccination may boost CD8+ T cell responses, although the risk of self-reactive T cell development may limit its use as a vaccine enhancing strategy.

In summary, our data shows that the ChAdOx1 nCoV-19 vaccine successfully expands spike-peptide specific CD8+ T cells in both younger adult and aged mice, agreeing with recent data showing that despite acquiring exhaustion markers such as PD-1, CD8+ T cells are capable of continued supernumerary cell division in the appropriate context [34]. Although, CD8+ T cells from aged mice produce fewer cytokines than their younger counterparts when directly stimulated with spike protein peptides [2]. This suggests that for future vaccines to successfully generate cytotoxic CD8+ T cell immunity in aged individuals, they must target epitopes which survive the age-associated contraction of T cell repertoire, and that the background environment into which the vaccine is introduced plays an unavoidably important role for CD8+ T cell responses.

Adenovirus-based vaccines are an attractive option for vaccinating the elderly, as ChAdOx1 nCoV-19 vaccination successfully expands antigen specific CD8+ T cells in aged mice, and this layer of cellular immunity is maintained to a greater extent than humoral immunity when viral variants occur [35].

Figures and legends:

Figure 1

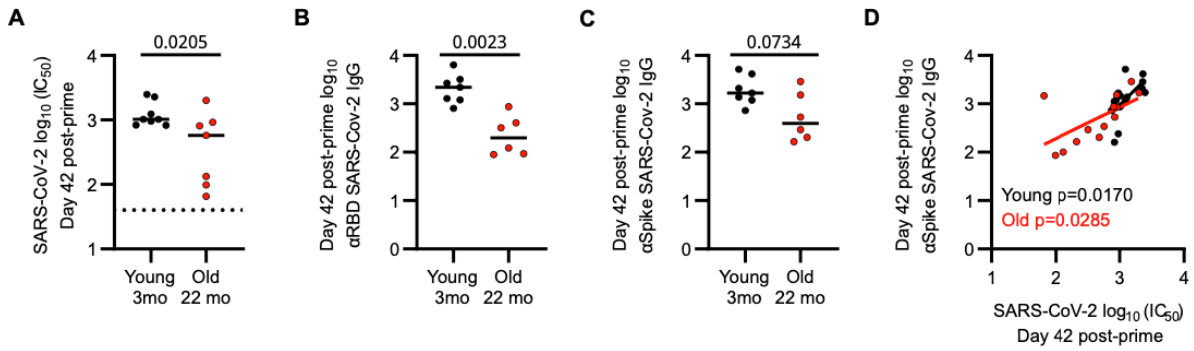

Figure 1 Advanced age negative affects serum immunity after ChAdOx1 nCoV-19 vaccination

Young (3 months old) and old (22 months old) mice were immunised with 50 µL of ChAdOx1 nCoV-19 (10^8 infectious units) intramuscularly. At 42 days post vaccination serum samples were analysed. **(A)** SARS-CoV-2 serum neutralising capacity expressed as reciprocal serum dilution required to inhibit pseudo-typed virus entry by 50% (IC50). Dotted line represents lower detection limits. **(B and C)** Serum anti-RBD **(B)** and anti-Spike **(C)** IgG antibodies. **(D)** IC50 vs anti-Spike IgG serum antibody. (A) young mice n=8, old mice n=7. (B and C) young mice n=7, old mice n=6. Data representative of two individual experiments. (D) young mice n=15, old mice n=13. Data combined from two individual experiments. (**A-C)**, a Mann-Whitney test was used. For **(D)** separate linear regressions were performed for young and old mice respectively.

Figure 2

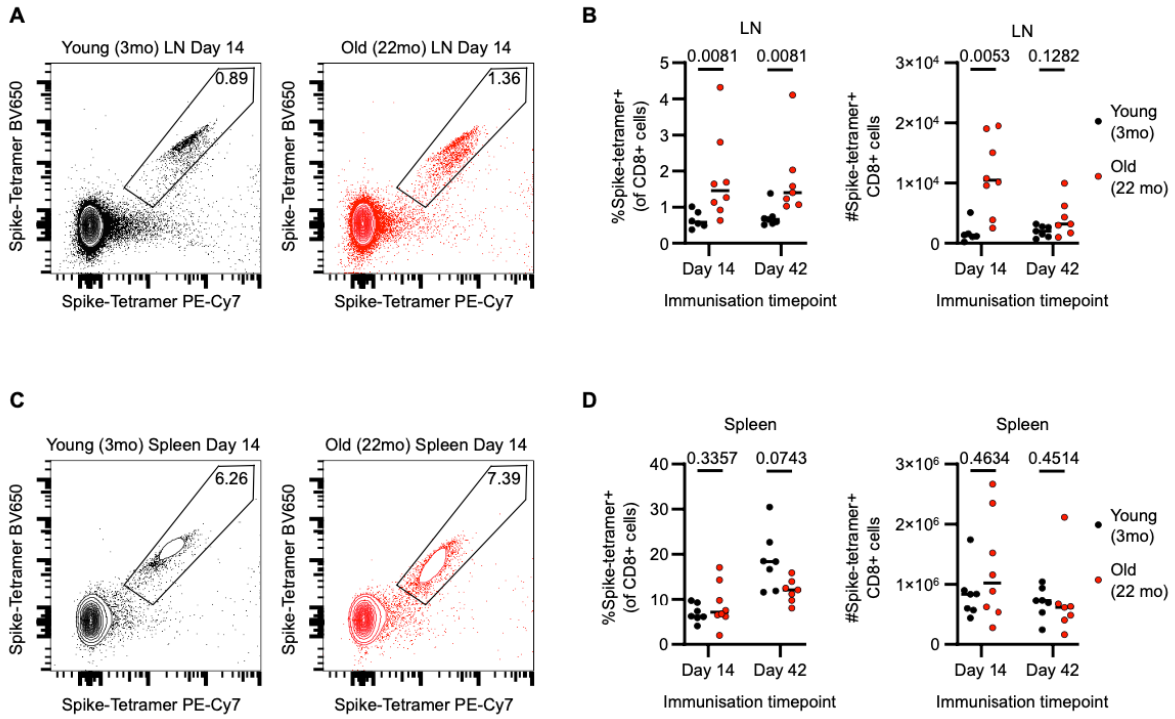

Figure 2 Aged mice generate comparable numbers of Spike-specific CD8+ T cells after ChAdOx1 nCoV-19 vaccination

Mice were immunised with 50 µL of ChAdOx1 nCoV-19 (10^8 infectious units) intramuscularly. At indicated timepoints, medial iliac lymph node (LN) and spleen samples were taken for analysis. **(A)** LN Spike-protein tetramer (SARS-CoV-2 S 539-546 - VNFNFNGL) binding CD8+ T cells were identified by flow cytometry analysis in both young and old mice. **(B)** Frequency (as a percentage of all CD8+ T cells) and number of Spike-protein specific CD8+ T cells were quantified in young and old mice in the LN at indicated timepoints. **(C)** Spleen Spike-protein tetramer (SARS-CoV-2 S 539-546 - VNFNFNGL) binding CD8+ T cells were identified by flow cytometry analysis in both young and old mice. **(D)** Frequency (as a percentage of all CD8+ T cells) and number of Spike-protein specific CD8+ T cells were quantified in young and old mice in the spleen at indicated timepoints. Each symbol represents a unique biological sample, n=7/8 per group. Data representative of two individual experiments. For **(B and D)** Multiple Mann-Whitney tests per row with multiple testing correction was used.

Figure 3

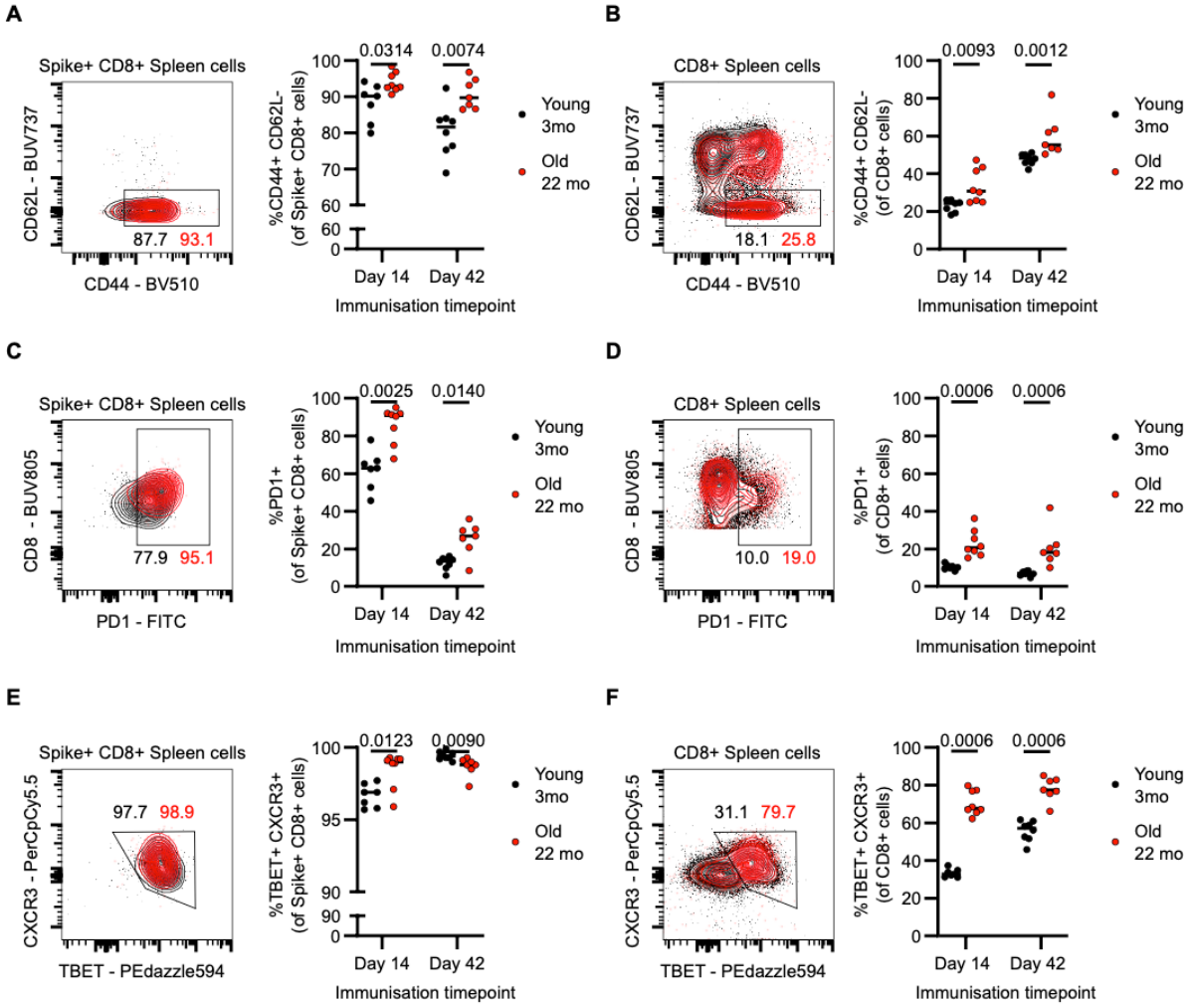

Figure 3 Splenic spike protein specific CD8+ T cells from aged mice have an altered phenotype.

Mice were immunised with 50 µL of ChAdOx1 nCoV-19 (10^8 infectious units) intramuscularly. At indicated timepoints, spleen samples from 3-month-old and 22-month-old mice were taken to analyse the frequency of various CD8+ T cell subsets by flow cytometry. **(A)** Spike-specific CD44+ CD62L- (T effector memory) phenotype. **(B)** Total CD44+ CD62L- (T effector) phenotype. **(C)** Spike-specific PD1 expressers. **(D)** Total PD1 expressers. **(E)** Spike-specific TBET+ CXCR3+ (activated) phenotype. **(F)** Total TBET+ CXCR3+ (activated) phenotype. Each symbol represents a unique biological sample, n=7/8 per group. Data representative of two individual experiments. For **(A-F)** Multiple Mann-Whitney tests per row with multiple testing correction was used.

Figure 4

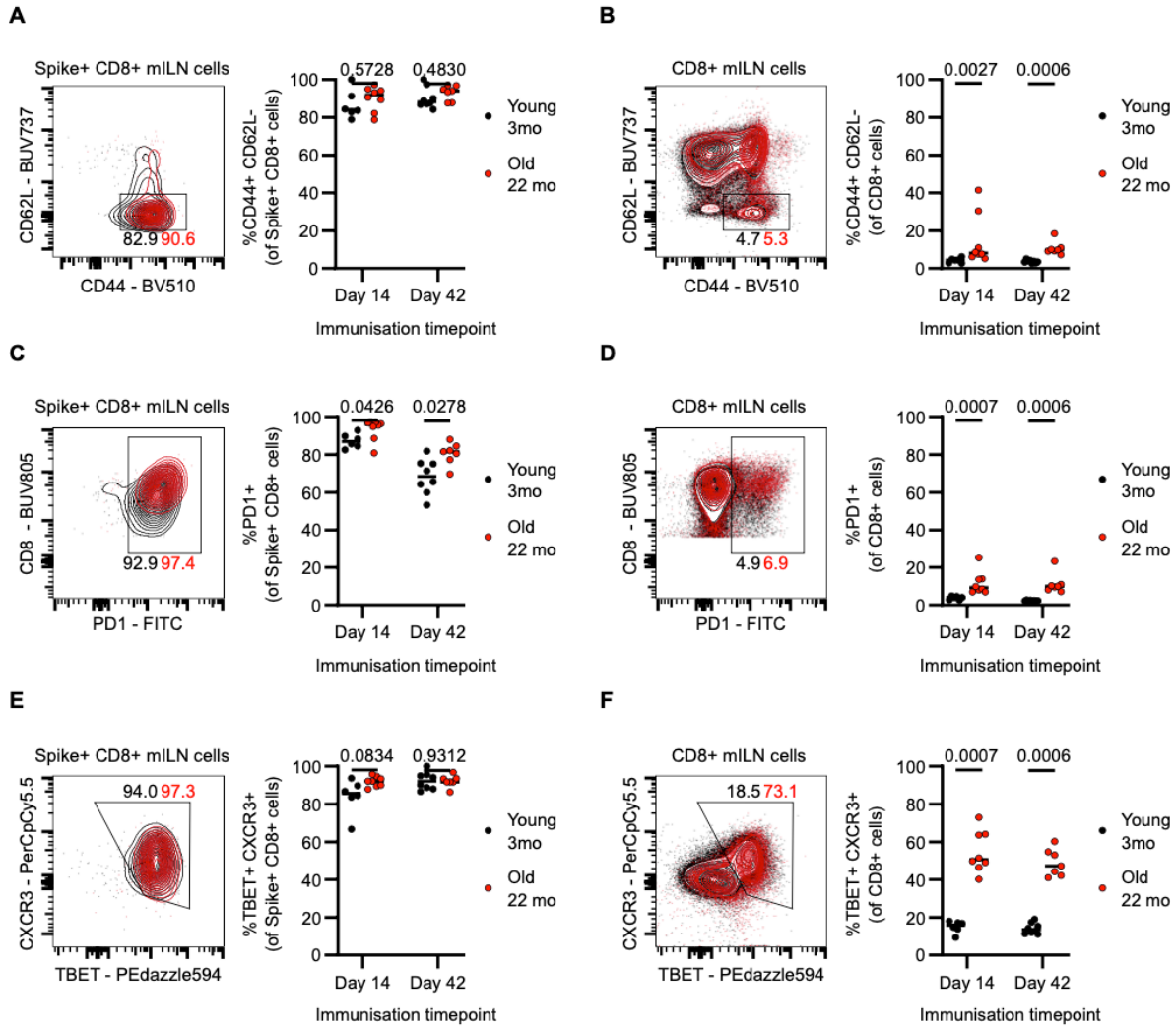

Figure 4 mILN spike protein specific CD8+ T cells from aged mice have an altered phenotype.

Mice were immunised with 50 µL of ChAdOx1 nCoV-19 (10^8 infectious units) intramuscularly. At indicated timepoints, medial iliac lymph node (mILN) samples from 3-month-old and 22-month-old mice were taken to analyse the frequency of various CD8+ T cell subsets by flow cytometry. **(A)** Spike-specific CD44+ CD62L- (T effector memory) phenotype. **(B)** Total CD44+ CD62L- (T effector) phenotype. **(C)** Spike-specific PD1 expressers. **(D)** Total PD1 expressers. **(E)** Spike-specific TBET+ CXCR3+ (activated) phenotype. **(F)** Total TBET+ CXCR3+ (activated) phenotype. Each symbol represents a unique biological sample, n=7/8 per group. Data representative of two individual experiments. For **(A-F)** Multiple Mann-Whitney tests per row with multiple testing correction was used.

Methods

Mouse housing, husbandry and immunisation.

C57BL/6Babr mice were bred and maintained in the Babraham Institute Biological Support Unit. No primary pathogens or additional agents listed in the FELASA recommendations were detected during health monitoring surveys of the stock holding rooms. Ambient temperature was ~19–21°C and relative humidity 52%. Lighting was provided on a 12hr light: 12 hr dark cycle including 15 min 'dawn' and 'dusk' periods of subdued lighting. After weaning, mice were transferred to individually ventilated cages with up to 5 mice per cage. Mice were fed CRM (P) VP diet (Special Diet Services) ad libitum and received seeds at the time of cage-cleaning as part of their environmental enrichment. All mouse experimentation was approved by the Babraham Institute Animal Welfare and Ethical Review Body. Animal husbandry and experimentation complied with existing European Union and United Kingdom Home Office legislation and local standards (PPL: P4D4AF812). Mice were immunised at 10-12 weeks old, and 95-101 weeks old for young and old groups respectively. Mice were immunised in the right quadriceps femoris muscle with 10^8 infectious units of ChAdOx1 nCoV-19 in 50 µL of phosphate buffered saline (PBS).

Micro-neutralisation test using lentiviral-based pseudotypes bearing the SARS-CoV-2 Spike.

Lentiviral-based SARS-CoV-2 pseudotyped viruses (pps) were generated in HEK293T cells as previously described [36]. Cells were seeded in 6 well dishes, before being transfected with SARS-CoV-2 spike, p8.91 (encoding HIV-1 gag-pol) and CSFLW in Opti-MEM along with 10 µL PEI transfection reagent overnight. Then, the transfection mix was replaced with 3 mL DMEM with 10% FBS (DMEM-10%) and incubated for 48 and 72 hours, then SARS-CoV-2 pps supernatants were pooled and centrifuged to remove cellular debris. Target HEK293T cells (transfected with human ACE2 expression plasmid) were seeded at a density of 2×10^4 in 100 µL DMEM-10% overnight. SARS-CoV-2 pps were titrated 10-fold on target cells. Sera were diluted 1:20 in serum-free media and added to a 96-well plate in triplicate and titrated 2-fold. A fixed titred volume of SARS-CoV-2 pps was added at a dilution equivalent to 10^5 signal luciferase units in 50 µL DMEM-10% and incubated with sera for 1 hour. Target cells expressing human ACE2 were then added at a density of 2×10^4 in 100 µL and incubated for 72 hours. Firefly luciferase activity was then measured with BrightGlo luciferase reagent and a Glomax-Multi+ Detection System (Promega, Southampton, UK).

Enzyme-linked immunosorbent assay (ELISA)

Standardised ELISA was performed to detect SARS-CoV-2 Spike or RBD – specific antibodies. MaxiSorp plates (Nunc) were coated with 100 ng/well protein overnight at 4 °C. Plates were washed with PBS + 0.05% Tween20, and blocked with Blocker™ Casein in PBS (Thermo Fisher Scientific) for 1 h at room temperature (RT). Sera (including positive, negative and internal control samples) diluted in casein were incubated for 2 hours at RT. Plates were washed, then AP-conjugated goat anti-mouse IgG or AP-conjugated goat anti-human IgG (Sigma-Aldrich) with pNPP substrate (Sigma-Aldrich) was added for 1h at RT. An arbitrary number of ELISA units were assigned to the positive control samples and OD values of each dilution were fitted to a 4-parameter logistic curve using SOFTmax PRO software. Sample ELISA values were calculated using the generated standard curve.

MHC-1 tetramer generation and flow cytometry

MHC-1 monomers were acquired from the NIH tetramer core, and conjugated to streptavidin following provided instructions; A 4:1 molar ratio was calculated for monomer:streptavidin incubation. Streptavidin was added to monomers in 10% increments, with each 10% added after 10

minutes, at room temperature in the dark. Once completely combined, reagents were left overnight at 4 °C before use.

Medial Iliac lymph nodes/spleens were pressed through a 70 µm mesh and washed through with FACS buffer (PBS containing 2% FBS and 1mM EDTA) to generate single cell suspensions. Cell numbers and viability were determined using a CASY TT Cell Counter (Roche). 2×10^6 cells were transferred into 96-well V bottom plates, which were then centrifuged and supernatant was removed. Cells were resuspended in 50µL of RPMI containing 200 nM Dasatinib (added to prevent T cell receptor internalisation). Cells were incubated for 15 minutes at 37 °C, before 50 µL RPMI + Dasatinib containing MHC-1 Spike-specific tetramers were added. Cells were then incubated for 105 minutes at room temperature in the dark. Cells were washed with FACS buffer, and stained with 100 µL of surface antibody mix for 2 hours at 4°C. Cells were washed twice with FACS buffer, and fixed with eBiosciences Foxp3/Transcription Factor Fixation reagent diluted as per manufacturer's instructions (#00-5323-00) for 30 min at 4°C. Cells were then washed with 1x Permeabilisation buffer (eBioscience #00-8333-56) twice and stained with intracellular antibody mix in permeabilisation buffer supplemented with 20% 2.4G2 hybridoma (ATCC hb-197) tissue culture supernatant at 4°C overnight. Cells were washed twice with permeabilisation buffer and once with FACS buffer and acquired on a 5-laser Cytek™ Aurora spectral flow cytometer. Cells for single colour controls were prepared in the same manner as fully stained samples. The antibodies used for surface and overnight staining are listed below. Manual gating of flow cytometry data was done using FlowJo v10.7 software (Tree Star).

Antibody/Fluorophore	**Supplier**	**Catalogue #**	**RRID**
Brilliant Violet 510™ anti-mouse/human CD44 Antibody	BioLegend	103044	AB_2650923
PE/Dazzle™ 594 anti-T-bet Antibody	BioLegend	644828	AB_2565677
BUV737 Rat Anti-Mouse CD62L	BD Biosciences	612833	AB_2870155
PerCP/Cyanine5.5 anti-mouse CD183 (CXCR3) Antibody	BioLegend	126514	AB_1186017
CD279 (PD-1) Monoclonal Antibody (RMP1-30), FITC	Thermo Fisher Scientific	11-9981-82	AB_465467
PE/Fire(TM) 640 anti-mouse CD4 antibody	BioLegend	100482	AB_2860585
BUV805 Rat Anti-Mouse CD8a	BD Biosciences	564920	AB_2716856
Brilliant Violet 650™ Streptavidin	BioLegend	405232	
PE/Cyanine7 Streptavidin	BioLegend	405206	

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Author contributions

Conceptualisation, W.S.F., M.A.L. and T.L.

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Investigation, W.S.F., N.T., J.N., A.J.S., S.D, D.W. and L.G.

Resource acquisition, T.L.

Writing – Original Draft Preparation, W.S.F, S.R., A.R. and M.A.L.

Writing – Review and Editing, all authors.

Project Administration, W.S.F., M.A.L. and T.L.

Funding Acquisition, M.A.L., D.B. and T.L.

Competing Interests

T.L. is named on a patent application covering ChAdOx1 nCoV-19. The remaining authors declare no competing interests. The funders played no role in the conceptualisation, design, data collection, analysis, decision to publish, or preparation of the manuscript.

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