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The impact of immunosenescence and senescence of immune cells on responses to infection and vaccination

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The impact of immunosenescence and senescence of immune cells on responses to infection and vaccination

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EDITORIAL
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Editorial: The Impact of Immunosenescence and Senescence of Immune Cells on Responses to Infection and Vaccination

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Keywords: immunosenescence, T cell repertoire, antigen-specific T cells, vaccines, pneumococcal responses

Editorial on the Research Topic

The Impact of Immunosenescence and Senescence of Immune Cells on Responses to Infection and Vaccination

Advancing age is characterized by changes in the innate and adaptive immune system termed immunosenescence, which contribute to the pathogenesis of various diseases, increased incidence of infections and reduced response to vaccinations. Antigen sensing, presentation and cytokine responses of innate cells are all altered with age leading to impaired responses to infection and vaccines. Aging affects individual T and B cells throughout their life-cycle, from alterations in hematopoiesis, maturation and homeostasis, to memory generation and effector functions as well as their interactions with other cell types, and the composition and repertoire of the adaptive immune cell compartments. Latent infection with Cytomegalovirus (CMV), has a profound impact on the aging immune system, particularly on the T cell compartment, but also other herpesviruses, e.g., Epstein-Barr virus (EBV) might play a role in T cell aging. Periodical reactivation of these viruses shapes the evolution of the T cell repertoire in aging leading to decreased diversity. The general dogma is that the T cell memory responses generated early in life remain stable as we age. Thus, one of the proposed strategies to improve vaccine responses in the elderly is to vaccinate early before these changes occur. However, Lanfermeijer et al. show that the T cell repertoire for CMV and EBV loses stability with age and becomes less diverse. They also demonstrate that CMV-infection is associated with a decreased diversity of EBV-specific $CD8^+$ T cells highlighting the impact of CMV on the T cell response to other pathogens. In the past, life-long repeated antigenic stimulation (e.g., in the context of CMV-infection) was thought to be the only driver of terminal differentiation of T cells. These terminally differentiated T cells, which accumulate with aging, exhibit characteristics of senescence and are frequently thought to be dysfunctional. However, recent data show that senescent-like T cells acquire alternative functions, e.g. Natural Killer (NK) cell characteristics, which are independent of antigen specificity, and that antigen-independent bystander activation of T cells by cytokines may drive senescence as well as expression of NK-like activity in T cells (Abbas and Akbar). Replicative and cellular senescence is frequently studied in cell types such as fibroblasts, but over the last years hallmarks of aging such as DNA damage, the senescence-associated secretory phenotype (SASP), mitochondrial dysfunction, protein homeostasis etc., have also been investigated in immune cells and have been identified as important players in T cell aging. Using mice with a deficiency in the DNA excision-repair gene *Ercc1*, Pieren et al. show accumulation of Tregs with an aging-related phenotype and reduced T-cell responsiveness.

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Immunosenescence contributes to increased incidence, morbidity and mortality of infections, e.g., of influenza, pneumococcal disease, herpes zoster and many more. He et al. investigate $CD4^+$ T cells specific for the immunogenic pneumococcal protein AliB and show that proliferation of pneumococcus-specific T cells as well as cytokine production is impaired in older adults, while regulatory T cells, that suppress T cell activation and induction of protective cytokines like IFN-γ and IL-17 accumulate. Lambert et al. studied $CD4^+$ T cell responses to pertussis in aging individuals. They also observed reduced frequencies of proliferating $CD4^+$ T cells against *Bordetella pertussis,* but this was not due to an increase in T regulatory cells. Instead, there was a deficiency in the T cell activation markers suggesting that several mechanisms are impacting $CD4^+$ T cell responses in the elderly. More importantly, they also showed that there is a gradual loss of memory against various pertussis epitopes decreasing the diversity of the pertussis repertoire similar to what was shown by Lanfermeijer et al. for EBV-specific $CD8^+$ T cells. The two studies clearly highlight the importance of studying antigen-specific T cell repertoire responses in aging. An insight into the mechanisms underlying the selective loss of certain epitopes with age is essential to design effective vaccines against the right epitopes. We also need to understand whether this loss is selective to certain phenotypes of memory T cells. The contribution of innate immune cells such as dendritic cells (DCs) and macrophages in maintenance of the T cell memory pool is also an understudied area. The impact of age on infectious diseases recently received increased attention as old age is also a major risk factor for severe COVID-19 disease. Farheen et al. summarize the impact of an aged immune system on susceptibility to SARS-CoV-2 infection and the risk for complications. At the same time, many -but not all-vaccines show less immunogenicity and clinical efficacy/effectiveness in older adults. Antibody responses to influenza and pneumococcal vaccination are generally lower in older adults leading to decreased protection for this age group. Crooke et al. report that inflammasome activation in response to influenza vaccination is preserved in monocyte-derived macrophages from older adults and does not explain the lower antibody responses observed in this age group. They also found increased expression of certain inflammasome-related genes in females compared to males highlighting the need to study sex-related differences in immune responses. Development of vaccine adjuvants and delivery systems that are effective in the elderly is the need of the hour especially with increased longevity. Bhalla et al. suggest a novel formulation of Liposomal Encapsulation of Polysaccharides (LEPS) to improve immune responses to pneumococcal vaccine and demonstrate protective effects of LEPS against invasive and pulmonary pneumococcal infections in aged mice. It will be interesting to see whether this platform is effective in generation of respiratory immunity against other pathogens. Several vaccines are specifically recommended for older adults (e.g., against influenza, pneumococcal disease and herpes zoster). In addition, vaccines recommended for all adults, such as regular booster vaccinations against tetanus/diphtheria/pertussis or other diseases depending on the epidemiological/geographical situation are relevant for older adults. The number of older travelers is on the rise and senior travelers have specific risks linked to their age, overall health and travel patterns. However, data on efficacy/effectiveness of travel vaccines in older adults are scarce. Ecarnot et al. summarize the risk of major vaccine-preventable travel-associated infectious diseases and vaccines recommended for older travelers.

In summary, aging leads to the accumulation of dysfunctional T cells along with a reduction of antigen-specific T cell repertoire. The loss of naïve T cells is considered a major culprit. The intrinsic changes in T cells themselves as well as age-associated changes in antigen presenting cells (APCs) required to prime T cell responses are areas that need further studies to improve immune responses. In this regard, information regarding tissue-resident immunity is also lacking in aging. The changes in mucosal immune response, also needs further studying since it is becoming clear that the tissue microenvironment shapes the immune response and older adults are at high risk for respiratory infections. Changes in matrix components and accumulation of dysfunctional or senescent matrix cells such as fibroblasts as well as immune cells are increasingly being recognized for their role in various age-associated diseases. Novel strategies are needed to rejuvenate the aging immune system in order to prevent infections and other age-associated diseases and to ensure optimal protection by vaccination. One such strategy might be the use of senolytics that can selectively remove dysfunctional aged cells. Cancer therapies as well as drugs altering the metabolic state of cells to induce apoptosis are potential senolytics. However, development of technologies that can target senescent cells for delivery of senolytics will be essential for the success of this approach.

AUTHOR CONTRIBUTIONS

AA and BW wrote the manuscript.

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Age and CMV-Infection Jointly Affect the EBV-Specific CD8$^+$ T-Cell Repertoire

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CD8$^+$ T cells play an important role in protection against viral infections. With age, changes in the T-cell pool occur, leading to diminished responses against both new and recurring infections in older adults. This is thought to be due to a decrease in both T-cell numbers and T-cell receptor (TCR) diversity. Latent infection with cytomegalovirus (CMV) is assumed to contribute to this age-associated decline of the immune system. The observation that the level of TCR diversity in the total memory T-cell pool stays relatively stable during aging is remarkable in light of the constant input of new antigen-specific memory T cells. What happens with the diversity of the individual antigen-specific T-cell repertoires in the memory pool remains largely unknown. Here we studied the effect of aging on the phenotype and repertoire diversity of CMV-specific and Epstein-Barr virus (EBV)-specific CD8$^+$ T cells, as well as the separate effects of aging and CMV-infection on the EBV-specific T-cell repertoire. Antigen-specific T cells against both persistent viruses showed an age-related increase in the expression of markers associated with a more differentiated phenotype, including KLRG-1, an increase in the fraction of terminally differentiated T cells, and a decrease in the diversity of the T-cell repertoire. Not only age, but also CMV infection was associated with a decreased diversity of the EBV-specific T-cell repertoire. This suggests that both CMV infection and age can impact the T-cell repertoire against other antigens.

Keywords: aging, cytomegalovirus, Epstein-Barr virus, T-cell repertoire, T cell

INTRODUCTION

CD8$^+$ T cells play an important role in the control and clearance of viral infections. One of the key components of a protective T-cell response is the recognition of viral epitopes via the T-cell receptor (TCR). T-cell receptors are formed via the random process of somatic V(D)J-recombination, leading to a large collection of TCRs with different specificities (Market and Papavasiliou, 2003). It is generally assumed that the diversity of the T-cell receptor repertoire is positively correlated with the level of protection against infectious diseases (Turner et al., 2009). The diversity of the total CD8$^+$ T-cell repertoire decreases with age (Britanova et al., 2014, 2016; Yoshida et al., 2017), which is mainly caused by a decrease in naive T-cell numbers (Britanova et al., 2014) as well as a decreased TCR diversity within the naive T-cell pool (Qi et al., 2014; Egorov et al., 2018). Together with less efficient priming of T cells (Briceno et al., 2016), this may explain why both CD8$^+$

T-cell protection against viral infections and vaccine efficacy decrease with age (Goronzy et al., 2001; Deng et al., 2004).

Although the level of TCR diversity within the *total* $CD8^+$ T-cell memory pool seems to remain stable with age (Qi et al., 2014), the composition of the memory T-cell pool keeps changing at the antigen-specific level. Exposure to new antigens during life leads to recruitment of new T-cell specificities into the memory pool, and existing memory T-cell clones may expand or contract. The diversity of T cells that are already present in the memory T-cell pool may be affected by the arrival of new memory T-cell specificities, due to competition for T-cell growth and survival factors. The relative stability of the diversity of the *total* $CD8^+$ T-cell memory pool therefore does not imply that *individual* antigen-specific T-cell repertoires are stably maintained with age. To study such changes in the memory T-cell pool, we investigated how the diversity of the antigen-specific TCR repertoires against cytomegalovirus (CMV) and Epstein Barr virus (EBV) changes with age.

Both CMV and EBV cannot be cleared from the body and repeatedly challenge the immune system, leading to high, and therefore easily detectable, frequencies of antigen-specific $CD8^+$ T cells over all ages in the majority of individuals (Khan et al., 2004; Sukdolak et al., 2013). Previous longitudinal studies focusing on the effect of aging on the antigen-specific repertoire have suggested that the T-cell repertoires against EBV and CMV remain relatively stable, at least during the first few years after primary infection, as the same T-cell clones were identified at different timepoints (Annels et al., 2000; Hadrup et al., 2006; Iancu et al., 2009; Klarenbeek et al., 2012). Consistent with this, several cross-sectional studies into the CMV and EBV-specific T-cell repertoires reported similar Vβ-skewing in young and older adults, and even identical TCR sequences between individuals of different age groups (Khan et al., 2002; Schwanninger et al., 2008; Cardenas Sierra et al., 2014). Although these studies have led to the view that antigen-specific T-cell repertoires are rather stable with age, it remains unknown, if the diversity of antigen-specific T-cell repertoires is maintained (Lanfermeijer et al., 2020).

The cellular immune response against CMV is even more pronounced than the response against EBV, and can reach up to 40% of the $CD8^+$ T-cell pool in the blood (Khan et al., 2002; Remmerswaal et al., 2015). Furthermore, CMV-infection leads to changes in the $CD8^+$ T-cell pool similar to those observed with aging, including the presence of large fractions of terminally differentiated cells (Almanzar et al., 2005; Chidrawar et al., 2009) and a more skewed and less diverse TCR repertoire (Khan et al., 2002; Nikolich-Zugich, 2008). It has been suggested that the large numbers of CMV-specific T cells can compete with non-CMV-specific T cells (Pawelec et al., 2005; Derhovanessian et al., 2009; Tu and Rao, 2016), leading to memory attrition (Sad and Krishnan, 2003). Studies on the effect of CMV-infection on non-CMV-specific T cells are not unambiguous, however. Several studies have shown that mice infected with murine CMV (MCMV) have impaired responses to heterologous infections (Cicin-Sain et al., 2012; Mekker et al., 2012; Smithey et al., 2012; Redeker et al., 2017). In contrast, another study observed a positive effect of MCMV infection on the diversity of the T-cell repertoire specific for a heterologous infection (Smithey et al., 2018). Studies in humans showed similarly contradicting results: while one study showed that CMV^+ individuals had lower absolute numbers of EBV-specific T cells than CMV^- individuals (Khan et al., 2004), another study found that the diversity of the non-CMV-specific memory T-cell repertoire was comparable in CMV^- and CMV^+ individuals, thereby suggesting that the memory T-cell pool simply expands to accommodate the large frequencies of CMV-specific T cells (Lindau et al., 2019). Thus, the effect of CMV infection on non-CMV-specific T-cell responses and their repertoire diversity, and how this is linked to aging, remains poorly understood (Lanfermeijer et al., 2020).

To gain insight into the maintenance of the repertoire of antigen-specific T cells, we studied the effect of aging on the phenotype and TCR repertoire composition of T cells specific for two immune-dominant CMV and EBV epitopes. In addition, we investigated how the EBV-specific T-cell repertoire is influenced by CMV-infection and how this is linked to aging. We observed that the richness of the CMV-specific and EBV-specific T-cell repertoire declines with age, independent of CMV serostatus. CMV infection led to a further decrease in diversity of the EBV-specific T-cell repertoire. This suggests that CMV infection and age both play an important role in the diversity of the antigen-specific T-cell repertoires.

METHODS AND MATERIALS

Study Design

Samples of healthy individuals covering a broad age range were combined from two cohorts. Samples of young adults ($n = 34$) between 18 and 52 years of age, from a cohort of unvaccinated controls or pre-vaccination participants, were used from a study carried out in 2009–2011 (the Pandemic influenza vaccination trial, Netherlands Trial Register NL1952) (Rosendahl Huber et al., 2018). The study was approved by the Central Committee on Research Involving Human Subjects of the Netherlands. Samples of older adults ($n = 57$), ≥60 years of age, were control samples from a study carried out in 2014–2015 (Influenza-like-illness-3, NL4666) (Kaaijk et al., submitted). This study was approved by the acknowledged ethical committee, METC Noord Holland. Both studies were carried out in accordance with the recommendations of Good Clinical Practice with written informed consent from all subjects, in accordance with the Declaration of Helsinki. See **Supplementary Figure 1** for a flowchart of the selection criteria of the donors used for the analysis.

Cytomegalovirus (CMV)-Specific and Epstein Barr Virus (EBV)-Specific Antibodies

For healthy young adults, CMV-specific antibody levels were measured using a commercial ELISA kit (IBL international GMBH) according to manufacturer's instructions. Participants with a CMV antibody level of ≥12 U/ml or higher were considered CMV^+, those with a level of ≤8 U/ml were considered CMV^-. None of the participants included in this study scored between the 8 and 12 U/ml range. For older healthy

adults, CMV-specific antibody levels and EBV-specific antibody levels were simultaneously measured in serum by an in-house-developed multiplex immunoassay (Tcherniaeva et al., 2018). Individuals with a CMV-specific antibody level of ≤4 RU (relative units)/ml were considered to be CMV^- and individuals with an antibody level >7.5 RU/ml were considered CMV^+. None of the participants included in this study had a CMV-specific antibody level between 4 and 7.5 RU/ml. Individuals were considered EBV- with an antibody level of ≤16 RU/ml, whereas those with an antibody level of >30 RU/ml were considered EBV^+. None of the older participants included in this study had an EBV−specific antibody level between 16 and 30 RU/ml. Note that the EBV-status of the younger individuals remained unknown, therefore only individuals with high EBV^{A2-GLC}-specific T-cell frequencies were used in our analysis.

PBMC and Serum Isolation

Peripheral blood mononuclear cells were obtained by Lymphoprep (Progen) density gradient centrifugation from heparinized blood, according to the manufacturer's instructions. PBMCs were frozen in 90% fetal calf serum and 10% dimethyl sulfoxide at −135°C until further use. Serum was isolated out of tubes with clot-activation factor and stored at −80°C until further use.

Antigen-Specific T Cells by Flow Cytometry

HLA-A2 positive healthy individuals were selected for subsequent EBV-specific and CMV-specific T cell analysis, by staining PBMCs for expression of HLA-A2 with the HLA-A2(BB7.2)-V450 antibody (BD Bioscience). From the HLA-A2 positive individuals, ±4 million PBMCs were stained using the HLA-class I dextramer containing the GLCTLVAML epitope of the BMLF1 protein of EBV (A*0201/GLCTLVAML-APC, Immudex) or the NLVPMVATV epitope of the pp65 protein of CMV (A*0201/NLVPMVATV-APC, Immudex), for 20 min at room temperature to assess their virus-specific T-cell frequencies.

Surface staining was performed for 30 min at 4°C with the following antibodies: Fixable Viability Staining-780 (BD bioscience), CD3 (SK7)-AF700(BD bioscience), CD8(RPA-T8)-BrilliantViolet510, CD45RO(UCHL1)-BrilliantViolet711, CD27(O323)-BrilliantViolet786, CCR7(150503)-BrilliantUV395 (BD bioscience), KLRG-1(13F12F2)-PE-Cy7 (eBioscience), PD-1(EH12.2H7)-PerCP Cy5.5, CD95(DX2)-BrilliantViolet421 (BD Biosciences), CD127(A019D5)-BrilliantViolet650, CD57(HCD57)-PE, and CXCR3(G025H7)-PE-Dazzle. All antibodies were purchased from Biolegend, unless stated otherwise. Acquisition was performed on a LSRFortessaX20 and data analysis was performed using FlowJo (Treestar). tSNE-analyses were performed using Cytobank (www.cytobank.org) (Kotecha et al., 2010) on 30 randomly selected dextramer-positive $CD8^+$ T cells per sample and labeled with epitope-specificity, age, and CMV-serostatus. The tSNE clustering was performed on all these data combined (including both antigen-specificities). Perplexity of the clustering was set at 100. Cofactors for ArcSinH transformation were calculated using the flowVS package for R (https://www.bioconductor.org/packages/release/bioc/html/flowVS.html). Both packages were slightly adapted to allow for FlowCytometric data analysis and integrated in an in-house developed pipeline.

Isolation of Antigen-Specific T Cells for T-Cell Receptor Analysis

$CD8^+$ T cells were isolated from PBMCs using a negative selection microbeads kit (Miltenyi Biotec) according to the manufacturer's protocol. Next, $CD8^+$ T cells were labeled at room temperature for 20 min with the A*0201/GLCTLVAML-APC dextramer and with the A*0201/NLVPMVATV-APC dextramer for CMV^+ individuals. Subsequently surface staining was performed using the following mAbs: CD3(UCHT1)-PerCP (Biolegend), CD4(OKT4)-BV510 (Biolegend), and CD8(RPA-T8)-FITC (Biolegend). $CD3^+CD4^-CD8^+$dextramer$^+$ cells were then sorted by FACS Melody (BD) directly into RNAlater (Ambion Inc. Applied Biosystems) and stored at −80°C for subsequent TCRβ clonotype analysis.

Preparing TCRβ cDNA Libraries for Sequencing

TCRβ analysis was performed as described previously (Shugay et al., 2014), with minor modifications. Briefly, mRNA was isolated with the RNA microkit (Qiagen) according to the manufacturer's protocol. Isolated mRNA was used for cDNA synthesis with 5′ RACE template switch technology to introduce a universal primer binding site, and unique molecular identifiers (UMIs) were added at the 5′ end of the cDNA molecules using the SMARTScribe Reverse Transcriptase (TaKaRa). cDNA synthesis was followed by an AMPure XP bead-based clean-up (Beckman Coulter). Purified cDNA molecules were amplified in two subsequent PCR steps (25 cycles in PCR1 and 20 cycles in PCR2) using the Q5® High-Fidelity DNA Polymerase (New England BioLabs), with an AMPure XP bead-based clean-up in between. PCR products were size-selected on gel and purified using the Nucleospin PCR clean-up kit (Machery-Nagel). The PCR products were sequenced via Illumina MiSeq paired end 2x250 nucleotide (nt) sequencing.

TCRβ Clonotype Analysis

Raw sequencing data were processed using the 12nt UMIs to correct for amplification biases and error-correction of reads. RTCR (Gerritsen et al., 2016) was used to identify both the UMI sequence and clonotype information from the reads. Because of the relatively small number of cells per sample, additional filtering steps were followed to minimize cross-sample contamination and biases introduced by errors in the UMI sequence. Sequences were only accepted if their UMI was observed in at least 40 sequencing reads. Sequences with identical UMIs in multiple samples were removed if they did not occur in at least 1,000 sequencing reads or if their absolute frequency was lower than 10% of the maximum frequency in the other samples. UMIs were clustered within each sample if they were within a Hamming distance of 3. More detailed information

about the processing and filtering of reads is provided in the **Supplementary Material**.

Clonotypes were defined by their CDR3 amino acid sequence and V and J segment. Our sequencing reads do not always allow to distinguish between very similar V-segments, e.g., TRBV12-3 and TRBV12-4, which are annotated as V12-3/4 in **Figure 3**, **Supplementary Figures 4, 5**, and **Supplementary Tables 1, 2A,B**.

For measuring diversity, the richness (defined as number of distinct clonotypes) of each sample was determined using normalized sample sizes (i.e., by iteratively sampling, without replacement, a given number of UMIs from the full set of UMIs that were identified in the sample). This approach accounts for the fact that the number of RNA molecules sampled may differ between cells. Diversity was calculated using the previously described Simpson's diversity index (Venturi et al., 2007). This index ranges between 0 and 1, with 0 representing minimal diversity and 1 representing maximal diversity. Sequence generation probabilities (CDR3+ V and J segments) were calculated using the default recombination model of OLGA (Sethna et al., 2019). Known antigen specificity of sequences was assessed using the VDJdb (Shugay et al., 2017; retrieved on 29 October 2020). Sequences from CMV^{A2-NLV}-and EBV^{A2-GLC}-specific samples were counted as a match if their V gene + CDR3 amino acid sequence + J gene was listed as a human TCRbeta sequence specific for the NLVPMVATV or GLCTLVAML epitope, respectively.

Statistical Analysis of Flow Cytometry Data

Differences between the groups (for example CMV^- compared to CMV^+) were assessed using Mann-Whitney *U*-tests. Correlations were tested with Spearman's rank correlation coefficient. For all analyses, p-values < 0.05 were considered statistically significant. Data were analyzed using GraphPad Prism 8.3 and SPSS statistics 22 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Characteristics of the Study Population

Healthy HLA-A2 positive individuals were on average 57.8 ± 19.0 years old ($n = 91$, range 21–82 years) and 57.1% of these individuals were CMV^+. Samples were obtained from two different cohorts, one containing young adults (21–52 years old) ($n = 34$), and one containing older adults (≥60 years old) ($n = 57$), of whom respectively 55.9 and 57.9% were CMV^+ (**Table 1**). No significant differences in age or sex were observed between CMV^- and CMV^+ individuals. **Supplementary Figure 1** gives a flowchart of the selection criteria of the donors used for the analysis.

TABLE 1 | Characteristics of the study population.

Healthy young adults	**Total ($n = 34$)**	**CMV^- ($n = 15$)**	**CMV^+ ($n = 19$)**	**Statistics**
Age (mean ± SD)	35.9 ± 10.3	35.3 ± 10.8	36.4 ± 10.1	ns
Sex (% women)	61.8%	53.3%	68.4%	ns
CMV-serostatus (CMV^+)	55.9%	.	.	.
Healthy older adults	**Total ($n = 57$)**	**CMV^- ($n = 24$)**	**CMV^+ ($n = 33$)**	**Statistics**
Age (mean ± SD)	71.2 ± 6.4	70.8 ± 6.6	71.5 ± 6.4	ns
Sex (% women)	43.9%	37.5%	48.5%	ns
CMV-serostatus (CMV^+)	57.9%	.	.	.

Overview of the sex and CMV-serostatus distribution of the young adults (Rosendahl Huber et al., 2018) and older individuals (Kaaijk et al., submitted). Differences in percentage of CMV serostatus between groups was tested with Chi-squared test (p = ns for all age groups). Differences in age between groups was tested with unpaired T-test. ·means not applicable.

Changes in the Phenotype of Antigen-Specific $CD8^+$ T Cells With Age

To study the association between age and the antigen-specific $CD8^+$ T-cell frequencies against EBV and CMV, we performed a dextramer-staining with one immuno-dominant epitope of CMV (NLVPMVATV) derived from the pp65 protein and one immune-dominant epitope of EBV (GLCTLVAML) derived from the BMLF-1 protein, both presented on the HLA-A2 molecule. We investigated the T-cell frequency against these two epitopes at different ages. The frequencies of CMV^{A2-NLV}-specific T cells tended to be slightly higher than the frequencies of EBV^{A2-GLC}-specific T cells [median of 0.220% vs. median of 0.120%; $P = 0.0990$ (ns)] (**Figure 1A**). We observed less inter-individual heterogeneity in the CMV^{A2-NLV}-specific T-cell frequencies of younger compared to older adults; in the older adults, in whom these frequencies ranged from very low (from 0.01%) to very high (up to 17%) (**Figure 1B**, left panel). EBV^{A2-GLC}-specific T-cell frequencies were not significantly associated with the age of the individuals (**Figure 1B**, right panel).

To assess the association between age and the phenotype of CMV^{A2-NLV} and EBV^{A2-GLC}-specific T cells, we performed a cluster analysis (tSNE) based on the expression of the memory T-cell markers CD27, CCR7, CD95, CD45RO, and CXCR3, on CD57 and KLRG-1, which are associated with a more differentiated phenotype and on the inhibitory receptor PD-1, and CD5, which plays a role in TCR signaling (Voisinne et al., 2018). For the tSNE analysis the same amount of antigen-specific T cells (i.e., 30) per sample was used. The very same clustering was applied on the samples in the four age groups and per epitope-specificity. We observed clear differences in the subset distribution between these groups for both CMV^{A2-NLV} and EBV^{A2-GLC}-specific T cells (**Figure 1C**). We identified 3 large clusters (1–3, **Figure 1C**, upper left panel), in which cluster 1 contains Central memory type markers $CCR7^{high}$, $CD27^{high}$, $KLRG\text{-}1^{low}$, $CD57^{low}$ cells, while cluster 3 contains the more differentiated cells, expressing $CCR7^{low}$, $CD27^{low}$, and $KLRG\text{-}1^{high}$. Cluster 2 forms an intermediate cluster based on the expression

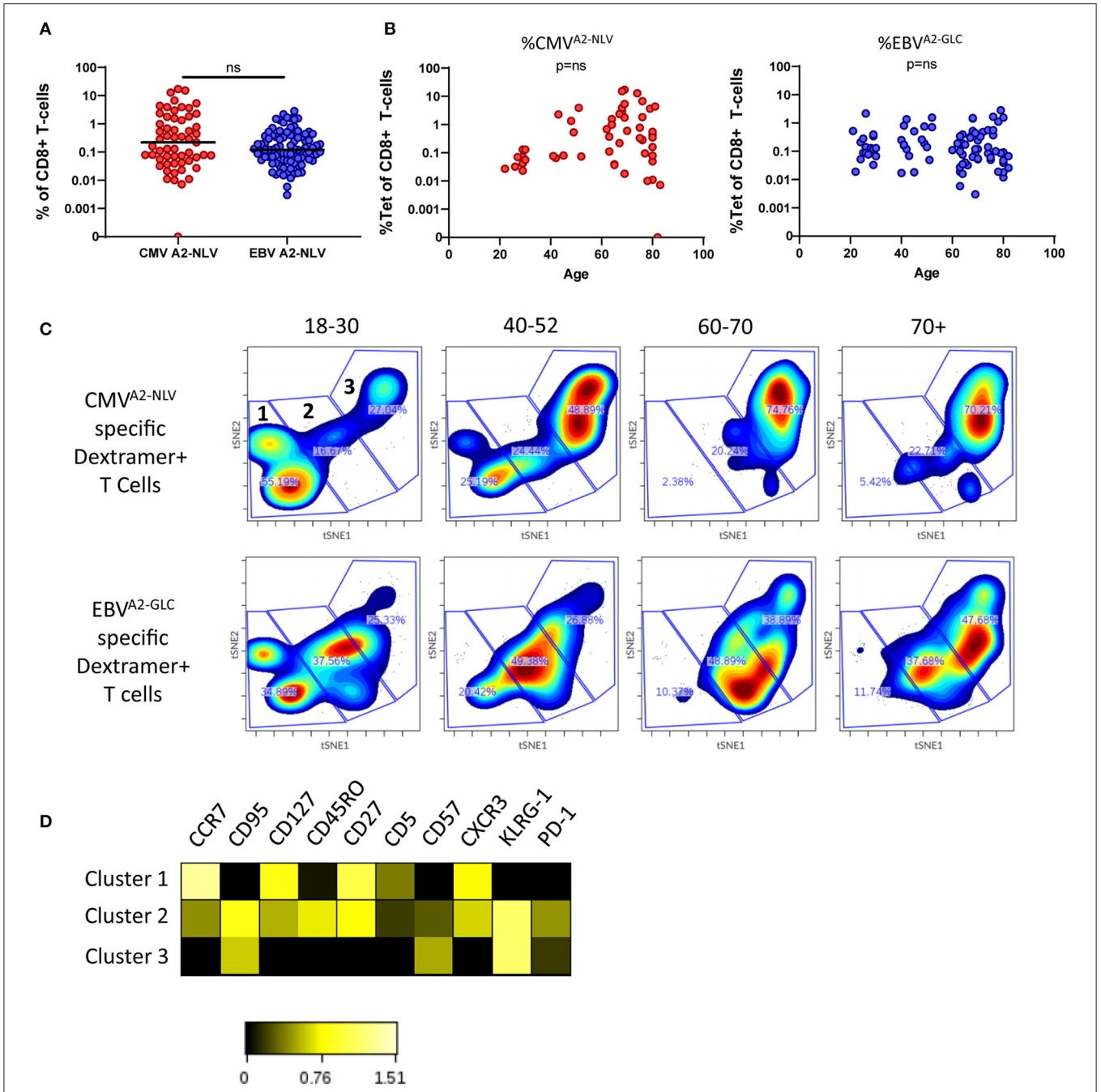

FIGURE 1 | Changes in the phenotype of antigen-specific CD8$^+$ T cells with age. **(A)** Percentage of CMV^{A2-NLV}-specific CD8$^+$ T cells (red, $n = 56$) and EBV^{A2-GLC}-specific CD8$^+$ T cells (blue, $n = 99$). Horizontal lines represent group median. **(B)** Percentage of CMV^{A2-NLV} (left) and EBV^{A2-GLC}-specific (right) CD8$^+$ T cells as a function of age. **(C)** t-SNE analysis of CMV^{A2-NLV}-specificand EBV^{A2-GLC}-specific CD8$^+$ T cells of donors ($n = 47$ and $n = 77$, respectively) categorized in four age groups. Clustering is based on MFI of CD5, PD-1, CD57, KLRG-1, CXCR3, CCR7, CD45RO, CD95, CD27, and CD127 of both epitopes. From each sample 30 cells were used. Three large clusters were manually identified. **(D)** Heatmap of expression of markers of the three t-SNE clusters. Clustering of t-SNE based on both CMV^{A2-NLV} and EBV^{A2-GLC}-specific CD8$^+$ T cells. Heatmap was based on the Log10 ratio of the median expression of the markers, normalized per marker to its column's minimum. Difference between epitopes was compared by Mann Whitney *U*-test. Correlations were tested with Spearman's rank correlation coefficient, ns stands for a non-significant *p*-value.

of these markers (**Figure 1D**). Despite relatively large inter-individual variation (**Supplementary Figure 2A**), our data suggest a shift from cluster 1 to cluster 3 for both CMV^{A2-NLV} and EBV^{A2-GLC}-specific T cells with age (**Figure 1C**, **Supplementary Figure 2A**). This shift in clusters occurs earlier and becomes more pronounced with age for CMV^{A2-NLV}-specific T cells than for EBV^{A2-GLC}-specific T cells.

Phenotypic Changes of CMV-Specific and EBV-Specific T Cells Are Differently Associated With Age

We next explored how these phenotypic changes associated with age by quantifying the expression of various markers in the individual samples of CMV^{A2-NLV}-specific and EBV^{A2-GLC}-specific T cells. This allowed us to use the expression data of all the dextramer$^+$ T cells. The composition of the memory population for the different ages based on conventional gating supported our observations of the cluster analysis. The memory subsets were defined based on the expression of CD27 and CD45RO in which the $CD27^-CD45RO^-$ subset is referred to as Temra cells. We found a positive association between age and the percentage of Temra cells for both CMV^{A2-NLV}-specific T cells [$p = 0.0032$, $r = 0.4167$, slope of 0.75%/year ($p = 0.0006$)] and, albeit to a lesser extent, EBV^{A2-GLC}-specific T cells [$p = 0.0010$, $r = 0.0.3796$, slope of 0.25%/year ($p = 0.1045$)] (**Figure 2A**). The fraction of Temra cells correlated positively with the frequency of CMV^{A2-NLV}-specific T cells, but not with the percentage of EBV^{A2-GLC}-specific T cells (**Supplementary Figure 3A**). For EBV^{A2-GLC}-specific T cells, the proportion of effector memory cells (Tem, $CD27^-CD45RO^+$) increased significantly with age ($p < 0.0001$, $r = 0.5001$) (**Supplementary Figure 3B**, right panel). The cluster analysis based on MFI from **Figure 1** also showed a gradual decrease in CCR7 expression with age, which was confirmed by plotting the geometric mean of the fluorescence intensity (gMFI) of CCR7 on a continuous scale for both CMV^{A2-NLV}-specific ($p < 0.0001$, $r = 0.0.4411$) and EBV^{A2-GLC}-specific T cells ($p = 0.0020$, $r = 0.3604$) (**Figure 2B**).

Next, we analyzed the expression of markers associated with a more differentiated phenotype and exhaustion-associated markers on the antigen-specific T cells more closely. Both CMV^{A2-NLV}- and EBV^{A2-GLC}-specific T cells are associated with high expression of the inhibitory marker KLRG-1. CD57 is a senescence marker known to be specifically highly expressed by CMV-specific T cells, compared to T cells against other chronic viruses (Hoji et al., 2007; van den Berg et al., 2019). We indeed found high percentages of KLRG-1^+ cells for both CMV^{A2-NLV}-specific (mean of 76.6%) and EBV^{A2-GLC}-specific T cells (mean of 79.8%), and the percentage of KLRG-1^+ cells was positively associated with age, both CMV^{A2-NLV}-specific ($p = 0.0007$, $r = 0.4722$) and EBV^{A2-GLC}-specific T cells ($p = 0.0847$, $r = 0.2046$) (**Figure 2C**). The percentage of $CD57^+$ T cells showed a positive trend with age for CMV^{A2-NLV}-specific T cells ($p = 0.0645$, $r = 0.2689$), while it was low across all ages for EBV^{A2-GLC}- specific T cells (**Figure 2D**). We observed a significant positive association between the frequency of CMV^{A2-NLV}-specific T cells and the percentage of KLRG-1^+, $CD57^+$ cells in the CMV^{A2-NLV}-specific T-cell population (**Supplementary Figure 3A**, upper panels). The frequency of EBV^{A2-GLC}-specific T cells, in contrast, was only positively associated with the percentage of KLRG-1^+ EBV^{A2-GLC}-specific T cells (**Supplementary Figure 3A**, lower panels), and not with the percentage of $CD57^+$ EBV^{A2-GLC}-specific T cells.

We also investigated the percentage of CMV^{A2-NLV}-specific and EBV^{A2-GLC}-specific T cells expressing PD-1, which in the context of chronic (active) infection is often used as an exhaustion marker (Jubel et al., 2020). We found no significant correlation between the percentage of PD-1^+ cells in the antigen-specific T-cell pool and age, both for EBV-specific and for CMV-specific T cells (**Supplementary Figure 3C**).

The CMV^{A2-NLV}-Specific Repertoire Is Less Diverse Than the EBV^{A2-GLC}-Specific Repertoire

We then investigated the TCR repertoire of CMV^{A2-NLV}-specific and EBV^{A2-GLC}-specific T cells in our samples by sequencing the TCRβ-chain. We used unique molecular identifiers (UMIs) to correct for sequencing errors and unequal PCR amplification, and performed an additional filtering procedure to exclude sequences that were likely due to contamination between samples or mutation in the UMI sequence (see **Supplementary Material** for more details). We proceeded with the samples in which at least 10 UMI-TCR pairs remained after filtering. The distribution of the TCRβ sequences per individual are shown in **Figure 3** (see **Supplementary Figure 4** for samples with <10 UMI-TCR pairs, all identified TCR sequences are provided in **Supplementary Tables 1, 2A,B**), with colors indicating the TCRβ sequences that are shared between individuals. We observed two different TCR sequences that were shared between individuals in the CMV^{A2-NLV}-specific repertoire samples, and eight in the EBV^{A2-GLC}-specific repertoire samples (**Supplementary Figures 5A,B**). A substantial fraction of the observed TCR sequences were also present in the VDJ database (VDJdb) of reported antigen-specific TCR sequences (Shugay et al., 2017) (**Supplementary Figure 5C**). We found that the sequences that were shared between individuals within our study or between an individual of our study and the VDJdb had an over 16-fold higher generation probability than those that were not shared (**Supplementary Figure 5D**). This supports the idea that the likelihood of TCR generation plays an important role in the presence, abundance and sharing of antigen-specific TCR sequences (Venturi et al., 2006, 2008; Elhanati et al., 2018).

To investigate the diversity of the antigen-specific T-cell repertoire, we used several measures of TCR diversity. The absolute richness, i.e., the total number of distinct clonotypes observed in a sample, was significantly higher for the EBV^{A2-GLC}-specific T cells than for the CMV^{A2-NLV}-specific T cells ($p = 0.0289$) (**Figure 4A**), even though the frequency of EBV^{A2-GLC}-specific T cells was lower than that of CMV^{A2-NLV}-specific T cells. However, the absolute richness is largely influenced by the total number of UMI-TCR pairs in a sample, i.e., the number of cDNA molecules that were sequenced. To overcome this potential bias, we therefore also used several alternative measures of TCR diversity. First, we calculated the unique clonotype ratio, by dividing the number of unique clonotypes by the total number of TCR sequences in each sample. Next, we calculated a normalized richness by counting the number of distinct TCR sequences in equally sized subsamples of the actual samples (by taking the mean richness of 10 randomly chosen subsamples). We also calculated the Simpson's diversity index as a sample size-independent

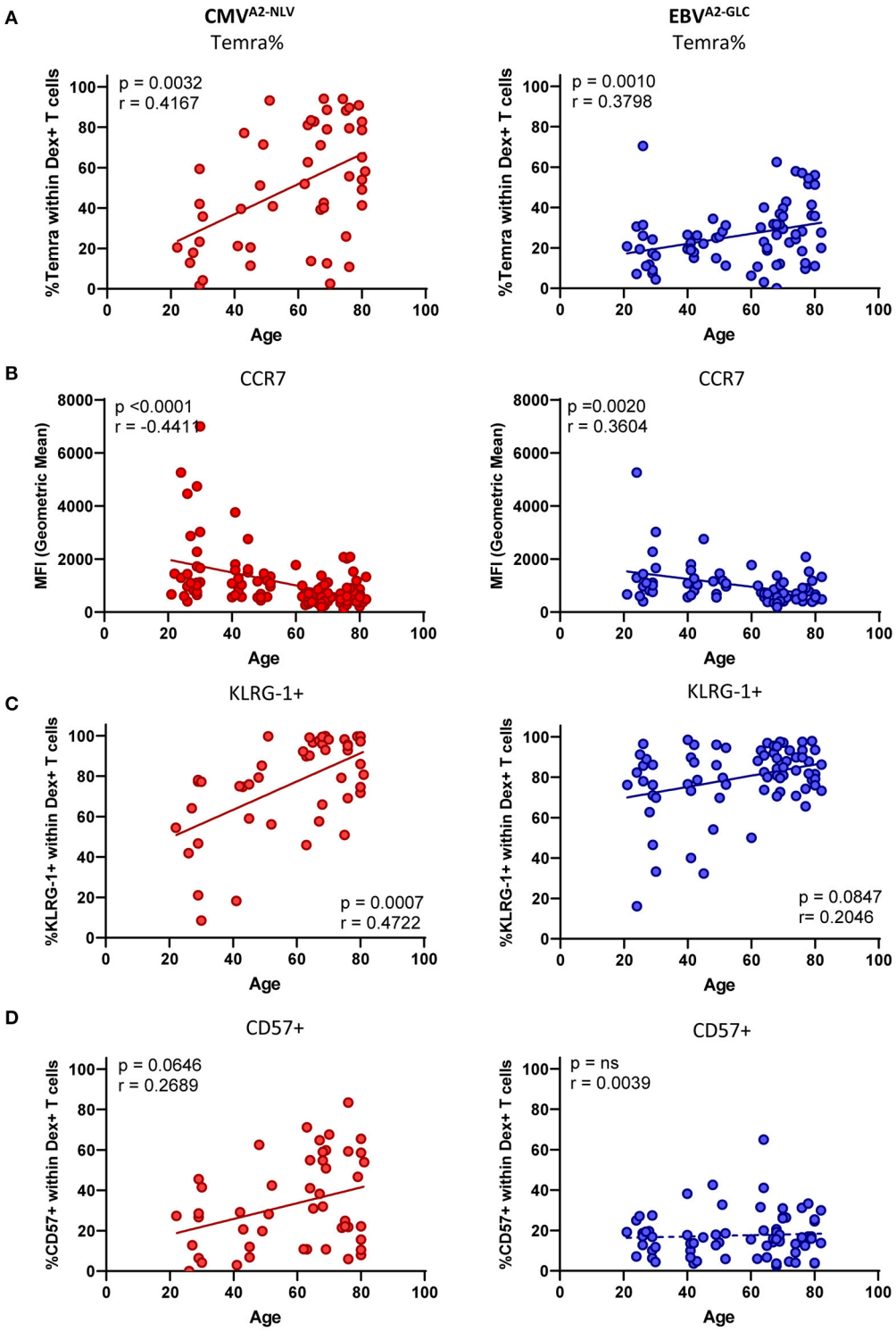

FIGURE 2 | The phenotype of CMV-specific and EBV-specific T cells is affected differently by age. **(A–D)** Phenotype analysis of both CMV^{A2-NLV}-specific (red) and EBV^{A2-GLC}-specific CD8$^+$ T cells (blue) plotted against age. **(A)** Percentage of Temra cells (CD27–, CD45RO–). **(B)** Geometric Mean of the fluorescent intensity of CCR7. Percentage of KLRG-1$^+$ **(C)** and CD57$^+$ **(D)** within Dextramer$^+$ CD8$^+$ T cells. Solid lines indicate a slope significantly ($p < 0.05$) different from a slope of 0, whereas a dotted line indicates no significant difference. Only donors with a sufficient T-cell response (at least 25 cells) were used for the phenotypical staining. Correlations were tested with Spearman's rank correlation coefficient.

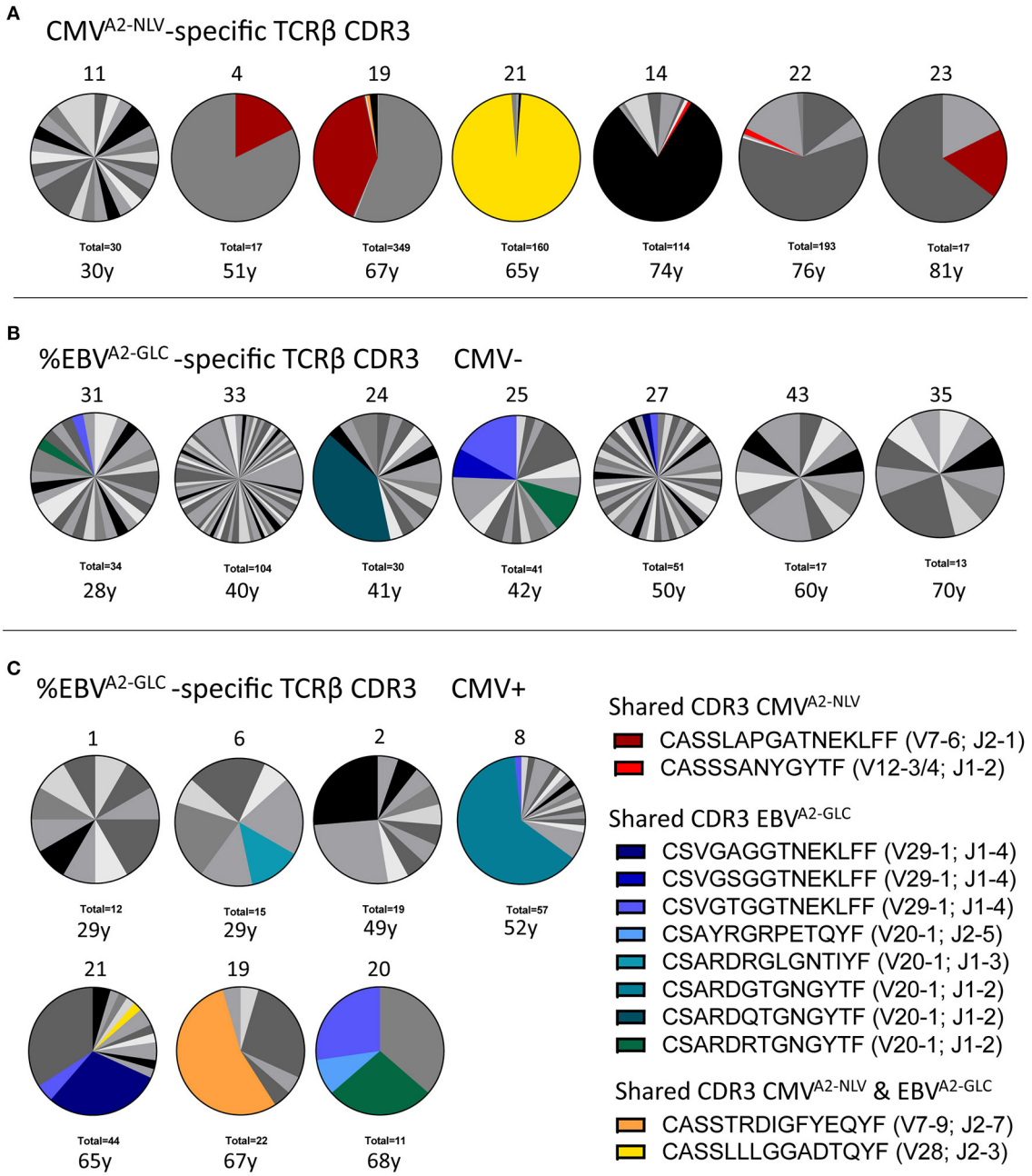

FIGURE 3 | Characterization of the antigen-specific TCRβ repertoire. **(A)** Distribution of TCRβ sequences in samples of CMV^{A2-NLV}-specific CD8$^+$ T cells ($n = 7$). **(B,C)** T-cell repertoire of EBV^{A2-GLC}-specific CD8$^+$ T cells of both CMV$^-$ **(B)** and CMV$^+$ individuals **(C)**. Each pie depicts the repertoire of a different sample, with its Donor ID on top and the total number of UMI-TCR pairs identified at the bottom, as well as the individual's age. Colors represent shared TCRβ sequences between donors. Gray scales depict unique TCRβ sequences. Note two shared sequences between the CMV^{A2-NLV} and EBV^{A2-GLC} sample of two single individuals (yellow and orange). As this sharing was limited to these single donors and involved a very abundant TCRβ in either one of the samples, we expect that this overlap occurred during the sorting of the cells, probably due to unspecific binding of the dextramer.

measure of the TCR diversity in each sample (Venturi et al., 2007). While richness quantifies the variety of different TCRs, Simpson's diversity index quantifies the evenness of the frequency distribution across the TCRs. Even after excluding differential sample sizes as a confounding factor, we found a higher TCR diversity in the EBV^{A2-GLC}-specific compared to the CMV^{A2-NLV}-specific T-cell repertoire (**Figure 4B**). We repeated these analyses on the subset of samples that contained at least 20 UMI-TCR pairs, which did not change the results qualitatively.

To understand what explains the higher diversity in some of the samples, we investigated the correlation between T-cell frequencies and the diversity of the repertoire for the CMV^{A2-NLV}-specific and EBV^{A2-GLC}-specific T-cell data combined. We found a negative correlation between all diversity measures and the frequency of antigen-specific T cells (**Figures 4C,D**). This suggests that individuals with high frequencies of antigen-specific T cells had large clonal expansions, leading to a decrease in TCR repertoire diversity.

Both Age and CMV-Infection Are Associated With a Lower Diversity of the $EBV^{A2\text{-}GLC}$-Specific T-Cell Repertoire

To investigate whether age is associated with the diversity of the antigen-specific T-cell repertoire, we analyzed the normalized richness and the Simpson's diversity index of the repertoire of both CMV^{A2-NLV}-specific and EBV^{A2-GLC}-specific T cells in relation to the age of the individuals. The normalized richness of the antigen-specific T-cell repertoires against both CMV^{A2-NLV} and EBV^{A2-GLC} showed a negative trend with age (p = ns, $r = 0.1786$ and $p = 0.0645$, $r = -0.5105$, respectively), although the decrease with age observed for the CMV^{A2-NLV}-specific samples was largely based on the datapoint of one young adult (**Figure 5A**). The Simpson's diversity index showed the same negative trend, although this was also not significant (p = ns for CMV^{A2-NLV} and $p = 0.0925$ for EBV^{A2-GLC}) (**Figure 5A**). This suggests that the diversity of the CMV^{A2-NLV}-specific and EBV^{A2-GLC}-specific T-cell repertoire decrease with age.

As there are indications in mice that CMV-infection can affect the T-cell response against heterologous virus infections (Cicin-Sain et al., 2012; Mekker et al., 2012; Redeker et al., 2017), we then stratified the EBV^{A2-GLC}-specific T-cell data according to the individuals' CMV status. We found a higher diversity of the EBV^{A2-GLC}-specific T-cell repertoire in CMV^- compared to CMV^+ individuals based on both normalized richness ($p = 0.0111$) and Simpson's diversity index ($p = 0.0070$) (**Figure 5B**). Linear regression analysis of these data suggests a lower diversity of the EBV^{A2-GLC}-specific T-cell repertoire in the presence of CMV over the entire observed age range (**Figure 5B**). A significant decrease in diversity of the EBV^{A2-GLC}-specific T-cell repertoire with age was only observed in CMV^+ individuals.

A likely explanation for the decreased diversity of the EBV^{A2-GLC}-specific T-cell repertoire in CMV^+ individuals could be memory attrition, i.e., large frequencies of CMV^{A2-NLV}-specific T cells outcompeting non-CMV-specific T cells. If there is indeed a role for memory attrition, one would expect that (1) the percentage of EBV^{A2-GLC}-specific T cells is lower in CMV^+ compared to CMV^- individuals, and (2) that the percentage of EBV^{A2-GLC}-specific T cells correlates negatively with the percentage of CMV^{A2-NLV}-specific T cells. CMV^+ individuals indeed had significantly lower percentages of EBV^{A2-GLC}-specific $CD8^+$ T cells compared to CMV^- individuals ($p = 0.0300$) (**Supplementary Figure 6A**). The lower fraction of EBV^{A2-GLC}-specific T cells was seen at all ages (**Figure 5C**). However, the percentage of EBV-specific T cells was not negatively associated with the percentage of CMV^{A2-NLV}-specific T cells. If anything, there was a trend toward a positive correlation between both antigen-specific T-cell frequencies ($p = 0.0598$, $r^2 = 0.2654$) (**Figure 5D**). Thus, the decreased frequency and diversity of EBV^{A2-GLC}-specific T cells in CMV^+ individuals does not seem to be due to memory attrition.

Based on the same t-SNE cluster analysis as shown in **Figure 1B**, we compared the EBV^{A2-GLC}-specific $CD8^+$ T-cell population of CMV^- ($n = 32$) and CMV^+ ($n = 40$) individuals (**Supplementary Figure 6B**). For this analysis, data from donors of all ages were pooled. For none of the three earlier identified clusters did we find any significant differences between CMV^+ and CMV^- individuals (**Supplementary Figure 6B**). As the t-SNE gates were rather rough, we wondered whether there would be any differences in the phenotype of EBV^{A2-GLC}-specific T cells when analyzing the data in more detail. Based on conventional gating, we observed no substantial CMV-related differences based on for example the percentage of $CD57^+$ or KLRG-1^+ cells. Only the percentage of PD-1^+ expressing EBV^{A2-GLC}-specific T cells was significantly higher in CMV^- as compared to CMV^+ individuals ($p = 0.0255$), while the EBV^{A2-GLC}-specific T-cell population in CMV^+ individuals had a significantly higher percentage of effector memory T cells than in CMV^- individuals ($p = 0.0106$) (**Supplementary Figure 6C**). Thus, CMV-infection may induce subtle changes in non-CMV-specific T cell populations, like those specific for EBV.

DISCUSSION

In this study, we investigated the effect of age and CMV-infection on the phenotype and diversity of the antigen-specific T-cell repertoire. We focused on CMV^{A2-NLV}-specific and EBV^{A2-GLC}-specific T cells, as these antigen-specific T cells are readily detectable in the T-cell pool at all ages. The antigen-specific T cells against both persistent viruses showed an age-related increase in the expression of several markers associated with a more differentiated phenotype, including KLRG-1, an increase in the fraction of terminally differentiated T cells and a decrease in the diversity of the antigen-specific T-cell repertoire. CMV-infection has also been proposed to reduce the diversity of the total memory T-cell pool (Khan et al., 2002; Emerson et al., 2017). However, the effect of CMV on the diversity of other antigen-specific T-cell repertoires remains poorly understood. Here we show that CMV infection is associated with a lower diversity of the EBV^{A2-GLC}-specific T-cell repertoire. Although the exact mechanism behind this association remains unknown, our data suggest that the decreased diversity of the EBV^{A2-GLC}-specific T-cell repertoire in CMV^+ individuals is not due to memory attrition. We found that antigen-specific T cells against CMV^{A2-NLV} and EBV^{A2-GLC} are different at the phenotypic level; CMV^{A2-NLV}-specific T cells have higher percentages of Temra cells, as defined by CD27- and CD45RO-, and higher expression of CD57 than EBV^{A2-GLC}-specific T cells. These findings are in line with other studies, showing that EBV^{A2-GLC}-specific T cells are predominantly $CD45RO^+$ (Kuijpers et al.,

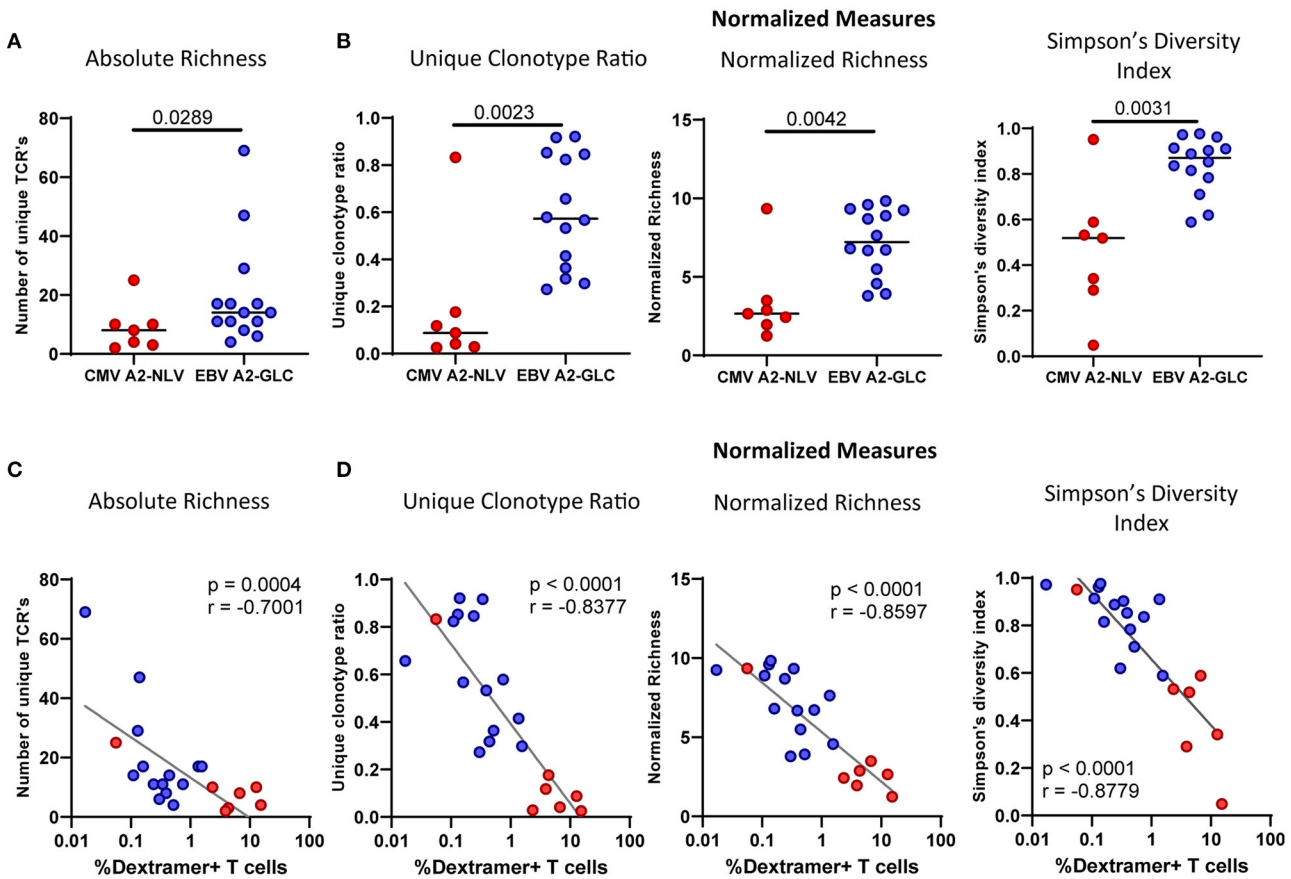

FIGURE 4 | The CMV^{A2-NLV}-specific TCRβ repertoire is less diverse than the and EBV^{A2-GLC}-specific TCRβ repertoire. Repertoire diversity calculations of the CMV^{A2-NLV} and EBV^{A2-GLC}-specific CD8^{+} T-cell repertoire. **(A)** Richness calculations based on the number of different TCRβ sequences. **(B)** Measures normalized for differential sample sizes: Unique Clonotype Ratio (left), normalized richness (middle), and Simpson's Diversity Index (right panel). **(C,D)** Correlation plot of the repertoire richness **(C)** and normalized diversity **(D)** and the frequency of the CMV^{A2-NLV}-specific (red) and EBV^{A2-GLC}-specific (blue) T cells. Horizontal lines in **(A,B)** show group medians. The unique clonotype ratio was calculated by dividing the number of unique clonotypes by the total number of TCR sequences. Normalized richness was calculated by counting the number of distinct TCR sequences in subsamples of 10 sequences. The mean of 1,000 subsampling iterations is shown. Differences between epitopes were compared by Mann Whitney. Correlations were tested with Spearman's rank correlation coefficient.

2003) and more often express CD27 (Appay et al., 2002). The presence of terminally differentiated CMV^{A2-NLV}-specific T cells is probably explained by the presence of large clonal expansions, which are typical for CMV infection (van den Berg et al., 2019).

The relatively high expression of markers associated with a more differentiated phenotype and the relatively low TCR repertoire diversity of the CMV^{A2-NLV}-specific T-cell population becomes even more pronounced in individuals at older age. Based on the high frequencies of CMV^{A2-NLV}-specific T cells in older adults, this age-effect is probably also linked to the presence of large clonal expansions consisting of terminally differentiated cells. It has been suggested that the increase in CMV-specific T-cell numbers with age is due to periodical infectious reactivation (van Boven et al., 2017). Although EBV^{A2-GLC}-specific T cells also show changes associated with age, these are less pronounced than for CMV^{A2-NLV}-specific T cells. This may be related to differences in cellular tropism between the two herpes viruses (Shenk, 2008; Hatton et al., 2014), or to possible differences in viral reactivation frequencies (Scheinberg et al., 2007; Thomasini et al., 2017).

It is tempting to speculate that the features of the T-cell responses against CMV^{A2-NLV} and EBV^{A2-GLC} that we observed are characteristic for the immune response to these two viruses in general. Although these two epitopes tend to be immunodominant, and thereby represent a fair share of the T-cell response in many individuals, it was recently shown that different combinations of HLA-alleles can influence the immunodominance of an epitope (Maleeva et al., 2019). This may also explain the large variation in antigen-specific T-cell frequencies that we observed in the four age groups. It remains to be investigated whether our results also apply to other epitopes for these two viruses, and to individuals in which these responses are less dominant.

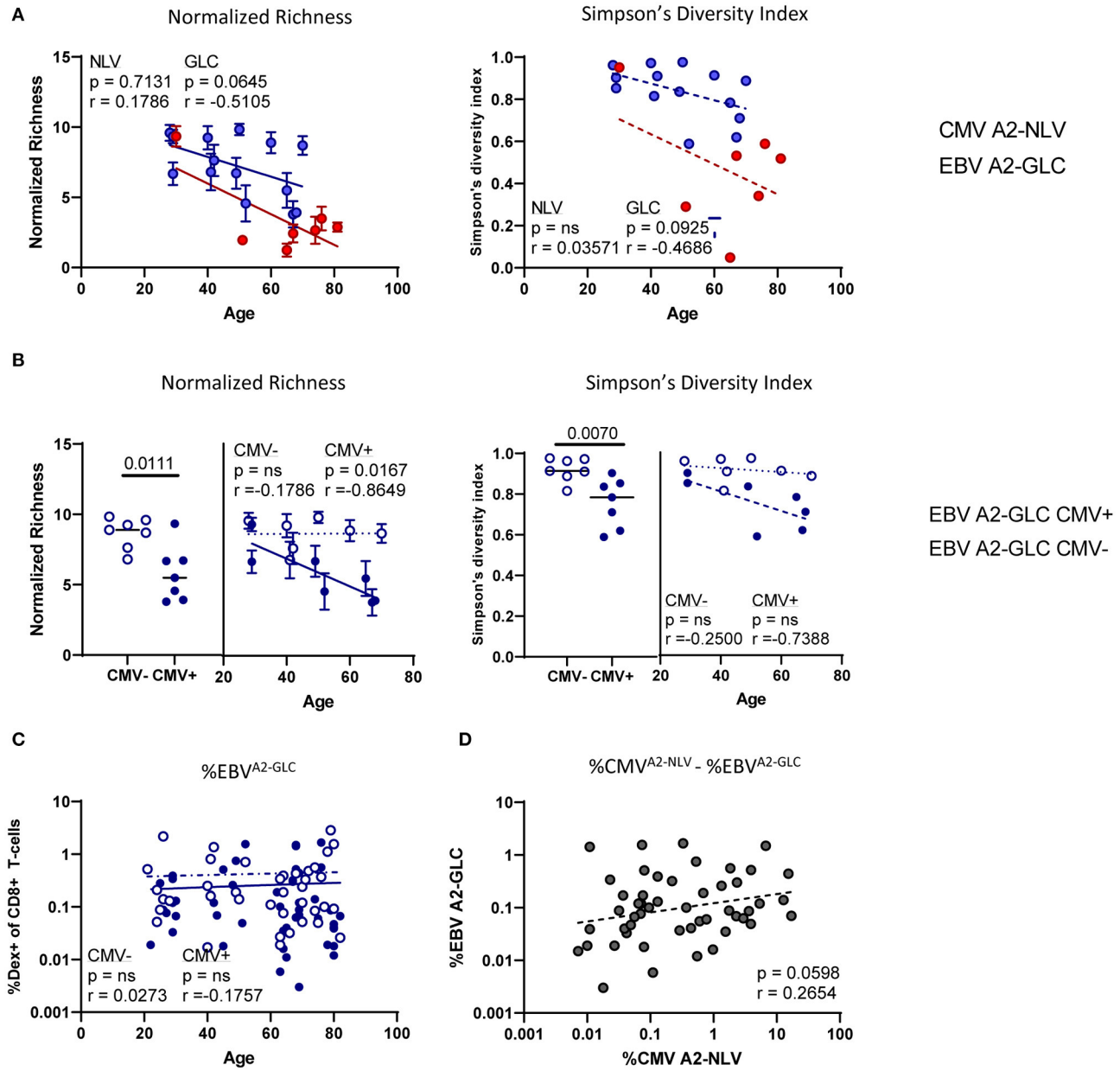

FIGURE 5 | The CMV^{A2-NLV}-specific TCRβ repertoire and the EBV^{A2-GLC}-specific TCRβ repertoire decrease with age, whereas the diversity of the EBV^{A2-GLC}-specific TCRβ repertoire is lower in the CMV^{+} individuals than in the CMV^{-} individuals. **(A)** Correlation plots of normalized richness score (left) and Simpson's diversity index (right panels) of CMV^{A2-NLV}-specific and EBV^{A2-GLC}-specific T-cell repertoires and age. Whiskers denote standard deviations over 1,000 subsampling iterations. **(B)** Normalized richness score and Simpson's diversity index of the EBV^{A2-GLC}-specific T-cell repertoire divided in CMV^{-} (blue, open circles) and CMV^{+} individuals (blue, filled circles). **(C)** Percentage of EBV^{A2-GLC}-specific CD8^{+} T cells in all CMV^{-} (blue open circles) and CMV^{+} individuals (blue filled circles). **(D)** Correlation of CMV^{A2-NLV} and EBV^{A2-GLC}-specific CD8^{+} T-cell frequencies within donors. Frequencies are log transformed for statistical analysis. Solid lines indicate a slope significantly ($p <$ 0.05) different from a slope of 0, whereas a dotted line indicates no significant difference. All correlations were tested by Spearman's Rank correlation coefficient.

Previous studies on the effect of aging on the antigen-specific T-cell repertoire have mostly focused on the maintenance of TCR sequences that are shared between individuals or that occur at different timepoints (Annels et al., 2000; Klarenbeek et al., 2012) or on a biased usage of Vβ-segments (Schwanninger et al., 2008). As these studies observed dominant T-cell clones both in a longitudinal setting and in a cross-sectional setting across different ages, they led to the view that the antigen-specific T-cell repertoires against CMV and EBV are relatively stable over time. Even though the T-cell repertoire analyses

were performed on a relatively small number of samples, our direct assessment of the TCR diversity of EBV^{A2-GLC}-specific T cells, and to a lesser extent of CMV^{A2-NLV}-specific T cells, showed a clear decrease in diversity with age, suggesting that the antigen-specific T-cell repertoires against these viruses are not as stable as previously thought. These seemingly contradicting conclusions may be due to the process of convergent contraction of the T cell repertoire, in which lower frequency clonotypes are lost over time, while only few T cells persist (Smith et al., 2020). Observations of these persisting T-cell clones over time would suggest that the antigen-specific T-cell repertoire is relatively stable, even though the richness and diversity of the repertoire may decrease with age. To study whether convergent contraction of the T-cell repertoire is indeed happening, a longitudinal study should be performed, focusing on the richness and diversity of the antigen-specific T-cell repertoire.

It remains unknown *why* the diversity of antigen-specific T-cell repertoires decreases with age and whether age is the real driver of the decrease in T-cell diversity or whether other factors play an important role. We cannot exclude the possibility that the older individuals of the study population had been infected at an older age, possibly leading to an antigen-specific T-cell repertoire of lower diversity because the diversity of the naive (precursor) pool is known to decrease with age (Britanova et al., 2014; Egorov et al., 2018). However, the recent finding that only a very small percentage of individuals seroconvert for CMV at later age (Samson et al., 2020), as well as the finding that more than 90% of the population is infected with EBV during adolescence (Balfour et al., 2013; Winter et al., 2020), makes this explanation unlikely. In our view, a more likely explanation for the reduced diversity in the antigen-specific T-cell repertoire of older individuals would be that older individuals have been infected for a longer time, and have lost more T-cell clones over time, for example due to exhaustion after restimulation (Lanfermeijer et al., 2020).

Our results indicate that CMV-infection is associated with a lower diversity of the EBV^{A2-GLC}-specific T-cell repertoire. As it is generally thought that T-cell receptor diversity is positively correlated with the level of protection against infectious diseases, one would expect that this decreased diversity would lead to a decreased EBV-specific T-cell response in CMV^+ individuals. If our findings also hold true for other antigens, this would suggest that CMV^+ individuals are less protected against other infections. This is in line with most mouse studies, which show a negative effect of CMV-infection on the T-cell efficacy against heterologous infections (Cicin-Sain et al., 2012; Mekker et al., 2012; Smithey et al., 2012; Redeker et al., 2017). *How* CMV-infection would lead to this lower diversity remains unknown. Although memory attrition has often been suggested to play a role, our data do not support this idea, as we observed a positive correlation between the frequencies of EBV^{A2-GLC}- and CMV^{A2-NLV}-specific T cells. The explanation for this positive correlation might be that some individuals are better T-cell responders than others. The observation that CMV-infection leads to lower frequencies of antigen-specific T cells is not surprising, as it is a relative measure, which is easily skewed by the high percentages of CMV-specific T cells. Likewise, in another study of our group it was shown that influenza-specific T-cell frequencies in older individuals were lower in CMV^+ compared to CMV^- individuals. Importantly, however, this did not result in lower influenza-specific IFNγ responses in CMV^+ individuals (van den Berg et al., in press), suggesting that CMV did not negatively impact the influenza-specific T-cell response; although sample sizes were unfortunately too small to confirm this based on the diversity of the influenza-specific TCR repertoire. It remains puzzling why these different results are observed between studies, and it would be interesting to understand whether e.g., the acute or chronic nature of a pathogen plays a role.

T cells isolated from blood represent only a small fraction of the total T-cell pool in the body, as it has been estimated that blood contains only 2 percent of all the T cells in the body (Westermann, 1990). Although many T cells travel through different compartments in the body, it remains to be investigated whether antigen-specific T-cell characteristics, like their phenotype and repertoire diversity, that are observed in the blood can be extrapolated to T cells in other sites of the body. The presence of tissue-resident T cells, which hardly circulate through the blood, complicates this even further. In the case of CMV and EBV, however, the blood may in fact be the most informative site to follow the antigen-specific T-cell response. It has previously been suggested that most CMV-specific T cells are present in the blood (Gordon et al., 2017). In line with this, a recent human study showed that most terminally differentiated memory $CD8^+$ T cells, including CMV-specific T cells, are confined to the intravascular circulation and do not circulate through the thoracic duct lymph (Buggert et al., 2020). Also for EBV, a bloodborne virus infecting B cells, the blood may in fact be the most representative compartment to study the antigen-specific T-cell response. It remains to be investigated whether the changes we observed in the antigen-specific T-cell repertoire in the blood also apply to antigen-specific T cells in other sites of the body. So far, studies have shown both a minimal overlap in the naïve T-cell pool between spleen and lymph nodes (Thome et al., 2016), as well as high degrees of overlap when focusing on the memory T-cell pool between blood and the thoracic duct lymph (Buggert et al., 2020), between peripheral blood and lymph nodes (Remmerswaal et al., 2015) and between spleen and lymph nodes (Thome et al., 2014).

In contrast to the commonly-held view that the antigen-specific T-cell repertoires against CMV and EBV are relatively stable with age, we here show that they both clearly decrease with age. Our data suggest that not only age but also CMV-infection is associated with the diversity of the EBV^{A2-GLC}-specific T-cell repertoire. Insights into how antigen-specific T-cell repertoires evolve with age and under the influence of other infections, like latent CMV, are important for the development of novel vaccination strategies to protect older adults against infectious diseases. One of the proposed strategies to prevent older adults is to induce protective immune responses through vaccination at a younger age. This would require stability of the induced immune response in order to provide protection later in life. Our data suggest that the antigen-specific T-cell repertoire is not as stable as previously thought.

Unfortunately, this implies that vaccination may also come too early. Ideally, one would like to vaccinate before the age-associated decline in naive T-cell repertoire diversity, but late enough to ensure that a substantial level of protection is maintained until later in life. Further research is needed to investigate why some cells are maintained while others are not, and to define the optimal moment of vaccination to protect the elderly.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Central Committee on Research Involving Human Subjects of the Netherlands and ethical committee, METC Noord Holland. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The original idea for this study was from DvB and JAMB. JL performed the majority of experiments, gathered data, and analysis. PdG performed data analysis. MH and MV performed experiments. JvB designed the original studies of the individuals. DvB and JAMB supervised the project. JL and PdG prepared figures and wrote manuscript with contributions and review from DvB, JAMB, and JvB. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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frontiers in Aging

ORIGINAL RESEARCH
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Compromised DNA Repair Promotes the Accumulation of Regulatory T Cells With an Aging-Related Phenotype and Responsiveness

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Decline of immune function during aging has in part been ascribed to the accumulation of regulatory T cells (Tregs) and decreased T-cell responses with age. Aside from changes to T cells that occur over a lifetime, the impact of intracellular aging processes such as compromised DNA repair on T cells remains incompletely defined. Here we aimed to define the impact of compromised DNA repair on T-cell phenotype and responsiveness by studying T cells from mice with a deficiency in their DNA excision-repair gene *Ercc1*. These *Ercc1* mutant (*Ercc1*$^{-/\Delta 7}$) mice show accumulation of nuclear DNA damage resulting in accelerated aging. Similarly to wild-type aged mice, *Ercc1*$^{-/\Delta 7}$ mice accumulated Tregs with reduced CD25 and increased PD-1 expression among their naive T cells. *Ercc1*-deficiency limited the capacity of Tregs, helper T cells, and cytotoxic T cells to proliferate and upregulate CD25 in response to T-cell receptor- and IL-2-mediated stimulation. The recent demonstration that the mammalian target of rapamycin (mTOR) may impair DNA repair lead us to hypothesize that changes induced in the T-cell population by compromised DNA repair may be slowed down or reversed by blocking mTOR with rapamycin. *In vivo* dietary treatment of *Ercc1*$^{-/\Delta 7}$ mice with rapamycin did not reduce Treg levels, but highly increased the proportion of CD25^{+} and PD-1^{+} memory Tregs instead. Our study elucidates that compromised DNA repair promotes the accumulation of Tregs with an aging-related phenotype and causes reduced T-cell responsiveness, which may be independent of mTOR activation.

Keywords: aging, ercc1, DNA damage, nucleotide excision repair, T cells, regulatory T cells, mTOR, rapamycin

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INTRODUCTION

The phenotype and functionality of T cells change during the course of aging, which contributes to aging-related pathology, increased susceptibility to infectious diseases, and reduced vaccine efficacy in the elderly (Goronzy and Weyand, 2013). The decline in T-cell function with age can in part be explained by changes to their phenotype and proliferative capacity, often referred to as T-cell exhaustion and/or T-cell senescence (Akbar and Henson, 2011). Additionally, we and others have

shown that FoxP3$^+$ regulatory T cells accumulate with age (Gregg et al., 2005; Nishioka et al., 2006; Sharma et al., 2006; Lages et al., 2008; Elyahu et al., 2019; Pieren et al., 2019) and it is thought that these cells impair protective immune responses by their suppressive capacity (Sharma et al., 2006; Lages et al., 2008; Garg et al., 2014). Insight into biological processes that contribute to decreased T-cell function and the accumulation of regulatory T cells (Tregs) with age is required to better understand the process of T-cell aging.

Characteristics of T-cell aging are mostly investigated on a chronological scale, i.e., changes that occur among T cells in relation to progressing time. Indeed, the exposure of antigens over a lifetime causes major alterations to the T-cell compartment (Farber et al., 2014). Aside from this standard pathway, aging is also driven by cell intrinsic processes such as the accumulation of nuclear DNA damage; a hallmark of aging and recently defined as the factor driving all other hallmarks of aging (López-Otín et al., 2013; Vermeij et al., 2016). Accumulation of DNA damage with age is a result of suboptimal DNA repair and is thought to be one of the drivers of cellular senescence (Herbig et al., 2006; Gorbunova et al., 2007; Akbar et al., 2016). This may also apply to aging of T cells, as T cells with a highly differentiated phenotype accumulate with age (Henson et al., 2009) and express higher levels of DNA damage (Lanna et al., 2014). However, to what extent characteristics of T-cell aging are explained by suboptimal DNA repair remains unclear. In this study, we defined the impact of compromised DNA repair on hallmarks of T-cell aging.

The endonuclease complex ERCC1-XPF mediates the repair of a broad variety of DNA lesions: 1) bulky helix-distorting lesions are removed by global-genome nucleotide excision repair; 2) lesions blocking transcription are removed by transcription-coupled repair; 3) DNA crosslinks are removed via interstrand crosslink repair; and 4) a subset of persisting double-strand DNA breaks are removed by the single-strand annealing pathway (Gregg et al., 2011; Marteijn et al., 2014). Mice with a deficiency in the DNA excision-repair gene *Ercc1* (*Ercc1*$^{-/\Delta 7}$) have one knock-out and one truncated allele of *Ercc1* and therefore show impaired DNA repair within the four aforementioned pathways, which results in the accumulation of nuclear DNA damage (Niedernhofer et al., 2006; Vermeij et al., 2016; Robinson et al., 2018). As a consequence, *Ercc1*$^{-/\Delta 7}$ mice show numerous age-related pathologies and signs of accelerated aging with a reduced average lifespan of only 20 weeks (Weeda et al., 1997; Dolle et al., 2011; Vermeij et al., 2016). *Ercc1*$^{-/\Delta 7}$ mice therefore provide an aging model that enables investigation of the impact of compromised DNA repair on T-cell phenotype and responsiveness.

Another important driver of cellular aging is the activation of the mammalian target of rapamycin (mTOR) (Johnson et al., 2013). mTOR is a well-known target in anti-aging research as inhibition of mTOR by rapamycin has been widely reported to slow down the process of aging in terms of life- and healthspan (Chen et al., 2009; Harrison et al., 2009; Bitto et al., 2016). Moreover, dietary supplementation of rapamycin in an encapsulated form (eRapa) to aged mice has been shown to reduce age-related changes observed in T-cells (Hurez et al., 2015). Interestingly, activation of the mTOR kinase mTORC1 has been reported to impair DNA damage response signaling, leading to accumulation of unrepaired DNA lesions (Xie et al., 2018). Moreover, mTORC1 activation may negatively interfere with the ataxia telangiectasia mutated (ATM) checkpoint that promotes DNA repair (Shen and Houghton, 2013). We therefore hypothesized that changes to the T-cell compartment induced by compromised DNA repair may be slowed down by *in vivo* blocking of mTOR by eRapa.

In this study, we defined the impact of compromised DNA repair on T-cell phenotype and T-cell responsiveness in *Ercc1*$^{-/\Delta 7}$ mice. Additionally, we assessed whether the impact of compromised DNA repair on T cells could be avoided by inhibition of mTOR by *in vivo* dietary treatment with eRapa. We present evidence suggesting that compromised DNA repair promotes the aging-related accumulation of Tregs and reduced T-cell responsiveness that we find in wild-type (WT) aged mice, which appears to be independent of mTOR activation.

MATERIALS AND METHODS

Mice

Young (2 months of age) and aged (22 months of age) wild type C57BL/6 female mice were purchased from Envigo (Venray, Limburg, Netherlands). Young and aged mice were maintained at the animal facilities of the Institute for Translational Vaccinology (Bilthoven, Utrecht, Netherlands). Generation and characterization of *Ercc1*$^{-/\Delta 7}$ mice has been previously described (Weeda et al., 1997). Breeding stocks of the parental strains, i.e., Ercc1$^{+/-}$ mice in a pure C57BL6J background and *Ercc1*$^{+/\Delta 7}$ mice in a pure FVB background were generated and maintened as described (Weeda et al., 1997; Dolle et al., 2011). Genetically uniform F1-hybrid C57BL6-FVB *Ercc1*$^{-/\Delta 7}$ mice were generated by combining both parental strains. Typical unfavorable characteristics, such as blindness in the FVB background or deafness in the C57BL6J background, do not occur in this hybrid background. Animals were housed in individual ventilated cages under specific pathogen-free conditions (20–22°C; 12 hr. light: 12 hr. dark cycle) and provided food and water *ad libitum*. Since *Ercc1*$^{-/\Delta 7}$ mice are smaller, food was administered within the cages and water bottles with long nozzles were used from ~2 weeks of age. Wild-type F1 *Ercc1*$^{+/+}$ littermates at the indicated ages were used as controls. Male and female *Ercc1*$^{-/\Delta 7}$ and *Ercc1*$^{+/+}$ mice were maintained at the animal facilities of the Erasmus Medical Center (Rotterdam, Zuid-Holland, Netherlands). Distribution of males and females per group; n = 3 males and n = 3 females in the group of *Ercc1*$^{+/+}$ mice, n = 3 males and n = 4 females in the group of *Ercc1*$^{-/\Delta 7}$ mice, and n = 3 males and n = 3 females in the group of *Ercc1*$^{-/\Delta 7}$ mice fed with eRapa.

Dietary Treatment With Rapamycin

Diets were based on AIN93G, using 2.3 g/kg choline chloride instead of choline bitartrate (Research Diet Services, Wijk bij Duurstede, the Netherlands). Microencapsulated rapamycin

(eRapa) and empty microcapsules (Eudragit S100) were obtained from Southwestern Research Institute (San Antonio, TX, United States). 42 mg eRapa, containing 10% Rapamycin, was added per kg AIN93G food mix, resulting in a 42ppm rapamycin supplemented diet. For the control diet, 38 mg empty microcapsules were added per kg AIN93G food mix. The diets were processed into pellets which were radiated with 9 kGy (Isotron, Ede, Netherlands). Supplemented eRapa and control diets were supplied *ad libitum* to the mice at 8 weeks of age for the remainer of their life as described previously (Birkisdottir et al., 2021).

Preparation of Single Cell Suspensions and Proliferation Labeling

Spleens of all mice were homogenized through a cell strainer to prepare single cell suspensions and red blood cells were lyzed on ice with ACK lysis buffer (0.155M NH4Cl; 10 mM KHCO3; 0.1 mM Na2EDTA, pH 7.2–7.4). Subsequently, splenocytes were resuspended in PBS to $10*10^6$ cells/mL and labeled with 0.5 μM CellTrace™ Violet (Invitrogen, Carlsbad, CA, United States) in PBS per milliliter of splenocyte suspension for 20 min at 37°C to track T-cell proliferation. Cells were washed in ice-cold RPMI-1640 medium (GIBCO, Thermo Fisher Scientific, Waltham, MA, United States) containing 10% fetal calf serum (FCS) (Greiner Bio-One, Kremsmünster, Austria).

Splenocyte Culture and *In Vitro* Stimulation

To investigate T-cell proliferation and upregulation of the activation marker CD25, splenic single cell suspensions were exposed to soluble anti-CD3 (0.019 μg/ml; clone 145-2C11, eBioscience, San Diego, CA, United States) alone or in the presence of soluble anti-CD28 (0.5 μg/ml; clone PV-1, Southern Biotech, Birmingham, AL, United States) or recombinant murine IL-2 (0.1 μg/ml; eBioscience). Stimuli were prepared in RPMI-1640 medium containing 10% FCS and then added to splenocyte suspensions ($4*10^5$ cells/well). Cells were cultured in 96-well U-bottom plates (CELLSTAR, Greiner Bio-One) at 37°C and 5% CO_2 for four days.

Immunofluorescence Labeling and Flow Cytometric Analyses

Splenic single cell suspensions were washed with PBS containing 2% FCS and labeled for 30 minutes at 4°C for a combination of cell surface markers with the following fluorescently labeled anti-mouse antibodies: anti-CD4-BUV395 (clone GK1.5), anti-CD44-V450 (clone IM7) (BD Horizon, Franklin Lakes, NJ, USA); anti-CD25-PE-Cy7 (clone PC61.5) (eBioscience); CD122-PE-Dazzle 594 (clone TM-beta1), and anti-PD-1-BV785 (clone 29F.1A12) (BioLegend, San Diego, CA, United States). Live/Dead™ Fixable Aqua Dead Cell Stain Kit (Invitrogen) was included in the cell surface labeling to assess cell viability. Cells were subsequently labeled intracellularly according to the FoxP3 Transcription Factor staining buffer set protocol (eBioscience) with anti-CD3zeta-FITC (clone H146–968) (Abcam, Cambridge, Cambridgeshire, United Kingdom) and anti-FoxP3-eFluor660 (clone 150D/E4) (eBioscience). Labeled cells were detected on a BD LSRFortessa X-20 (BD Biosciences, Franklin Lakes, NJ, United States). Data analysis was performed using FlowJo software (Tree Star, Ashland, OR, United States).

Dimensionality Reduced Analyses

Dimensionality reduced analysis (viSNE) of flow cytometry data was performed in Cytobank (www.cytobank.org) (Amir et al., 2013). Cell density maps show clustering of $CD4^+$ and $CD4^-$ naive and memory T-cell populations that were generated from pooled flow cytometry datafiles of $Ercc1^{-/\Delta 7}$ mice (n = 7) and pooled flow cytometry datafiles of the different $Ercc1^{+/+}$ mice (n = 6). The number of cells included in the viSNE analysis were equal between $Ercc1^{-/\Delta 7}$ and $Ercc1^{+/+}$ mice; $2.3*10^5$ naive $CD4^+$ T cells, $1.4*10^5$ naive $CD4^-$ T cells, $3.3*10^4$ memory $CD4^+$ T cells, and $2.2*10^4$ memory $CD4^-$ T cells. Clustering was based on expression of FoxP3, CD25, CD122, and PD-1. Expression of the designated cellular markers in the heatmaps was based on their ArcSinh5-transformed median expression.

Statistics

Statistical analyses were performed using GraphPad Prism 7 software (La Jolla, CA, United States). The appropriate parametric or non-parametric tests were used based on the tested normality of distribution of the data by Kolmogrov-Smirnov test and Shapiro-Wilk test. Dependent on the number of comparisons and the normality of distribution of the data, unpaired Student *t* Test, Mann-Whitney *U* test, or One-way ANOVA test followed by Holm-Sidak multiple comparisons test were performed as indicated in the figure legends. Data presented in bar graphs are expressed as the mean ± standard deviation (SD). For all analyses, p values < 0.05 were considered statistically significant.

RESULTS

Compromised DNA Repair Contributes to Increased Proportions of Memory T Cells

With age, the total T-cell population among lymphocytes decreases, whereas the proportion of memory cells within the T cell population increases (Farber et al., 2014; Duggal et al., 2019; Pieren et al., 2019). We first assessed whether compromised DNA repair contributes to these aging-related changes. We compared the T-cell populations in spleens of accelerated aging $Ercc1^{-/\Delta 7}$ mice (n = 7, 4 months of age) with those in spleens of littermate control $Ercc1^{+/+}$ mice (n = 6, 4 months of age) (**Figures 1A–C**). As a reference we did similar analyses in WT young (n = 6, 2 months of age) and WT aged (n = 6, 22 months of age) mice (**Figures 1D–F**).

Ercc1-deficient mice did not significantly show a decrease of $CD3^+$ T-cell frequency among live spleen lymphocytes (**Figure 1A**) that occured in normal WT aging (**Figure 1D**). Subsequently, we analyzed the frequency of memory cells by expression of the memory marker CD44 among $CD4^+$ T cells and $CD4^-$ T cells. Of note, $CD3^+$ $CD4^-$ T cells can be considered $CD8^+$ T cells as this subset mainly comprises $CD8^+$ T cells and a

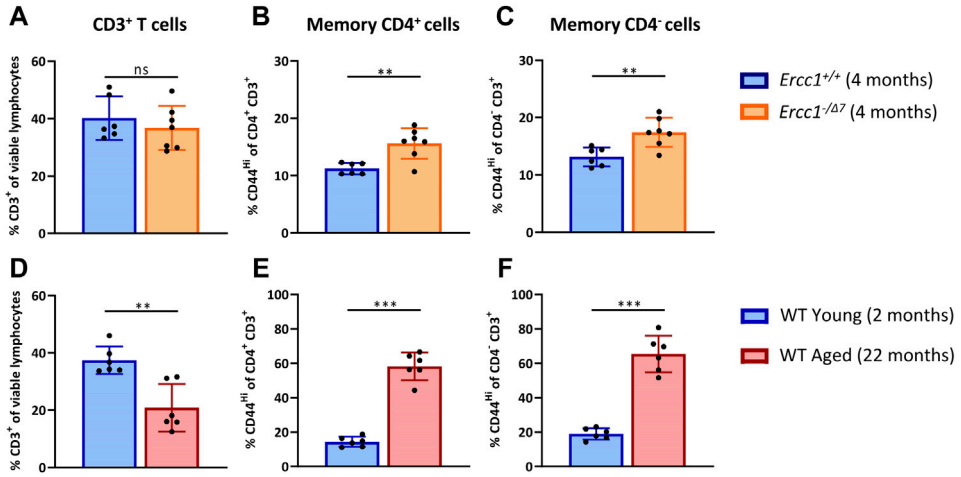

FIGURE 1 | Compromised DNA repair contributes to increased proportions of memory T cells. The frequencies of **(A)** total T cells ($CD3^+$ cells of viable lymphocytes), **(B)** memory $CD4^+$ T cells ($CD44^{Hi}$ of $CD4^+CD3^+$ cells), and **(C)** memory $CD4^-$ T cells ($CD44^{Hi}$ of $CD4^-CD3^+$ cells) were determined in the spleen of *Ercc1*$^{+/+}$ (n = 6, 4 months of age) and *Ercc1*$^{-/\Delta7}$ (n = 7, 4 months of age) mice, as well as in **(D–F)** WT young (n = 6, 2 months old) and aged mice (n = 6, 22 months old). Bar graphs show mean ± SD; $^{**}p < 0.01$, ns = not statistically significant for the difference between groups using unpaired Student's *t* test, two-tailed.

very low proportion of $CD4^-$ $CD8^-$ cells in *Ercc1*$^{-/\Delta7}$ mice that we analyzed in an additional data set (3% on average) (**Supplementary Figure S1**), similar to previous observations in WT mice (4% on average) (Pieren et al., 2019). Although *Ercc1*$^{-/\Delta7}$ mice did not reach the major increase of memory $CD4^+$ and $CD4^-$ T cell frequencies found in WT aged mice (**Figures 1E,F**), the proportion of memory T cells was higher in *Ercc1*$^{-/\Delta7}$ mice compared to control *Ercc1*$^{+/+}$ mice (**Figures 1B,C**). Thus, our data indicate that the rise in proportion of memory T cells among $CD4^+$ and $CD4^-$ T cells found in WT aged mice can in part be attributed to compromised DNA repair.

Compromised DNA Repair Promotes Accumulation of FoxP3$^+$ Tregs Within the Naive CD4$^+$ T-Cell Subset

Elevated frequencies of Tregs among the $CD4^+$ T-cell pool is a major hallmark of aging. Age-related differences in T-cell subsets have largely been ascribed to the shifted balance of naive T cells toward memory T cells during aging rather than an aging-related effect within these cell subsets. Indeed, Tregs in aged WT mice showed a shift toward increased numbers of memory cells in the Treg pool (**Supplementary Figure S2** (Raynor et al., 2015). Although *Ercc1*$^{-/\Delta7}$ mice showed elevated frequencies of memory cells in the overall T-cell pool (**Figures 1B,C**), they did not show a different memory cell frequency among Tregs compared to control *Ercc1*$^{+/+}$ mice (**Supplementary Figure S2**). However, the impact of aging may be reflected beyond the mere shift in balance of naive/memory T cells and may be defined by changes within naive and memory T cell populations (Pieren et al., 2019). Indeed, we previously reported that naive $CD4^+$ T cells of WT aged mice are enriched with FoxP3$^+$ Tregs that express an aging-related phenotype characterized by increased expression of PD-1 and lower expression of CD25 (Pieren et al., 2019). Here we asked whether this major hallmark of aging in WT aged mice can be attributed to compromised DNA repair. We applied dimensionality reduction (viSNE) to form phenotypically distinct clusters based on simultaneous expression of different aging-related molecules within $CD4^+$ naive ($CD44^{Lo}$) and memory ($CD44^{Hi}$) T cells of *Ercc1*$^{-/\Delta7}$ mice and *Ercc1*$^{+/+}$ mice (**Supplementary Figure S3**, gating strategy). Cluster formation was based on the combined expression of FoxP3, CD25, CD122, and PD-1 as aging-related markers previously used to reveal aging-related clusters of T cells (Pieren et al., 2019).

viSNE analysis of T cells of *Ercc1*$^{+/+}$ (n = 6 pooled) and *Ercc1*$^{-/\Delta7}$ mice (n = 7 pooled) showed four phenotypically distinct clusters within the naive $CD4^+$ T-cell subset (**Figure 2A**). Cluster 1 highly expressed the Treg marker FoxP3 (**Figure 2B**) and the proportion of this cluster was higher in *Ercc1*$^{-/\Delta7}$ mice compared to *Ercc1*$^{+/+}$ mice (16.2 vs. 10.8%) (**Figure 2C**). Accumulation of Tregs within the naive $CD4^+$ T-cell subset was indeed significantly higher in individual *Ercc1*$^{-/\Delta7}$ mice compared to control *Ercc1*$^{+/+}$ mice (**Figure 2D**), which closely resembled findings in WT aged mice (**Supplementary Figure S4**). Additionally, accumulated Tregs of *Ercc1*$^{-/\Delta7}$ mice comprised significantly lower $CD25^+$ (**Figure 2E**) and higher PD-1^+ cell frequencies (**Figure 2F**) and expression levels per cell (MFI) (**Supplementary Figures S5 and S6**) compared to *Ercc1*$^{+/+}$ mice, which is similar to observations in WT aged mice (Pieren et al., 2019). Thus, compromised DNA repair appears to contribute to the accumulation of Tregs within the naive $CD4^+$ T-cell compartment with an aging-related phenotype that resembles previous findings in WT aged mice (Pieren et al., 2019).

FoxP3$^+$ Tregs Within the Memory CD4$^+$ T-Cell Subset in Mice With Compromised DNA Repair

WT aged mice do not accumulate Tregs within the memory $CD4^+$ T-cell pool ((Pieren et al., 2019) and **Supplementary Figure S4**) and

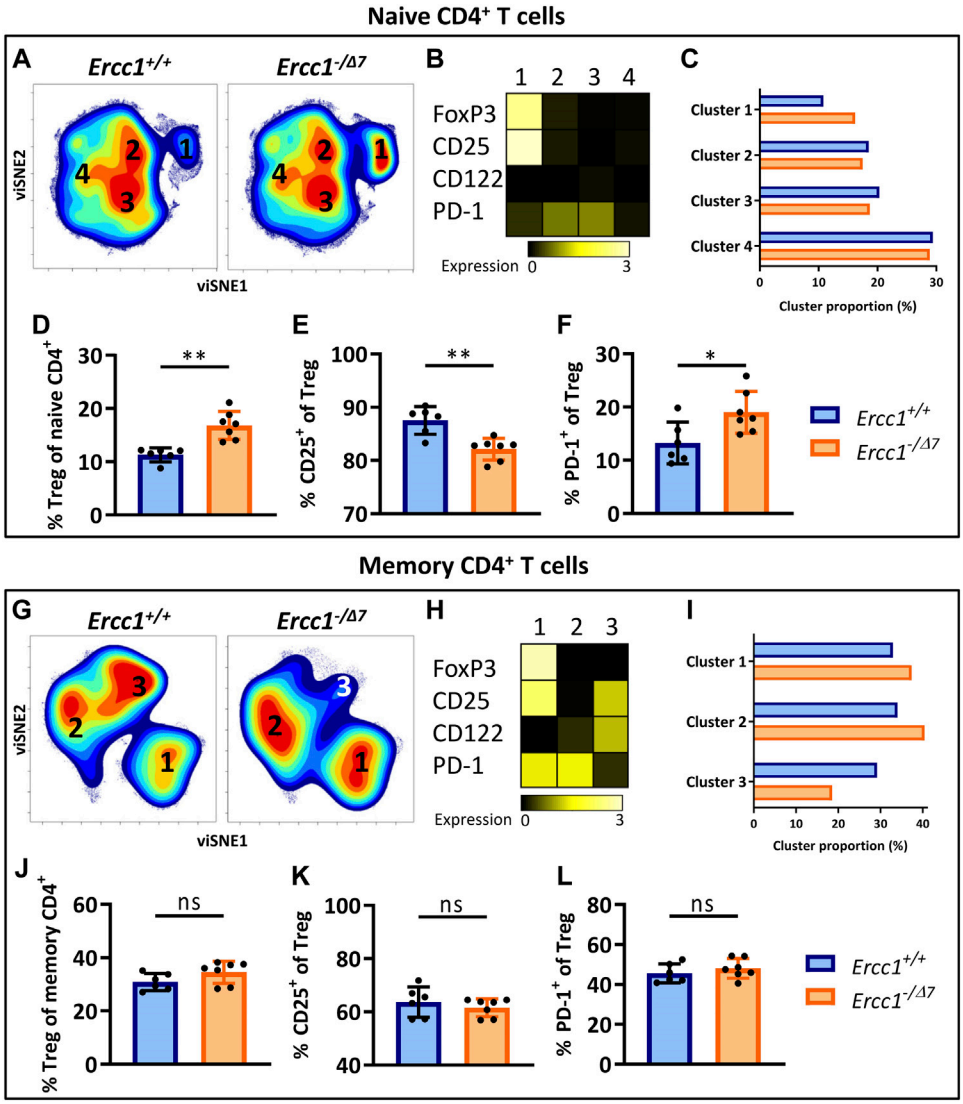

FIGURE 2 | Compromised DNA repair promotes accumulation of FoxP3$^+$ Tregs within the naive CD4$^+$ T-cell subset. Naive (CD44^{Lo}) and memory (CD44^{Hi}) cells were identified within the CD4$^+$ T-cell population. Cell density maps of dimensionality reduced single-cell data by viSNE show clustering within **(A)** naive CD4$^+$ T cells and **(G)** memory CD4$^+$ T cells of pooled flow cytometry datafiles of *Ercc1*$^{+/+}$ mice (n = 6) and pooled datafiles of *Ercc1*$^{-/\Delta7}$ mice (n = 7). Numbers in the density maps correspond to the cluster numbers above the heat maps **(B,H)**. These heat maps depict the Arcsinh-transformed median expression of the indicated markers. Bar graphs **(C,I)** indicate the proportion of each cluster within the total viSNE for *Ercc1*$^{+/+}$ (blue) and *Ercc1*$^{-/\Delta7}$ (orange) mice. Bar graphs **(D–F, J–L)** show the frequency of **(D)** naive and **(J)** memory FoxP3$^+$ regulatory T cells in individual *Ercc1*$^{+/+}$ and *Ercc1*$^{-/\Delta7}$ mice, as well as the frequency of **(E,K)** CD25$^+$ and **(F,L)** PD-1$^+$ cells within these naive and memory Treg subsets. Bar graphs show mean ± SD; **p < 0.01, ns = not statistically significant for the difference between *Ercc1*$^{+/+}$ and *Ercc1*$^{-/\Delta7}$ mice using parametric unpaired Student's t test or non-parametric Mann-Whitney test, two-tailed, dependent on the tested normality of distribution of the data.

we assessed whether *Ercc1*$^{-/\Delta7}$ mice resemble these findings. viSNE analysis of memory (CD44^{Hi}) CD4$^+$ T cells of *Ercc1*$^{-/\Delta7}$ and *Ercc1*$^{+/+}$ mice showed three phenotypically distinct clusters (**Figure 2G**) with cluster 1 indicating FoxP3-expressing Tregs (**Figure 2H**). Whereas the proportion of cluster 1 containing Foxp3$^+$ cells was higher in *Ercc1*$^{-/\Delta7}$ mice compared to *Ercc1*$^{+/+}$ mice (37.4 vs. 32.9%) (**Figure 2I**), the frequency of Foxp3$^+$ Tregs within the memory CD4$^+$ T-cell subset did not show a difference between individual *Ercc1*$^{-/\Delta7}$ and *Ercc1*$^{+/+}$ mice (**Figure 2J**). In contrast to previous findings in WT aged mice (Pieren et al., 2019), *Ercc1*-deficiency did not show altered frequency and expression of CD25 and PD-1 on Tregs within the memory CD4$^+$ T-cell subsets (**Figures 2K,L**; **Supplementary Figures S5 and S6**). These findings suggest that compromised DNA repair may not significantly contribute to phenotypical changes of Tregs within the memory CD4$^+$ T-cell pool.

The Aging-Related Phenotype of Naive Th Cells in Mice With Compromised DNA Repair

We next investigated whether compromised DNA repair contributes to phenotypical changes within naive (CD44^{Lo})

helper T cells (Th cells). viSNE-clusters 2, 3, and 4 identified within the naive CD4$^+$ T-cell pool do not express FoxP3 (**Figures 2A,B**), indicating that these cells are naive Th cells. The minimal difference in proportion of clusters 2, 3, and 4 between *Ercc1*$^-$ and *Ercc1*$^{+/+}$ mice (**Figure 2C**) indicates that *Ercc1*-deficiency did not profoundly affect the phenotype of naive Th cells based on the markers we included. Indeed, *Ercc1*$^{-/\Delta 7}$ mice did not show the increased frequencies or expression of PD-1$^+$ cells among naive Th cells (**Supplementary Figure S6**) that we previously found in WT aged mice (Pieren et al., 2019). Thus, these findings suggest that *Ercc1*-deficiency may not have a significant impact on expression of CD122, CD25, and PD-1 by the naive Th population and therefore might not explain the aging-related changes based on these markers within the naive Th-cell subset in WT aged mice.

Compromised DNA Repair Promotes the Aging-Related Phenotype of Memory Th Cells

Next, we assessed the impact of compromised DNA repair on the phenotype of memory (CD44^{Hi}) Th cells. These cells were represented by the FoxP3-negative clusters 2 and 3 within the memory CD4$^+$ T cells in our viSNE analyses (**Figures 2G,H**). Cluster 2 was more abundant in *Ercc1*$^{-/\Delta 7}$ compared to *Ercc1*$^{+/+}$ mice (40.4 vs. 34.0%), which points toward more *Ercc1*$^{-/\Delta 7}$ memory Th cells expressing PD-1 as reported previously in WT aged mice(Pieren et al., 2019). Indeed, a trend toward higher PD-1$^+$ memory Th-cell frequency and higher expression of PD-1 on *Ercc1*$^{-/\Delta 7}$ memory Th cells compared to *Ercc1*$^{+/+}$ cells was found (**Supplementary Figure S6**). Further, we observed a lower proportion of cluster 3 (18.6 vs. 29.2%) in *Ercc1*$^{-/\Delta 7}$ compared to *Ercc1*$^{+/+}$ mice (**Figure 2I**), which is reflected in lower CD122 expression and CD122$^+$ memory Th-cell frequency in *Ercc1*$^{-/\Delta 7}$ mice (**Supplementary Figure S5**) and corresponds to earlier findings in WT aged mice (Pieren et al., 2019). Together, these data indicate that *Ercc1*-deficiency imposes an aging-related phenotype on memory Th cells that has been reported based on the expression of PD-1 and CD122.

Aging-Related Changes in Naive and Memory Tc Cells in Mice With Compromised DNA Repair

CD3$^+$ cells that do not express CD4 can be considered CD8$^+$ cytotoxic T cells (Tc cells), as this subset mainly comprises CD8$^+$ T cells ((Pieren et al., 2019), **Supplementary Figure S1**). Clustering of naive (CD44^{Lo}) Tc cells by viSNE showed relatively comparable cell-density plots, expression of phenotypical markers, and cluster proportions (**Figures 3A–C**) between *Ercc1*$^{-/\Delta 7}$ and *Ercc1*$^{+/+}$ mice, which suggests a low impact of *Ercc1*-deficiency on naive Tc cells. Indeed, we observed no phenotypical difference in expression and cell frequencies of naive Tc cells (**Figures 3D,E**; **Supplementary Figures S5 and S6**) apart from a slight decrease in the frequency of PD-1$^+$ Tc cells of *Ercc1*$^{-/\Delta 7}$ mice (**Figure 3F**). These findings are in contrast with increased PD-1$^+$ cell-frequencies found among naive Tc cells of WT aged mice (Pieren et al., 2019).

Memory (CD44^{Hi}) Tc cells of WT aged mice do not differ from those of WT young mice (Pieren et al., 2019). In contrast, viSNE analysis of *Ercc1*$^{-/\Delta 7}$ memory Tc cells showed an increase in cluster 1 (21.5 vs. 12.7%) and 2 (35.7 vs. 25.6%), and a decrease in cluster 3 (38.4 vs. 56.9%) compared to *Ercc1*$^{+/+}$ mice (**Figures 3E–G**). These differences were reflected in a reduced frequency of CD122$^+$ cells and an increased frequency of PD-1$^+$ cells among the memory Tc cells of *Ercc1*$^{-/\Delta 7}$ mice compared to *Ercc1*$^{+/+}$ mice (**Figures 3I,J**), which is also shown in the expression of these markers on a per cell basis (**Supplementary Figures S5 and S6**). Together, these data indicate that the impact of compromised DNA damage repair on naive and memory Tc cells may not explain findings on aging of Tc cells in WT aged mice.

Compromised DNA Repair Promotes the Accumulation of Regulatory Cells Within the Memory Tc-Cell Subset

PD-1$^+$CD122$^+$ Tc cells are regulatory Tc cells (Tc reg) (Dai et al., 2010; Elizondo et al., 2019) and these cells mainly accumulate within the naive Tc-cell subet of WT aged mice (Pieren et al., 2019). In contrast, PD-1$^+$CD122$^+$ Tc reg cells did not accumulate within the naive Tc-cell subset of *Ercc1*$^{-/\Delta 7}$ mice (**Supplementary Figure S7**), but rather within their memory Tc-cell subset. Moreover, the frequency of CD25$^+$ naive and memory Tc reg cells did not differ between *Ercc1*$^{-/\Delta 7}$ and *Ercc1*$^{+/+}$ mice. Thus, compromised DNA repair does not explain findings on Tc reg cells in WT aged mice.

eRapa Reduces Memory T Cells That Are Induced by Compromised DNA Repair

Inhibition of mTOR by rapamycin reduces aging-related T-cell changes in WT mice (Hurez et al., 2015). Moreover, the mTOR pathway is thought to impair the DNA damage response (Xie et al., 2018). We therefore hypothesized that *in vivo* dietary treatment of *Ercc1*$^{-/\Delta 7}$ mice with eRapa may slow down changes to the T-cell compartment induced by compromised DNA repair. To this end, 8 week old *Ercc1*$^{-/\Delta 7}$ mice were fed eRapa for the remainder of their life (Birkisdottir et al., 2021) [42 ppm, reported previously as a life- and healthspan extending dose (Miller et al., 2014)].

eRapa-fed *Ercc1*$^{-/\Delta 7}$ mice showed decreased proportions of CD3$^+$ T cells among live spleen-derived lymphocytes compared to control-fed *Ercc1*$^{+/+}$ and *Ercc1*$^{-/\Delta 7}$ mice (**Figure 4A**), consistent with previous reports in WT mice (Hurez et al., 2015). Interestingly, eRapa reduced the proportion of memory CD4$^+$ and CD4$^-$ T cells toward levels found in control-fed *Ercc1*$^{+/+}$ mice (**Figures 4B,C**), suggesting that the increase of memory T cells induced by compromised DNA repair may in part be dependent on mTOR activation.

eRapa Increases the Proportion of Memory Tregs but Does Not Reduce the Accumulation of Total Tregs

eRapa did not prevent the rise in proportion of FoxP3$^+$ Tregs within the overall CD3$^+$ T cell pool in *Ercc1*$^{-/\Delta 7}$ mice

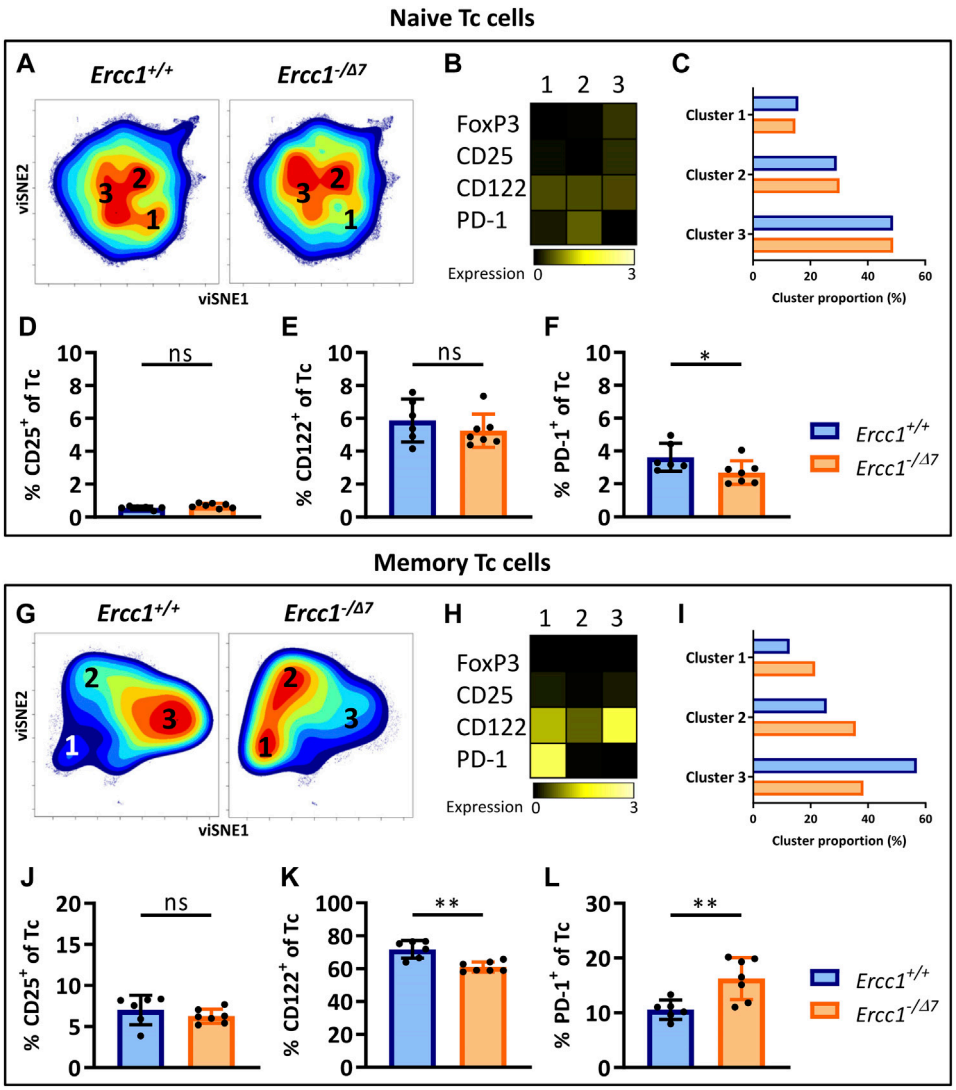

FIGURE 3 | Aging-related changes in naive and memory Tc cells in mice with compromised DNA repair. Naive ($CD44^{Lo}$) and memory ($CD44^{Hi}$) cells were identified within the $CD4^-$ T-cell population (Tc cells). Cell density maps of dimensionality reduced single-cell data by viSNE show clustering within **(A)** naive Tc cells and **(G)** memory Tc cells of pooled flow cytometry datafiles of *Ercc1*$^{+/+}$ mice (n = 6) and pooled datafiles of *Ercc1*$^{-/\Delta7}$ mice (n = 7). Numbers in **(B,H)** the density maps correspond to the cluster numbers above the heat maps. These heat maps depict the Arcsinh-transformed median expression of the indicated markers. Bar graphs **(C,I)** indicate the proportion of each cluster within the total viSNE for *Ercc1*$^{+/+}$ (blue) and *Ercc1*$^{-/\Delta7}$ (orange) mice. Bar graphs show the frequencies of **(D,J)** $CD25^+$ **(E,K)** $CD122^+$, and **(F, L)** PD-1^+ cells among naive and memory Tc cells in individual *Ercc1*$^{+/+}$ and *Ercc1*$^{-/\Delta7}$ mice. Bar graphs show mean ± SD; *$p < 0.05$, **$p < 0.01$, ns = not statistically significant for the difference between *Ercc1*$^{+/+}$ and *Ercc1*$^{-/\Delta7}$ mice using parametric unpaired Student's *t* test or non-parametric Mann-Whitney test, two-tailed, dependent on the tested normality of distribution of the data.

(**Figure 4D**). However, we observed that eRapa decreased the proportion of Tregs within the naive $CD4^+$ T-cell pool of *Ercc1*$^{-/\Delta7}$ mice and strongly increased the proportion of Tregs within the memory $CD4^+$ T-cell pool of these mice (**Figures 4E,H**). eRapa did not prominently reverse the aging-related lower $CD25^+$ and higher PD-1^+ cell frequencies found among naive Tregs of *Ercc1*$^{-/\Delta7}$ mice (**Figures 4F,G**). In contrast, eRapa highly increased the frequency of $CD25^+$ and PD-1^+ cells among memory Tregs, which was not observed in *Ercc1*$^{-/\Delta7}$ mice without eRapa (**Figures 4I,J**). Together, these data suggest that accumulation of Tregs with an aging-related phenotype by compromised DNA repair may be independent of mTOR activation.

Aging-Related Reduction of $CD122^+$ Memory Th-Cell Frequencies can Be Restored by eRapa

One of the few observations in the Th-cell subset of *Ercc1*$^{-/\Delta7}$ mice that reflected findings in WT aged mice (3) was a decreased frequency of $CD122^+$ cells among memory Th cells (**Supplementary Figure S6**). eRapa restored the proportion of

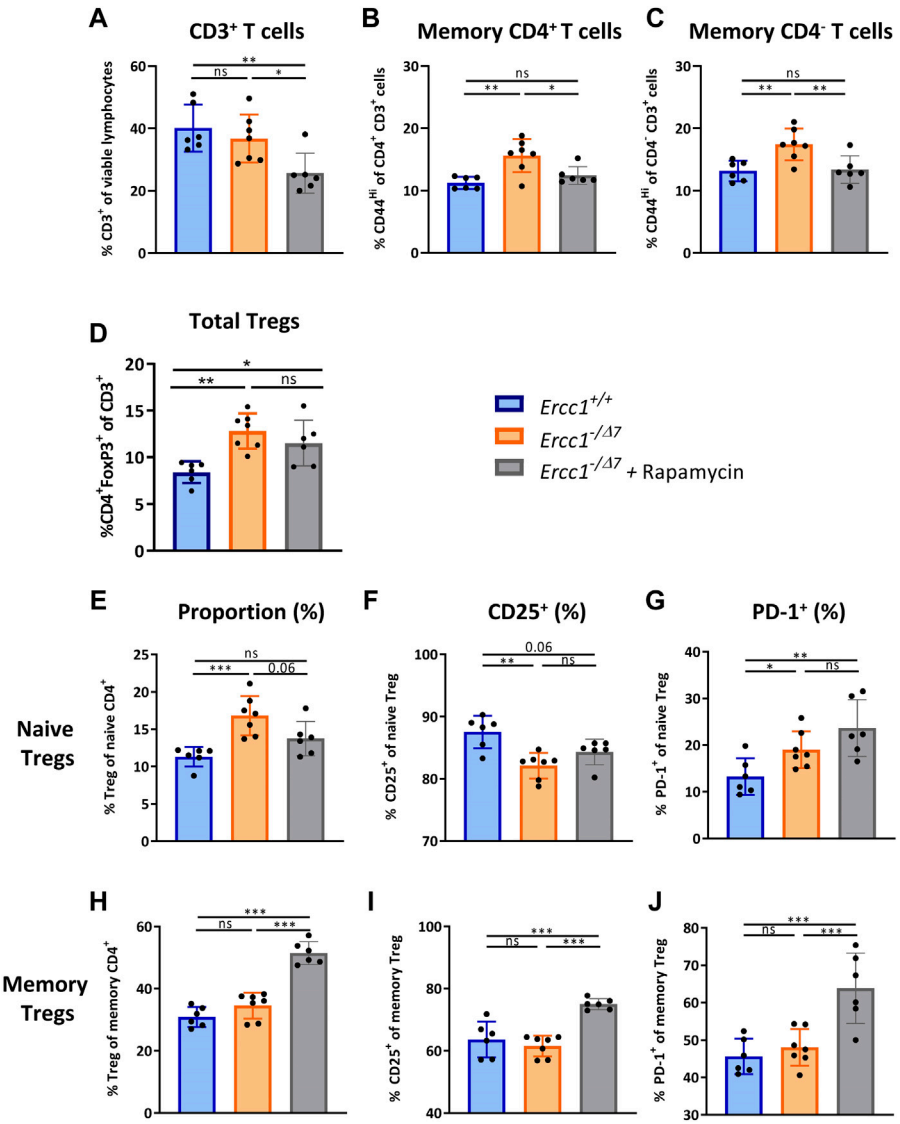

FIGURE 4 | eRapa increases the proportion of Tregs within the memory CD4$^+$ T-cell subset. The frequencies of **(A)** total T cells (CD3$^+$ cells of viable lymphocytes) **(B)** memory CD4$^+$ T cells (CD44^{Hi} of CD4$^+$CD3$^+$ cells) **(C)** memory CD4$^-$ T cells (CD44^{Hi} of CD4$^-$CD3$^+$ cells), and **(D)** total Tregs (CD4$^+$FoxP3$^+$ of CD3$^+$) were determined in the spleens of *Ercc1*$^{+/+}$ (n = 6, blue bars), *Ercc*$^{-/\Delta7}$ (n = 7, orange bars), and *Ercc1*$^{-/\Delta7}$ treated with eRapa (n = 6, gray bars). The frequencies of **(E)** naive Tregs (FoxP3$^+$ cells of CD44^{Lo}CD4$^+$CD3$^+$), and **(H)** memory Tregs (FoxP3$^+$ cells of CD44^{Hi}CD4$^+$CD3$^+$), and their respective **(F,I)** CD25$^+$ and **(G,J)** PD-1$^+$ cell frequencies were also determined in these mice. Bar graphs show mean ± SD; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns = not statistically significant for the difference between groups using one-way ANOVA corrected with Holm-Sidak test for multiple comparisons.

CD122$^+$ memory Th cells to levels found in *Ercc1*$^{+/+}$ mice (**Supplementary Figure S8**). This finding shows that eRapa may reverse this aging-related aspect of memory Th cells and suggests that CD122 expression by memory Th cells is dependent on mTOR activation.

Compromised DNA Repair Limits T-Cell Receptor/Interleukin-2-Mediated Treg Proliferation and Activation

Reduced proliferation and reduced upregulation of the activation marker CD25 in response to cellular stimulation are hallmarks of declined T-cell responsiveness at older age (Jiang et al., 2007; Pieren et al., 2019). To investigate the consequences of compromised DNA repair on T-cell responses, we exposed total spleen cells of *Ercc1*$^{+/+}$ and *Ercc1*$^{-/\Delta7}$ mice, or young and aged WT mice to anti-CD3 alone to mimic stimulation of the T-cell receptor (TCR), or in combination with anti-CD28 or interleukin-2 (IL-2) as a co-stimulator. After four days, we measured T-cell responsiveness by proliferation and upregulation of the activation marker CD25. Additionally, we stimulated T cells of eRapa-fed *Ercc1*$^{-/\Delta7}$ mice to assess whether eRapa would prevent consequences of compromised DNA repair on T-cell responsiveness.

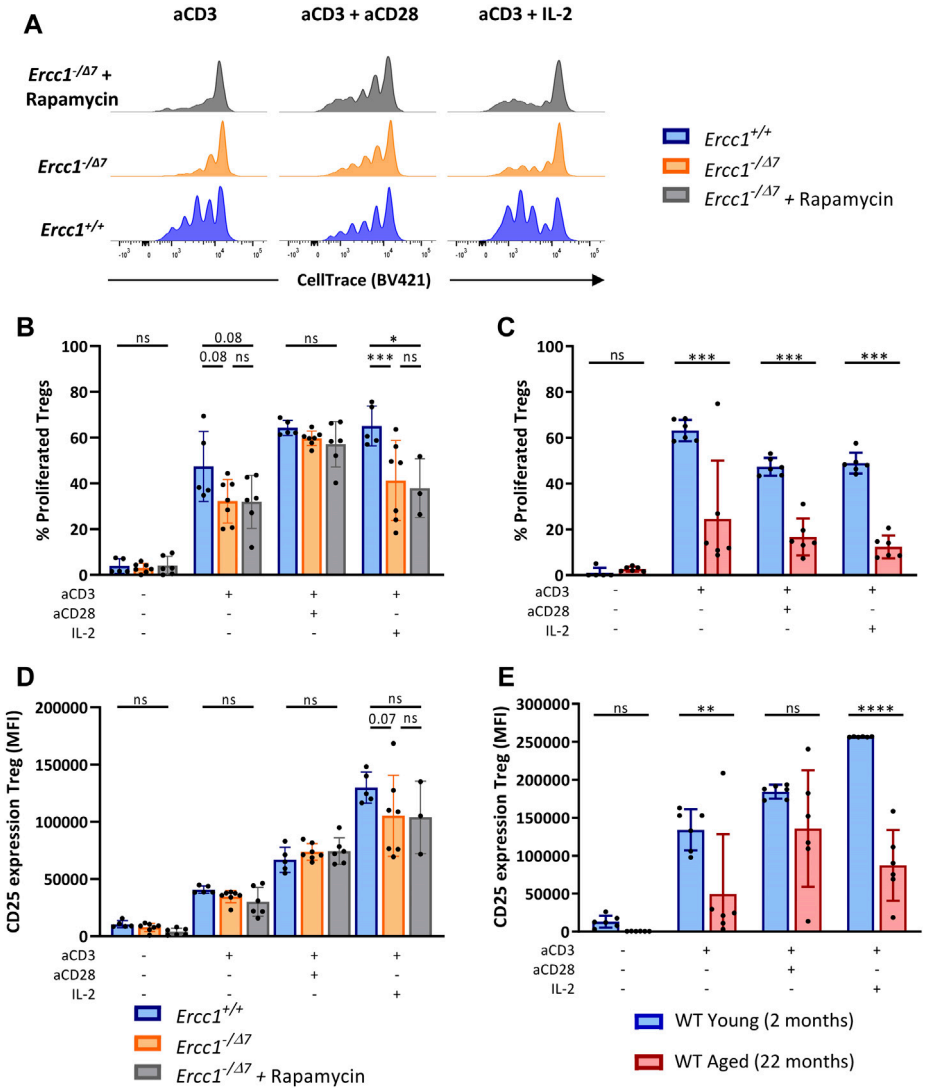

FIGURE 5 | Compromised DNA repair limits T-cell receptor/Interleukin-2-mediated Treg proliferation and activation. Total splenocytes of WT young (n = 6) and aged (n = 6) mice, and *Ercc1*$^{+/+}$ (n = 5), *Ercc1*$^{-/\Delta7}$ (n = 7), and *Ercc1*$^{-/\Delta7}$ mice treated with rapamycin (n = 3–6) were exposed to anti-CD3 alone or in combination with anti-CD28 or IL-2 for four days. Treg proliferation was traced by CellTrace labeling **(A)**; dilution of CellTrace label intensity indicates cellular proliferation. Bar graphs show **(B)** Treg proliferation of *Ercc1*$^{-/\Delta7}$ and *Ercc1*$^{-/\Delta7}$ mice and of **(C)** WT young and aged mice. Bar graphs show CD25 expression by Tregs of **(D)** *Ercc1*$^{-/\Delta7}$ and *Ercc1*$^{-/\Delta7}$ mice and **(E)** WT young and aged mice. Bar graphs show mean ± SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = not statistically significant for the difference between groups using parametric one-way ANOVA corrected with Holm-Sidak correction for multiple comparisons. Due to the low number of eRapa-fed *Ercc1*$^{-/\Delta7}$ mice (n = 3), statistical significance of this group was determined by non-parametric Mann-Whitney *U* Test.

We observed that compromised DNA repair in part explains diminished Treg proliferation in WT aged mice, as Tregs of *Ercc1*$^{-/\Delta7}$ mice showed a trend toward reduced proliferation in response to anti-CD3 alone and significantly reduced proliferation in response to anti-CD3 with IL-2 (**Figures 5A,B**). However, Tregs of WT aged mice also showed reduced proliferation in response to anti-CD3 combined with co-stimulation by anti-CD28 (**Figure 5C**), whereas CD28-mediated co-stimulation appeared to remain intact in Tregs from *Ercc1*$^{-/\Delta7}$ mice. eRapa did not restore the reduction of Treg proliferation observed in *Ercc1*$^{-/\Delta7}$ mice (**Figures 5A,B**). Additionally, Tregs of *Ercc1* mice showed a trend toward reduced CD25 upregulation in response to anti-CD3 with IL-2, although the variation within the group of *Ercc1*$^{-/\Delta7}$ mice was relatively high (**Figure 5D**). These findings in part reflect observations in WT aged mice, as WT aged mice also showed reduced CD25 expression in response to anti-CD3 in the absence of exogenous IL-2 (**Figure 5E**). eRapa did not restore Treg CD25 expression levels in *Ercc1*$^{-/\Delta7}$ compared to *Ercc1*$^{+/+}$ mice (**Figure 5D**). It has to be noted that the number of eRapa-fed *Ercc1*$^{-/\Delta7}$ mice stimulated with anti-CD3 with IL-2 was low (n = 3) due to a limited number of splenocytes available for the different assays and these results should therefore be approached

with caution. Together, our findings indicate that compromised DNA repair contributes to reduced Treg responsiveness to anti-CD3 and IL-2 observed with WT aging, which may be independent of mTOR activation.

Compromised DNA Repair Limits T-Cell Receptor/Interleukin-2 Mediated Th- and Tc-Cell Responsiveness

Th and Tc cells of *Ercc1*$^{-/\Delta 7}$ mice showed a trend toward reduced proliferation in response to anti-CD3 with IL-2, whereas Th and Tc cells of WT aged mice showed reduced proliferation also in response to anti-CD3 with anti-CD28 (**Supplementary Figure S9**). Despite the small number of mice, eRapa did not appear to restore proliferation of Th and Tc cells from *Ercc1*$^{-/\Delta 7}$ mice in response to anti-CD3 with IL-2 (**Supplementary Figure S9**). Tc cells but not Th cells of *Ercc1*$^{-/\Delta 7}$ mice showed reduced CD25 expression induced by response to anti-CD3 with IL-2, despite the individual response within each group being highly variable. In contrast, both cell types showed reduced CD25 induction in WT aged mice (**Supplementary Figure S9**). *In vivo* treatment of *Ercc1*$^{-/\Delta 7}$ mice with eRapa did not affect stimulation-induced CD25 expression by their Th cells (**Supplementary Figure S9**). Thus, compromised DNA repair may partly explain impaired Th- and Tc-cell responses found in WT aged mice.

DISCUSSION

Aging has a significant effect on T-cell phenotype and responsiveness, which contributes to pathology, increased susceptibility to infectious diseases, and reduced vaccine efficacy in the elderly (Goronzy and Weyand, 2013). Aside from well described T-cell aging phenomena that occur over time, the contribution of intracellular processes that drive T-cell aging such as compromised DNA repair remain incompletely defined. Here we provide novel insights into T-cell aging as we show which parts of T-cell aging can be attributed to compromised DNA repair. Moreover, we show that the majority of the changes to T cells induced by compromised DNA-repair could not be reversed by inhibition of mTOR activation.

Accumulation of Tregs during aging has been described in mice and humans (Pieren et al., 2019; Lages et al., 2008; Sharma et al., 2006; Gregg et al., 2005; Nishioka et al., 2006; Elyahu et al., 2019) and is thought to limit protective immune responses. However, the underlying cause of Treg accumulation with age remains unclear. Here we discovered that compromised DNA repair contributes to the accumulation of Tregs observed during aging. Similar to WT aged mice, Treg accumulation in *Ercc1*$^{-/\Delta 7}$ mice was explained by an increase in the proportion of Tregs within the naive CD4$^+$ T-cell subset and not within the memory CD4$^+$ T-cell subset, as defined by other approaches (**Supplementary Figure S2**, Raynor et al., 2015). These findings indicate that propagation of memory Treg cells during aging may be a mechanism that occurs over time and complements the rise of Tregs in the naïve pool that occurs in both aging wild type mice and *Ercc1*$^{-/\Delta 7}$ mice. Moreover, Tregs of *Ercc1*$^{-/\Delta 7}$ mice showed an age-related phenotype characterized by increased expression of PD-1 and decreased expression of CD25, consistent with previous findings in WT mice (Elyahu et al., 2019; Pieren et al., 2019). Together, our data indicate that compromised DNA repair may be a contributing factor in promoting Treg accumulation previously observed to occur during the process of aging.

An important question that now remains is how compromised DNA repair results in the accumulation of Tregs. As possible explanation for this finding, we speculate that compromised DNA-damage repair in cells that make up the micro-environment of T cells significantly contributes to the phenotypical and functional changes found within the T cell pool with age. Senescent cells develop during the process of aging in multiple organs partly due to intracellular accumulation of DNA damage (Rodier et al., 2009; Pereira et al., 2019). These cells are known to secrete a collection of pro-inflammatory factors collectively known as the senescence-associated secretory phenotype (SASP). Accumulation of senescent cells therefore results in an inflammatory micro-environment (Rodier et al., 2009). Interestingly, it has been reported that the cytokine IL-6 is part of the pro-inflammatory SASP and promotes the accumulation of Tregs in WT aged mice (Raynor et al., 2015). Moreover, it is known that *Ercc1*$^{-/\Delta 7}$ mice also accumulate senescent cells and show increased levels of the SASP, including higher levels of IL-6 in several organs and serum (Chen et al., 2013; Karakasilioti et al., 2013; Vermeij et al., 2016; Kim et al., 2020). Based on these and our findings, we speculate that accumulation of Tregs with age is mediated by pro-inflammatory factors like IL-6 secreted by senescent cells that result from accumulation of DNA damage over life. Accumulation of Tregs in response to IL-6 induced by DNA-damage may be an attempt by the immune system to counteract pro-inflammatory conditions that occur during aging.

Diminished T-cell proliferation is a hallmark of T-cell aging (Jiang et al., 2007; Pieren et al., 2019). Our data show that compromised DNA repair hampers TCR- and IL-2-mediated T-cell proliferation. Reduced expression of the IL-2 receptor (IL-2R) chains CD25 and CD122 may explain reduced IL-2-mediated proliferation (Nakamura et al., 1994; Liao et al., 2013). Indeed, memory Th and Tc cells of *Ercc1*$^{-/\Delta 7}$ mice showed reduced CD122$^+$ cell frequencies and expression levels. Moreover, *Ercc1*$^{-/\Delta 7}$ Tregs and Tc cells showed reduced upregulation of CD25 expression after stimulation. Compromised DNA repair did not impact all T-cell stimulatory pathways since co-stimulation via CD28 in the presence of CD3 stimulation could trigger proliferation. This was in contrast to findings in WT aged mice as these mice show impaired proliferation in response to CD28 and CD3 stimulation. Together, our findings indicate that defects in IL-2-mediated T-cell proliferation observed with age can be attributed to compromised DNA repair. Conversely, compromised DNA repair did not hamper CD28-mediated T-cell proliferation. Therefore, impaired CD28-mediated proliferation likely develops via a mechanism other than *Ercc1*$^{-/\Delta 7}$-mediated compromised DNA repair.

As mTORC1 negatively interferes with the ATM checkpoint that promotes DNA damage repair (Shen and Houghton, 2013), the mTOR pathway may be linked to DNA damage response

signaling (Xie et al., 2018), and eRapa reduces aging-related phenotypical T-cell changes in WT mice (Hurez et al., 2015) we investigated whether rapamycin could slow down the aging-related T-cell changes imposed by compromised DNA repair. *In vivo* treatment with eRapa did not slow down the accumulation of Tregs in *Ercc1*$^{-/\Delta 7}$ mice, suggesting that compromised DNA repair promotes the accumulation of Tregs independent of mTOR. Our findings concur with a previous study in eRapa-fed WT mice that shows no change to Treg numbers (Hurez et al., 2015). In contrast, Neff et al. report a decrease in Treg numbers after eRapa supplementation (Neff et al., 2013). These conflicting results may be explained by the difference in Treg characterization. Whereas we and others (Hurez et al., 2015) characterize Tregs by expression of the Treg master transcription factor FoxP3 (Hori et al., 2003), Neff et al. assessed Tregs as CD25$^+$ cells without including FoxP3 (Neff et al., 2013). WT aging lowers the frequency and expression of CD25 on FoxP3$^+$ Tregs (Nishioka et al., 2006; Pieren et al., 2019), which we also observed in *Ercc1*$^{-/\Delta 7}$ mice. Therefore, characterization of Tregs based on CD25 only may underestimate the total amount of Tregs present.

eRapa could not prevent decreased proliferation of T cells we observed in *Ercc1*$^{-/\Delta 7}$ mice. These findings are in contrast to previously observed findings in eRapa-fed WT mice, where eRapa improved T-cell proliferation (Hurez et al., 2015). An explanation might be that we used whole spleen cultures that allow interactions between different subsets of T cells, whereas Hurez *et al.* studied an isolated subfraction of CD4$^+$ T cells. Our spleen cell cultures included eRapa-induced CD25$^+$ and PD-1$^+$ memory Tregs. CD25$^+$ and PD-1$^+$ memory Tregs of WT aged mice have been shown to comprise a Treg subset with enhanced suppressive capacity (Elyahu et al., 2019), which may suggest suppression of Th- and Tc-cell proliferation in our cultures by these eRapa-induced memory Tregs. Generation of CD25$^+$ and PD-1$^+$ memory Tregs by eRapa may be explained by upregulation of the memory marker CD44 on naive Tregs, since *in vivo* treatment with rapamycin can induce expression of CD44 and PD-1 FoxP3$^+$ Tregs (Chaoul et al., 2015). Alternatively, memory FoxP3$^-$ CD4$^+$ T cells may have upregulated FoxP3 following eRapa. However, this is less likely as Foxp3$^-$ effector T cells and CD4$^+$CD25$^-$ cells exposed *in vitro* to rapamycin do not develop into FoxP3$^+$ Tregs (Battaglia et al., 2005; Charbonnier et al., 2019). Future studies on the effects of eRapa on proliferation of isolated T-cell fractions and whole-cell cultures will be important to provide full insight in the immune modulatory properties of eRapa.

Our data show that compromised DNA repair contributes to increased frequencies of memory T cells, but not to the extent found in WT aged mice. Since antigens are a major driving force behind the formation of memory T cells (Farber et al., 2014), the higher level of inflation of memory T cells found in WT aged mice is likely explained by the higher antigenic exposure over their longer lifetime. These findings indicate that compromised DNA repair partly contributes to aging-related phenotypic T-cell alterations and that aging of the T-cell pool is a diverse process that is driven by multiple factors in addition to DNA-damage. Interestingly, eRapa decreased the frequency of memory T cells found in *Ercc1*$^{-/\Delta 7}$ mice at the benefit of a rise in the proportion of naive T cells, which is consistent with a previous report in WT mice (Hurez et al., 2015). Since higher frequencies of naive cells at young age are linked to better T-cell mediated protection, we predict that the eRapa-induced rise of the naive T cell frequency may contribute to improved T-cell mediated protection. This is supported by studies showing rapamycin improved T-cell function against pathogens (Ferrer et al., 2010) and antigen-specific T-cell responses (Araki et al., 2009).

Although our data indicate that compromised DNA damage repair contributes to aging of the immune system, there are limitations regarding our study. First, although *Ercc1*-deficient mice show compromised DNA repair in at least four repair pathways (Vermeij et al., 2016), it does not account for deficiencies in other DNA-repair pathways, such as nonhomologous end-joining or base-excision repair (Hoeijmakers, 2009). It is therefore possible that defects in DNA-repair mechanisms other than those mediated by *Ercc1*-deficiency have their own characteristic impact on aging-related T-cell changes. Second, although endogenous nuclear DNA damage accumulation in *Ercc1*$^{-/\Delta 7}$ mice has been confirmed in liver and kidney cells (Robinson et al., 2018), it is unknown whether T cells of *Ercc1*$^{-/\Delta 7}$ mice also accumulate DNA damage. Therefore, it remains to be established whether T-cell changes we ascribe to *Ercc1*-deficiency are due to T-cell intrinsic accumulation of DNA damage, or due to DNA damage present in the microenvironment these T cells reside in. Additionally, the inflammatory profile affecting T cells might differ between normal aging by response to antigen exposure over time, senescence-driven aging, and DNA damage-accelerated aging. Finally, we used a 42 ppm eRapa dose in our *in vivo* experiments as this dose was previously reported to further expand the health- and lifespan of WT mice compared to lower eRapa dosages (Miller et al., 2014). Compared to other studies investigating the effect of eRapa on T-cell phenotype and function in WT mice (Neff et al., 2013; Hurez et al., 2015), our dose is 3-fold higher. As different doses of rapamycin show different outcomes of lifespan in WT mice (Harrison et al., 2009; Neff et al., 2013), it is likely that changes within the T-cell population are also dose-dependent. Additionally, to what extent the 42 ppm dose of eRapa inhibits mTORC1 and mTORC2 signals remains unknown and warrants future immune studies, although recently, we did show suppression of the mTORC1 downstream effector pS6 in *Ercc1* mice by this dose of eRapa (Birkisdottir et al., 2021).

Our study uses a limited set of markers to identify T-cell subsets that may be affected. We focused on some basic aging-related T-cell subsets we and others previously identified to be subject to aging. However, the complex immune system expresses a plethora of other aging-related markers and subsets of cells that may be affected by *Ercc1*-deficiency. Thus, our data warrant future studies to unravel the impact of deficiencies of DNA-repair mechanisms on the many other aging-related molecules and T-cell subsets as well as other leukocyte subsets. For instance, markers such as CD62L that identify different memory subsets, could shed a light on central memory, effector memory, or virtual memory T-cells as well as Treg subsets. In addition, although the CD3$^+$CD4$^-$ population is a useful proxy for the analyses CD8$^+$ T cells (without labeling CD8) that is not impacted

by aging in our studies, the work we present here does not fully exclude that also minor populations of $CD4^{-}CD8^{-}$ or $CD4^{+}CD8^{+}$ T cells may be affected by *Ercc1*-deficiency. Lastly, the *Ercc1* model does not allow a fair comparison of absolute T-cell subset numbers between $Ercc1^{-/\Delta 7}$ and $Ercc1^{+/+}$ mice, as the body and organ weights of $Ercc1^{-/\Delta 7}$ mice are significantly lower (Dolle et al., 2011). Smaller sizes of the spleen and the thymus in $Ercc1^{-/\Delta 7}$ mice may potentially influence the output, homeostasis, and differentiation of T cells. However, the size of spleen and thymus relative to total body weight is comparable between $Ercc1^{-/\Delta 7}$ and $Ercc1^{+/+}$ mice and thymic involution rates appear similar between these genotypes (Dolle et al., 2011), which may suggest minimal influence of smaller organ size on T-cell subsets.

Collectively, this study reveals a novel and pivotal role for compromised DNA repair in promoting accumulation of Tregs with an aging-related phenotype. Although smaller mTOR-mediated effects by eRapa may be missed by the relatively small group sizes in our study, compromised DNA repair appears to impose accumulation of naive Tregs through a mechanism that may be independent of mTOR activation. Our study indicates that preventing DNA-damage over the course of life may help to prevent accumulation of immunosuppressive Tregs hampering protective immunity at old age. Moreover, our study warrants further studies of biological processes that may underlie aging-related immune defects in order to better understand the process of aging.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethical Committees of: National Institute for Public Health and the Environment (RIVM) (DEC no. 201400042) Erasmus Medical Center (DEC no. 139-12-13).

AUTHOR CONTRIBUTIONS

DP, NS, WV, MD, and TG designed the study. DP, NS, SI, BN, CV, RB, and TG performed experiments. DP and NS analyzed the data. DP, NS, WV, MD, and TG interpreted the data. DP and TG DP, WV, MD, and TG edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fragi.2021.667193/full#supplementary-material

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MINI REVIEW
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Induction of T Cell Senescence by Cytokine Induced Bystander Activation

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As people around the world continue to live longer, maintaining a good quality of life is of increasing importance. The COVID-19 pandemic revealed that the elderly are disproportionally vulnerable to infectious diseases and Immunosenescence plays a critical role in that. An ageing immune system influences the conventional activity of T cells which are at the forefront of eliminating harmful foreign antigens. With ageing, unconventional end-stage T cells, that exhibit a senescent phenotype, amass. These senescent T cells deviate from T cell receptor (TCR) signaling toward natural killer (NK) activity. The transition toward innate immune cell function from these adaptor T cells impacts antigen specificity, contributing to increased susceptibility of infection in the elderly. The mechanism by which senescent T cells arise remains largely unclear however in this review we investigate the part that bystander activation plays in driving the change in function of T cells with age. Cytokine-induced bystander activation may offer a plausible explanation for the induction of NK-like activity and senescence in T cells. Further understanding of these specific NK-like senescent T cells allows us to identify the benefits and detriments of these cells in health and disease which can be utilized or regulated, respectively. This review discusses the dynamic of senescent T cells in adopting NK-like T cells and the implications that has in an infectious disease context, predominately in the elderly.

Keywords: ageing, immunosenecence, bystander activation of T cells, senescent T cells, NK-like CD8(+) T cells

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INTRODUCTION

Until recently, T cells were considered to only differentiate towards an end-stage through repeated life-long antigen activation, for example, by persistent viruses such as cytomegalovirus (CMV) (Vasto et al., 2007; Fulop et al., 2013; Tu and Rao, 2016). These end-stage T cells, that exhibit characteristics of senescence, accumulate during ageing and have been shown to exhibit low proliferative activity after activation and were therefore considered to be dysfunctional (Deursen, 2014). It was assumed therefore, that age-associated decline of immunity or immune senescence, was attributed in part to the development of deficient T cell function over time. However instead of being dysfunctional, senescent-like T cells, especially within the CD8 compartment, lose key components of their T cell receptor (TCR) signalling apparatus while concomitantly acquiring Natural Killer (NK cell) characteristics (Pereira et al., 2020). This indicates that as T cells differentiate towards a proliferative end-stage, they acquire a new functional perspective that is independent of their antigen specificity. Therefore, T cell function is re-focussed rather than lost during ageing and in this review, we discuss that antigen-independent bystander activation of T cells by cytokines may drive

senescence as well as expression of NK-like activity in T cells. We also explore potential beneficial and immune-pathogenic roles for these cells in health and disease, especially those that induce intense inflammatory responses.

THE PHENOTYPIC AND FUNCTIONAL CHARACTERISTICS OF SENESCENT T CELLS

Although end-stage T cells exhibit many characteristics of senescence, they are not senescent by classical definition as they can be re-induced to proliferate by blocking key signalling pathways (Goronzy and Weyand Nat Rev. Immunol. 2020). Nevertheless, in this review we refer to end-stage T cells as senescent T cells for simplicity. Senescent T cells in both the CD4 and CD8 compartments can be identified and isolated by the loss of cell surface expression of co- stimulatory molecules such as CD27 and CD28 while acquiring the expression of KLRG1 and CD57 (Covre et al., 2020). In addition, these cells have short telomeres, low telomerase activity, low proliferative capacity and express senescence related molecules including Atm, γH2AX, the cyclin inhibitor p16, sestrins, and p38 MAP kinase (Pereira et al., 2020). These senescent T cells are also characterised by their high levels of expression of senescence-associated β-galactosidase (SA-βGal) activity that can be measured by flow-cytometry (Martinez-Zamudio et al., 2021). Senescent T cells can also re-express CD45RA and despite the loss of proliferative capacity, these cells have potent cytotoxic activity and secrete pro-inflammatory cytokines such as TNFα and IFNγ after activation (Akbar et al., 2016; Pereira et al., 2020).

SENESCENT T CELLS ACCUMULATE DURING AGEING AND IN INFLAMMATORY DISEASES

Senescent T cells increase during ageing, due in part to responses to lifelong infection with viruses such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) (Nikolich-Žugich J, 2018). An abundance of senescent T cells that have pro-inflammatory potential are found in age-related diseases including rheumatoid arthritis (Goronzy et al., 2013; weyand et al., 2017), Alzheimer's Disease (Panossian et al., 2002; Franco et al., 2006; Gate et al., 2020), and cardiovascular diseases (Yu et al., 2015; Youn et al., 2019). Senescent T cells correlate with lesion size in cutaneous leishmaniasis (CL), a disease characterized by intense inflammatory responses and the formation of destructive cutaneous lesions (World Health Organization/Department of control of neglected tropical diseases, 2015; Covre et al., 2019). During acute infection with *L. braziliensis* senescent CD4 and CD8 T cells home to the skin by upregulating the skin homing receptor CLA (Covre et al., 2019). There, these senescent T cells can initially control the infection. However, the potent inflammatory activity of senescent T cells also contributes to the development of skin pathology in CL, even after parasites have been cleared (Covre et al., 2020). Senescent T cells can also infiltrate and accumulate in the kidneys of patients with Systemic Lupus Erythematosus (Tahir et al., 2015). It is possible therefore, that the inflammatory potential of senescent T cells may contribute to the immunopathology that is associated with inflammatory diseases (Covre et al., 2020). As senescent T cells may also associate with inflammatory diseases, understanding how these populations are generated and how their functional activity is controlled is vital to clarify how these cells may switch from being beneficial to becoming detrimental for health.

SENESCENT T CELLS ACQUIRE CHARACTERISTICS OF NATURAL KILLER CELLS

Optimal T cell activation requires co-stimulatory signals *via* CD28 and CD27 and the loss of these cell surface receptors by senescent T cells suggests their decreased ability for T cell activation (Pereira et al., 2020). In addition, senescent $CD4^+$ T cells lose expression of key components of the TCR signalosome such as LCK, LAT, and phospholipase C gamma (PLC- γ) (Lanna et al., 2014; Lanna et al., 2017; Pereira et al., 2020) that underscores their hypo- responsiveness to TCR signaling. Despite this loss, these cells express elevated levels of cytotoxic granules and perforin and secrete abundant levels of pro-inflammatory cytokines after activation (Akbar et al., 2016). The senescence program in $CD4^+$ and $CD8^+$ T cells is regulated by stress sensor molecules known as the sestrins (Lanna et al., 2017; Pereira et al., 2020). Sestrin expression in T cells is induced by signalling processes that may arise from either DNA damage or nutrient deprivation (Lanna et al., 2017).

Interestingly, senescent but not non-senescent T cells that express sestrins also express both stimulatory and inhibitory NK receptors and acquire the ability to perform TCR independent functional responses (Pereira et al., 2020). The function of activating (NKG2D) and inhibitory (NKG2A) on senescent $CD8^+$ T cells has been demonstrated previously (Pereira et al., 2020) and these cells can also express additional killer inhibitory receptors (KIR) but their function on senescent $CD8^+$ T cells has not been investigated (Pereira et al., 2020). Sestrin inhibition restores TCR signalosome expression, proliferative and telomerase activity as well as re-inducing the expression of CD28 (Lanna et al., 2017; Pereira et al., 2020) while concomitantly decreasing both NKG and KIR receptor expression by these T cells (Pereira et al., 2020). Therefore, senescent T cells exhibit a reversible senescence program but can be induced to exhibit preferentially T cell or NK related functional activity by selective sestrin inhibition. The NK-like function of TEMRA cells is likely to be regulated by a balance of stimulatory and inhibitory NKR signalling.

The biological significance of the acquisition of NK receptors and functions by senescent T cells is unclear but there is an expansion of these cells in older individuals and especially in centenarians (Derhovanessian et al., 2013; Pawelec, 2014; Michel et al., 2016) suggesting that this may be a beneficial adaptation to ensure broad and rapid effector function during ageing. The

potential beneficial and detrimental consequences of bystander activation of T cells have been discussed in excellent previous reviews (Kim and Shin, 2019). The reprogramming of senescent T cells from TCR to NKR functional activity could allow $CD8^+$ T cells to specifically target persistent pathogens including CMV and the EBV and also malignant cells during ageing (Khan et al., 2004; Jackson et al., 2017; Pawelec et al., 2017).

THE INDUCTION OF SENESCENCE CHARACTERISTICS IN T CELLS BY CYTOKINES

Surprisingly a literature survey suggests that a large proportion of senescent T cells may accumulate because of bystander activation by cytokines and not from antigen-specific stimulation *in vivo*. In this regard, type-1 interferons (IFN-1) plays a critical role in driving T cell senescence. Cytomegalovirus (CMV)-specific $CD4^+$ T cells in healthy donors exhibit a greater extent of a senescent phenotype compared to other virus-specific T cells populations in the same individuals (Fletcher et al., 2005). This may be due in part to the ability of CMV to activate the secretion of large quantities of IFN-α by plasmacytoid dendritic cells, which inhibits telomerase activity and the loss of costimulatory molecules by activated $CD4^+$ and $CD8^+$ T cells *in vitro* (Fletcher et al., 2005; Lanna et al., 2013). Observations in hepatitis C virus-infected individuals on IFN-α therapy support this theory as they develop increased proportions of circulating $CD8^+$ T cells with a $CD28^-$ phenotype (Manfras et al., 2004). Interestingly, TNF-α is also induced at high concentrations *via* CMV stimulation and this cytokine can also downregulate co-stimulatory molecule, i.e., CD28 (Billadeau et al., 2003). IFN-α may also contribute to telomerase inhibition and telomere erosion in $CD4^+$ T cells during recall antigen challenge in the skin *in vivo* (Reed et al., 2004). The possible mechanisms for inhibition of telomerase activity by this cytokine include the reduction of transcription and translation of human telomerase reverse transcriptase (hTERT) in stimulated $CD8^+$ T cells (Lanna et al., 2013) and also the downregulation of NF-kB which drives hTERT transcription (Lanna et al., 2013). In other studies, inactivation of the IFN pathway has been found to extend the lifespan of *Terc* knockout mice indicating that DNA-damage response- induced IFN signalling as a key mechanism that links senescence and premature ageing (Yu et al., 2015). However, it is not clear at present if these cytokines regulate sestrin expression by senescent T cell populations.

Of note, IFN-1 signalling induces extensive T cell proliferation *in vivo* during infection with lymphocytic choriomeningitis virus (LCMV) (Tough et al., 1996; Dumont et al., 1986). Therefore, during viral infection, a proportion of T cell proliferation may be induced by bystander activation by IFN-1 (Buchmeier et al., 1980). Crucially, IFN-1 may not directly stimulate the proliferation (Zhang et al., 1998) but instead induce IL-15 that drives the proliferative activity in $CD8^+$ T cells (Zhang et al., 1998; Tough et al., 2000). Memory $CD8^+$ T cells may also be activated in a TCR—independent manner by other cytokines including IL-12 (Xiao et al., 2009), IL-18, or a combination of these (Lotze et al., 1981; West et al., 2011; Newby et al., 2017). In addition to the ability of IL-15 to induce proliferation after antigen stimulation *in vivo* (Kim and Shin, 2019), it can also induce NK activity in T cells (Kennedy et al., 1998; Castillo et al., 2012). This suggests that there may be interplay between cytokine-induced T cell senescence (through extensive proliferation) and NK receptor expression by these cells. Collectively these observations suggest that senescent T cells may also arise from bystander activation and not only from repeated antigen-specific T cell stimulation *in vivo*.

THE INDUCTION OF NATURAL KILLER RECEPTORS AND FUNCTION IN T CELLS BY CYTOKINES

In 1985, Rosenberg and colleagues discovered that high-dose IL-2 incubation induces NK activity in $CD8^+$ T cells, NK cells and invariant NK cells (Rosenberg et al., 1985; West et al., 2011). This was the beginning of the era of lymphokine (cytokine)-induced killer cells (CIK) (Fagan and Eddleston, 1987; Hartwing and Korner, 1990). More recently, CIK cells have been shown to be end-stage $CD8^+$ T cells that are derived from a $CD3^+CD56^-CD8^+$ T cell population (Franceschetti et al., 2009). These cells express polyclonal TCR Vβ chains and acquire the expression of CD56, NKG2D and exhibit granular lymphocyte morphology. The cytokine activated killer cells have a $CD45RA^+CCR7^-$ phenotype that is congruent with terminally differentiated (senescent) human memory T cells that re-express CD45RA (T_{EMRA}) (Franceschetti et al., 2009). This supports the possibility that bystander activation of T cells may induce both senescence as well as NK receptor expression, but it is not clear if both outcomes are induced by the same mechanism.

IL-15 (but not IL-18) has been shown to confer NKG2D-mediated cytolytic function upon memory $CD8^+$ T cells (Tough et al., 1999; Bastidas et al., 2014). NKG2D in $CD8^+$ T cells can mediate costimulatory activity in the presence of TCR engagement (Groh et al., 2001: Jamieson et al., 2002; Roberts et al., 2001; Maasho et al., 2005). However, NKG2D signaling can also occur in the absence of TCR engagement (Lanier et al., 2015). In freshly isolated $CD8^+$ $TCR\alpha\beta^+$ intraepithelial lymphocytes (IELs) obtained from patients with active celiac diseases or IELs preactivated with IL-15, the cells displayed NKG2D-mediated cytotoxicity without TCR interaction (Roberts et al., 2001; Meresse et al., 2004). More recently, Hepatitis A virus (HAV)-infected cells obtained from acute hepatitis A (AHA) patients produced IL-15 which drove TCR-independent activation of memory $CD8^+$ T cells via upregulation of NK receptors, NKG2D and NKp30 (Kim et al., 2018). IL-15 signalling stimulates the upregulation of NKG2D expression directly (Roberts et al., 2001; Meresse et al., 2004; Correia et al., 2011; Kim et al., 2018). However, NKG2D expression in bystander-activated $CD8^+$ T cells is likely to be regulated through the coordinated interaction of many pro-inflammatory cytokines such as IFN-1 and IL-15, and TCR signalling (Kim and Shin, 2019).

IL-15 has also been found to drive the expression of NKG2A on mature T cells (Ponte et al., 1998; Mingari et al., 1998). NKG2A is a killer inhibitory receptor that commonly acts to block NK cell activity. However, NKG2A can also exert an inhibitory effect on TCR-mediated function on cytotoxic T cells. Therefore, the extent to which NKG2D or NKG2A is expressed by T cells or the differential expression of their ligands on target cells may potentially dictate whether there will be activation or inhibition of cytotoxicity.

THE NATURAL KILLER RECEPTOR SIGNALLING COMPLEX IN SENESCENT CD8$^+$ T CELLS

An important question is how signalling pathways in senescent CD8$^+$ T cells are re-wired to enable NK-like cytotoxic activity. Expression of NKG2D on the cell surface requires its association with adaptor proteins that stabilize the immunoreceptor complex and provide it with signalling activity (Franceshetti et al., 2009; Pereira et al., 2020). Immunoprecipitation studies showed that NKG2D associates with both the adaptor molecules DAP10 and DAP12. DAP10 contains a YxxM motif that activates PI3K leading to co-stimulation after TCR activation (Leong et al.: Gibbert et al., 2012), while DAP12 has an ITAM-motif that can recruit and activate Zap70-Syk to trigger cytokine release and cytotoxicity (Dunn et al., 2007; Zhu et al., 2008; Micco et al., 2013). Previous studies showed that in human CD8$^+$ T cells, NKG2D is predominantly associated with DAP10 (Dunn et al., 2007; Zhu et al., 2008; Gibbert et al., 2012; Micco et al., 2013; Leong et al., 2014) which allows it to act as a costimulatory signal for the TCR. More recently, expression of DAP12 was found exclusively in senescent CD8$^+$ T cells and was necessary and sufficient to mediate NKG2D- dependent cytotoxicity (Pereira et al., 2020). However, it is not known if DAP12 expression is also regulated by bystander cytokine activation.

Immunoprecipitation and imaging cytometry experiments indicated that DAP12, sestrin 2 and Jnk were co-localized in a complex with NKG2D in senescent but not in non-senescent CD8$^+$ T cells (Pereira et al., 2020). Therefore, senescent T cells express a new signalling complex that include the sestrins that enable them to mediate NKG2D mediated cytotoxicity. However, the development of this new NK complex may be reversible since sestrin blockade can downregulate NKG2D and DAP12 but increase TCR related signalling molecules expression (Pereira et al., 2020). It will be important to determine the relationship between bystander activation, TCR activation and sestrin expression in senescent T cells. It remains to be determined how the sestrins interact with KIR receptors in senescent T cells.

THE RELATIONSHIP BETWEEN SENESCENT T CELLS AND VIRTUAL MEMORY T CELLS

Virtual memory (VM) CD8$^+$ T cells share certain characteristics with senescent CD8$^+$ T cells. VM CD8$^+$ T cells constitute up to 20% of the CD8 T cell population in murine lymphoid organs (Hogquist et al., 2014). These cells are generated by homeostatic mechanisms in response to self-antigens as well homeostatic cytokines, i.e., IL-7 and IL-15 (Hogquist et al., 2014). One of the key differences between VM cells and "true" memory T cells is that the former arise in germ free (GF) as well as "antigen free" mice, (Haluszczak et al., 2009), a situation that is unlikely to occur in humans. Comparably to senescent (TEMRA) CD8$^+$ T cells (Callender et al., 2018), VM T cells have diminished proliferative potential in aged mice and humans, although they are highly proliferative in young individuals (Quinn et al., 2018). Interestingly, VM T cells accumulate during ageing and molecular analysis has revealed that aged VM T cells present a profile consistent with cellular senescence including reduced proliferative capacity, dysregulated MAPK signaling, and an elevated survival *in vitro* and *in vivo* with elevated expression of Bcl-2 (Quinn et al., 2018; Quinn et al., 2019). In mice, VM cells are characterized as CD44^{high}CD122^{high}CD49a^{lo} and EOMES positive (Haluszczak et al., 2009). Human VM T cells have not been well-defined, however like senescent T cells, they may arise as a result of bystander activation by cytokines (e.g. IL-15) (White et al., 2016) express NK receptors including NKG2A and KIR (Jacomet et al., 2015), accumulate with age, and show features of senescence (Quinn et al., 2018). This may indicate that VM T cells are a subset of or perhaps equivalent to the senescent CD8$^+$ (TEMRA) cell population, especially in older individuals.

EVIDENCE FOR BYSTANDER ACTIVATION OF HETEROLOGOUS T CELLS DURING VIRAL SPECIFIC IMMUNE RESPONSES

These observations raise the possibility that there is bystander activation of T cells (**Figure 1**) by cytokines during specific immune responses to viruses *in vivo*. During acute hepatitis A virus (HAV) infection, IL-15 instead of the TCR activates CD8$^+$ T cells that are specific for pathogens other than HAV itself, including human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), influenza A virus respiratory syncytial virus, and vaccinia virus (Kim et al., 2018). As described above, the secretion of IL-15 could be secondary to the production of IFN-1 during the infection. The bystander activated CTLs in HAV infected individuals express high levels of cytotoxic molecules, i.e., NKG2D and GZMB (Kim et al., 2018). Bystander activation induced by IL-15 has also been indicated during human immunodeficiency virus (HIV) infection (Bastidas et al., 2014). The T cell repertoire of untreated HIV-infected patients showed that the TCR diversity of cycling effector memory CD8$^+$ T cell reflects that of the complete effector memory CD8$^+$ population and the activation of these cells is driven by non-specific activation (Younes et al., 2016). It has also been shown that bystander activation of CD8$^+$ T cells occurs in the primary stages of HIV infection and activation markers, CD38 and HLA-DR, are upregulated in total CD8$^+$ T cells and notably in the CD8$^+$ T cells specific for HIV-unrelated viruses, i.e., HCMV, EBV, respiratory syncytial virus, and lymphadenopathy associated virus (LAV) (Dalod et al., 1999;

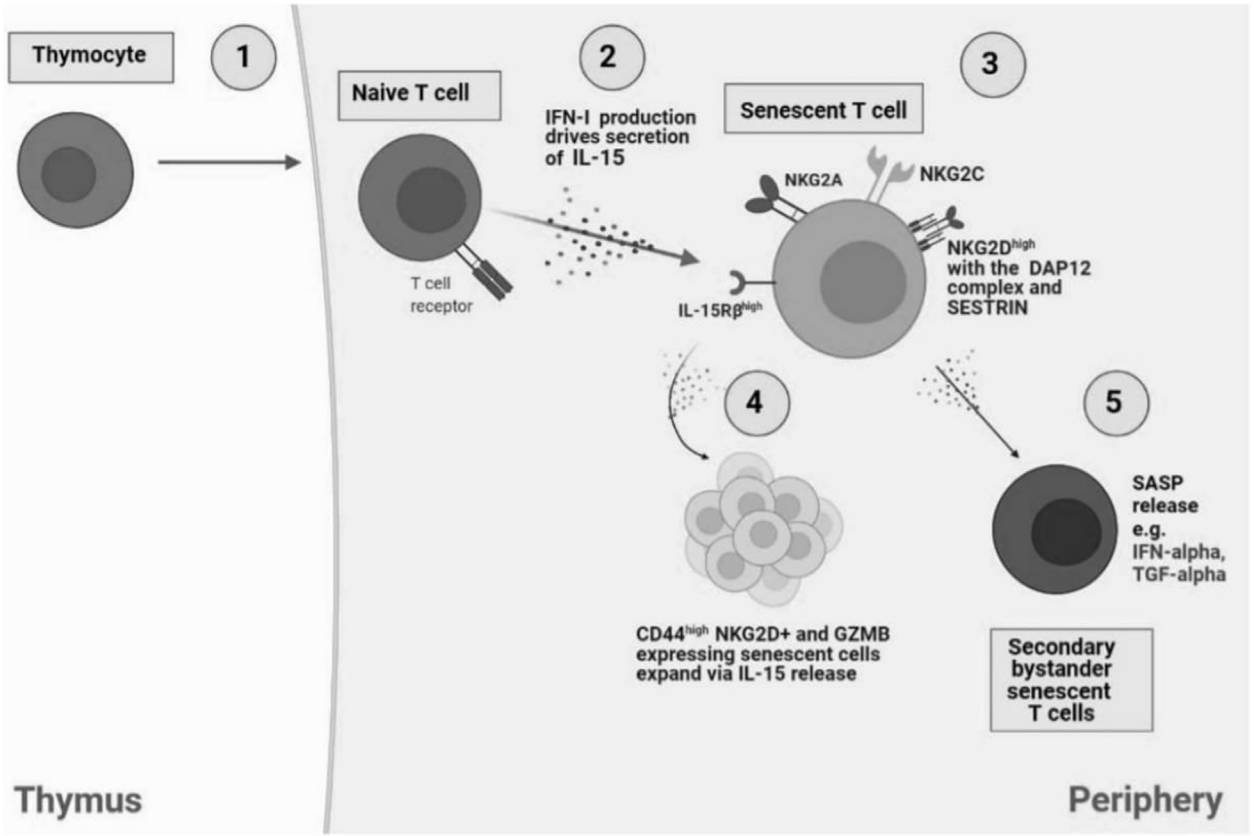

FIGURE 1 | Bystander activation of NKR expressing senescent T cells. 1) In the thymus, IL-14 release from thymocytes drives cell differentiation into innate CD8 single positive thymocytes which migrate to the periphery to generate into memory T cells. However, naïve CD8+ T cells have the capacity to differentiate to senescent T cells in an antigen-independent manner, through cytokine stimulation, also termed bystander activation. 2) Nalve T cells produce IFN-1 which drives IL-15 secretion in neighbouring immune cells and the subsequent establishment of senescent T cells. 3) Senescent T cells express NK receptors including NKG2A, NKG2C, and notably NKG2D with the adaptor molecule, DAP12, driven by SESTRIN. 4) IL-15 secretion drives the expansion of NKG2D and CD44+ expressing senescent cells. 5) SASP release by senescent T cells, i.e., IFN-α and TGF-α can drive generation of secondary bystander senescent T cells.

Doisne et al., 2004; Kohlmeier et al., 2011). Other viral infections including Hepatitis B and influenza A also induce extensive bystander activation of T cells (Kim and Shin, 2019). This highlights the severity of antigen-independent activation of CD8 T cells in contributing to immunopathology. Therefore, it is important to further understand cytokine bystander activated T cells and how they may be regulated as a potential therapeutic to alleviate severe iimmunopathology during viral diseases.

THE INDUCTION OF NATURAL KILLER LIGAND EXPRESSION ON NON-T CELLS BY CYTOKINES

While bystander cytokines can induce NKG2D expression in T cells, it begs the question of whether they can also induce the expression of NK ligands on different cell types. This could be a potential mechanism for non-specific tissue damage that leads to immunopathology. IFN-1 has been shown to induce the expression of MHC class I polypeptide–related sequence A (MICA) and UL16 binding protein (ULBP2) molecules that are MHC-related molecules and can both bind to and activate cells by NKG2D (Katlinskaya et al., 2015). Muscle biopsy specimens acquired from polymyositis (PM) patients show an upregulation of MICA/B correlating with IL-15 expression by muscle cells and the identification of $CD8^+$ $NKG2D^+$ cells within inflammatory infiltrates (Ruck et al., 2015). This suggests that NKG2D/IL-15 signaling pathway may induce muscle destruction in patients *via* $CD8^+$ $NKG2D^+$ cells targeting the MICA/B ligands that are upregulated by IL-15. Respiratory syncytial virus (RSV) can raise IL-15 production levels in respiratory epithelial cells *via* the NFkB pro-inflammatory pathway (Zdenrghea et al., 2012). This in turn leads to an upregulation of MICA on the surface of epithelial cells which can be eliminated *via* NK cell killing (Zdenrghea et al., 2012). In another study, IFN-y was shown to upregulate the expression of MICA on the surface of human corneal epithelial cells (HCEC) that led to enhanced HCEC apoptosis *via* NKG2D presenting NK and $CD8^+$ T cells (Wu et al., 2018). Furthermore, it has been shown that TNFα and IL-1β promote MICA expression on endothelial cells surface (Chaeuveau et al., 2014). Several key pathways are found to enhance MICA expression after cytokine treatment including

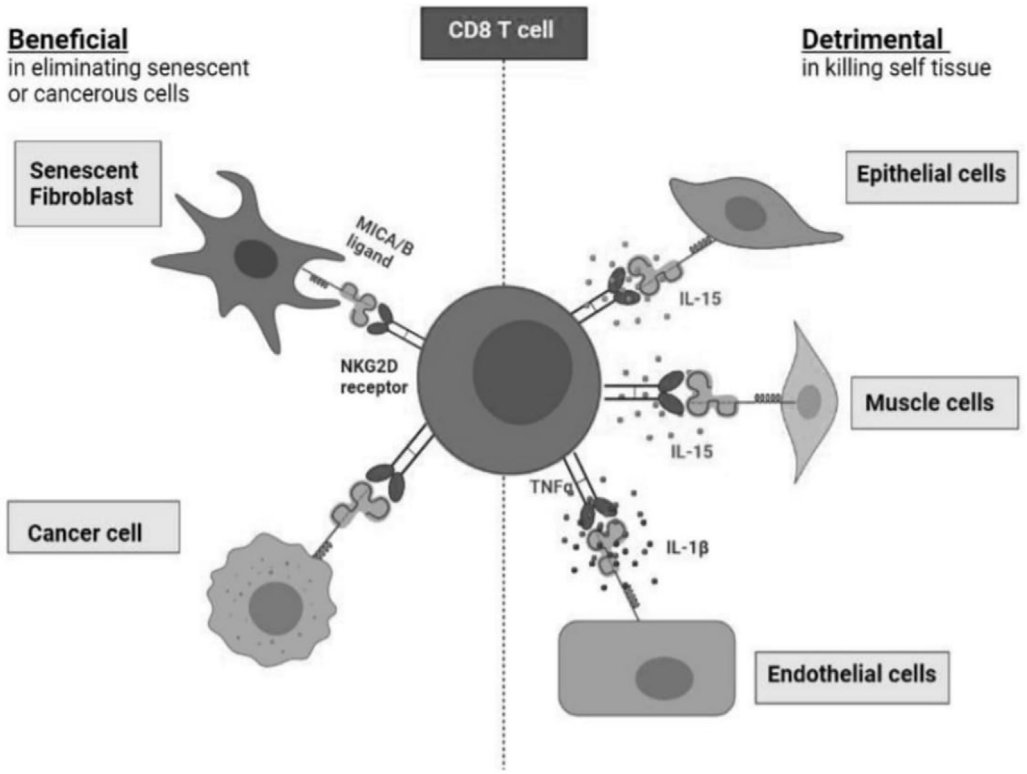

FIGURE 2 | The benefits and detriments of NKR expression on healthy $CD8^+$ T cells. Healthy $CD8^+$ T cells expressing NKRs harness cytotoxic targeting of many cell types. NKR expression allows for the elimination of senescent fibroblasts as well as cancer cells which present the MICA/B ligands. However, NKR expression on $CD8^+$. T cells can drive healthy tissue destruction *via* targeting of endothelial cells, muscle cells, and epithelial cells which also present MICA/B which is facilitated by various cytokines: IL-15, TNFα and IL-1β.

the NFkB and mitogen-activated protein kinase pathways JNK, ERK1/2, and p38 (Fitau et al., 2006; Lin et al., 2012; Chaeuveau et al., 2014). Collectively these observations suggest that bystander activation and inflammation in tissues may both upregulate NK ligands on certain cell types and in certain circumstances although the mechanism for this is unknown at present.

THE IMMUNOSURVEILLANCE OF SENESCENT CELLS IN TISSUES

NK cells provide broad antigen independent protection against malignant and infected cells (Paul and Lal, 2017). It is likely that senescent T cells, especially within the CD8 compartment, may have a similar protective role (Pereira et al., 2020) (**Figure 2**). In addition to this, both NK cells and $CD8^+$ T cells can also recognize and eliminate senescent non-lymphoid cells in tissues (Covre et al., 2020). Senescent non-lymphoid cells increase in all tissues during ageing and these populations can be recognised and eliminated by cells of the immune system, including senescent T cells (Covre et al., 2020). Different immune cell types including macrophages, neutrophils, natural killer (NK) cells, $CD8^+$, and $CD4^+$ T cells have been implicated in the surveillance of senescent tissue cells (Heonicke et al., 2012; Burton et al., 2018; Pereira et al., 2019).

Senescent cells become immunogenic by expressing stimulatory ligands like MICA/B that bind to NKG2D and activate NK cells killing (Gasser et al., 2005; Krizhanovsky et al., 2008; Sagiv et al., 2016). Moreover, by secreting cytokines, senescent cells can recruit immune cells into tissues and enable senescent cell clearance (Iannello et al., 2013). Functionally, we have shown that both NK and $CD8^+$ T lymphocytes can target senescent cells through a NKG2D-dependent mechanism. Despite expressing high levels of NKG2D, the simultaneous presence of the inhibitory receptor NKG2A on senescent NK and $CD8^+$ T cells may compromise their ability to eliminate senescent tissue cells. There is therefore a balance between activating and inhibitory receptor signalling on the same cell that has to be taken into consideration (Pereira et al., 2019). The expansion of $NKG2A^+$ $CD8^+$ T cells with age may explain why the immune system is less effective at eliminating senescent cells in old subjects, thus allowing them to accumulate compared to young individuals (Pereira et al., 2019).

However, NKR expression on $CD8^+$ T cells can drive healthy tissue destruction *via* targeting of endothelial cells (Chauveau et al., 2014), muscle cells (Ruck et al., 2015), and epithelial cells (Zdrenghea et al., 2012; Wu et al., 2018) which also present

MICA/B which is facilitated by various cytokines: IL-15, TNFα, IFN-γ, and IL-1β.

SENESCENT T CELLS IN HEALTH AND DISEASE

In this article we have discussed data suggesting that bystander activation by cytokines during immune responses can induce senescence in T cells that are unrelated to the infectious agent. This bystander activated (senescent) T cells are also induced to express NK receptors by these cytokines. The NK ligands on tissues can be themselves induced by inflammatory cytokines or are also constitutively expressed on senescent cell populations. While the senescent T cell population may have a broad protective role against tumours and infected cells, they may also be able to recognize and eliminate senescent cells from tissues. Previous studies indicate that the elimination of senescent tissue cells promotes health and alleviates age-related pathology (Baker et al., 2011). In these situations, bystander activation induced senescent T cells may promote health. However, in situations where there is extensive inflammation, these cells may have a destructive role because the cytokines would induce NK ligand expression on tissues. In this regard, persistent activation during the acute stage of leishmaniasis drives T cell senescence and homing of these cells to the skin. These senescent T cells secrete pro-inflammatory cytokines, exhibiting a senescence-associated secretory phenotype (SASP) that generates exacerbated inflammation. The exaggerated inflammation contributes to the progression of skin lesions and tissue destruction which has been shown in both mucosal leishmaniasis (ML) and cutaneous leishmaniasis (CL) patients (Bacellar et al., 2002; Antonelli et al., 2005; Crosby et al., 2014). The proportions of senescent T cells in the circulation and in the skin of these patients correlate with skin lesion size suggesting their contribution to the skin pathology that is observed.

More recently, infection with the SARS-CoV-2 virus that causes severe respiratory disease has led to a world-wide pandemic (coronavirus disease 2019, COVID-19; Merad et al., 2020). This infection mostly induces mild to moderate symptoms in younger individuals but induces devastating morbidity and mortality in some older individuals. A key hallmark of severe COVID-19 is exuberant inflammation in the respiratory tract of patients (Merad et al., 2020). As the proportion of senescent T cells with NK function increase during ageing and there is also extensive inflammation in tissues especially the lungs of the infected patients, the scene may be set where these T cells may recognize and destroy NK-ligand expressing cells in the lung epithelium and endothelial cells in this tissue (Akbar and Gilroy, 2020). A fundamental unanswered question is the nature of the trigger that may induce a potentially beneficial re-structuring of function in T cells during ageing and senescence to become highly destructive. An answer to this question would also be important for a wide range of other disease also where senescent T cells and extensive inflammation co-exist.

AUTHOR CONTRIBUTIONS

ANA conceived the presented idea of the article and AAA investigated and collated the research in support of the research theme, with the encouragement of ANA. ANA verified the research and edited the manuscript. AAA created diagrams to illustrate the findings of the research. ANA supervised the findings of this work. All authors contributed to the final manuscript.

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REVIEW
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Vaccines and Senior Travellers

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Background: International tourist travel has been increasingly steadily in recent years, and looks set to reach unprecedented levels in the coming decades. Among these travellers, an increasing proportion is aged over 60 years, and is healthy and wealthy enough to be able to travel. However, senior travellers have specific risks linked to their age, health and travel patterns, as compared to their younger counterparts.

Methods: We review here the risk of major vaccine-preventable travel-associated infectious diseases, and forms and efficacy of vaccination for these diseases.

Results: Routine vaccinations are recommended for older persons, regardless of whether they travel or not (e.g., influenza, pneumococcal vaccines). Older individuals should be advised about the vaccines that are recommended for their age group in the framework of the national vaccination schedule. Travel-specific vaccines must be discussed in detail on a case-by-case basis, and the risk associated with the vaccine should be carefully weighed against the risk of contracting the disease during travel. Travel-specific vaccines reviewed here include yellow fever, hepatitis, meningococcal meningitis, typhoid fever, cholera, poliomyelitis, rabies, Japanese encephalitis, tick-borne encephalitis and dengue.

Conclusion: The number of older people who have the good health and financial resources to travel is rising dramatically. Older travellers should be advised appropriately about routine and travel-specific vaccines, taking into account the destination, duration and purpose of the trip, the activities planned, the type of accommodation, as well as patient-specific characteristics, such as health status and current medications.

Keywords: older adults, vaccines, travel, immunization, seniors 65 and over

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INTRODUCTION

The number of older travellers is on the rise. The World Tourism Organization estimates that tourist arrivals increased by 4% in 2019, to reach a record 1.5 billion, continuing a trend that has been evident over the last decade (United Nations World Tour, 2021). Many of these travellers are older individuals, and although age in itself is not a contra-indication to travel, there are specificities in older persons that merit consideration in the context of travel. The natural, age-related decline in physiological function known as immunosenescence means that the body is more susceptible to external stressors, and less well able to mount an adequate immune response in case of infection. This phenomenon affects both innate (natural) and adaptive (acquired) immunity and is characterized by

two main features, namely an impaired ability to respond to new antigens, and an impaired ability to sustain memory response after infection (Goronzy and Weyand, 2013). Consequently, older people may be at higher risk of infection, and more likely to experience a severe and/or complicated course of disease. Vaccine responses in older individuals often fail to generate sufficient protection and exhibit reduced efficacy (Allen et al., 2020). For some vaccines, it has been shown that immune responses develop more slowly in older adults [e.g., in hepatitis A (Van Der Meeren et al., 2015) and hepatitis B (Weinberger et al., 2018), among others (Weinberger et al., 2010)]. Therefore, the time between administration of the vaccine and the start of travel is of particular importance for older adults. In parallel, a faster decline in antibody titers is observed with increasing age (Weinberger et al., 2008). It also makes a difference whether primary (first) or booster vaccinations are applied. Kaml et al. demonstrated that booster vaccinations against tetanus, pertussis and diphtheria resulted in a less robust response in subjects over 65 years of age compared to younger individuals, whereas the immune response to all three components of the vaccine was better in those older patients who had been previously vaccinated against Tdap (Kaml et al., 2006). This reinforces the concept of regular booster vaccinations throughout life (Weinberger et al., 2008). There may also be a background of frailty or chronic disease, which raise their own challenges in the context of travel, particularly if traveling to areas where the healthcare system is of a lower standard.

Several diseases that pose a risk to travellers may be effectively prevented by vaccination. However, changing immune response and the presence of comorbid conditions may change the risk-benefit ratio of certain vaccines. We review the risk of major vaccine-preventable travel-associated diseases, and the forms and efficacy of vaccination for these diseases.

ROUTINE VACCINATION AND TRAVEL

Irrespective of travel plans, the vaccine status of older individuals should regularly be monitored and updated, during routine contact with healthcare professionals. Elderly individuals should be advised about the vaccines that are recommended, available and (in many countries) reimbursed for their age group in the framework of the national vaccination schedule. The person's status with regard to diphtheria–tetanus–pertussis (DTP), measles–mumps–rubella (MMR) and herpes zoster vaccination should be investigated, and where possible, recorded in a personal health record, and the patient advised to receive initial vaccination (if not previously immunized) or booster doses, as appropriate. Tetanus in particular is of special relevance for persons planning to travel. Indeed, in a study performed among 331 adults aged 18–60 years attending travel vaccine clinics in France and who had all been vaccinated according to national recommendations, Launay et al. reported that the percentage of subjects with antibodies to tetanus and diphtheria decreased with age (Launay et al., 2009). Similarly, they also reported that the mean time since the last booster dose increased with age, to reach over 9 years (9.4 years, 95% CI, 6.8–12.1) in those aged 50 to 60. In addition, they found that the total number of diphtheria vaccine (primary vaccination and boosters) doses received was significantly lower in older individuals (p <0.001), and only 62.7% (95% CI 50.8–74.6) of those aged 50–60 years had anti-diphtheria antibodies at a level considered to be seroprotective (i.e., ≥0.1 IU/ml). Weinberger et al. also reported that recall response to diphtheria and tetanus vaccination was insufficient in older individuals in their study of 252 persons aged above 60 years who received a booster vaccination against tetanus and diphtheria, and a subgroup (n = 87) that received a second booster 5 years later (Weinberger et al., 2013). Although almost all participants had protective antibody concentrations at 4 weeks after the first vaccination, antibodies fell below levels considered to be seroprotective in 10% (for tetanus) and 45% (for diphtheria) after 5 years. Protection was restored in almost all vaccinees after the second vaccination (Weinberger et al., 2013). These findings underline the need for regular and well-documented booster shots of recommended vaccines in adults throughout the life course, independently of travel plans. This is of particular importance in view of the latest epidemiological data regarding diphtheria, showing an upward trend in the number of cases worldwide 2017, after a steady decline from 2000 to 2016, suggesting that progress in the fight against diphtheria worldwide has stalled (Clarke et al., 2019). The WHO South-East Asia region has reported the majority of global diphtheria incidence each year since 2000, and within the WHO South-East Asia region, India, Nepal, and Indonesia together accounted for 96–99% of cases (Clarke et al., 2019). Population migration or political instability in certain regions likely contributed to creating conditions favorable to outbreaks, with the largest most recent outbreaks reported in the Rohingya refugee population in Bangladesh, and in Yemen and Venezuela (Clarke et al., 2019).

Influenza Vaccination

Routine influenza vaccination should be advised and administered annually. In the European Union, all countries recommend the influenza vaccine for individuals aged over 65 years, independently of travel. According to the most recent log scale diagram by Steffen (2018), influenza is the most incident vaccine-preventable disease in travellers. Due to physiological immune-senescence, vaccine efficacy among older adults is significantly lower (37% in those aged 65 years and older vs. 51% in those aged 18–64 years) (Belongia et al., 2016). These numbers have also been confirmed by the Canadian Serious Outcome Surveillance (SOS) Network, although the authors also demonstrated that, despite this rather low efficacy, the number of deaths prevented by the influenza vaccine is around 75% for those aged 65 years and older (Nichols et al., 2018). There are different vaccines available in different parts of the world. For example, there are separate vaccines for the Northern and Southern hemispheres, although their strains are often similar (Lambach et al., 2015). There is a live-attenuated intranasal influenza vaccine, not available in Europe, which is not recommended for people aged 65 or older (Grohskopf et al., 2018). There are several licensed vaccines for seniors, i.e., a trivalent inactivated high-dose vaccine, a quadrivalent

inactivated adjuvanted vaccine and a quadrivalent recombinant vaccine, particularly suitable for those with an egg allergy (Grohskopf et al., 2018). A high-dose quadrivalent inactivated influenza vaccine (HD-IIV4; Fluzone High-Dose Quadrivalent, Sanofi Pasteur) was licensed by the Food and Drug Administration in 2019 for the prevention of influenza in persons aged 65 years and older (Chahine, 2021). In a phase III trial in the United States, it was shown to be well tolerated and to induce non-inferior immune responses compared to those induced by the trivalent high-dose vaccine for the shared strains (Chang et al., 2019). The preferred vaccine is the adjuvanted formula, because the presence of MF59 enhances the response by the senescing immune system through the recruitment and activation of immune cells at the injection site. In addition, MF59-adjuvanted vaccine was shown to induce a stronger booster response than the non-adjuvanted vaccine, and also provided broader serological protection against drifted strains that circulated in the 2 years after vaccination (Ansaldi et al., 2008). MF59 has also been shown to induce local stimulation and recruitment of dendritic cells (DCs), and subsequent increased uptake of antigen by DCs (Coffman et al., 2010). Through this increased recruitment of immune cells, facilitating migration of DCs into tissue-draining lymph nodes, the MF59 adjuvant helps to prime adaptive immune responses (Seubert et al., 2008). Finally, in a head-to-head comparison of standard-dose and enhanced vaccines among 1861 adults aged 65–82 years, Cowling et al. reported that recipients of enhanced vaccines showed improved humoral and cell-mediated immune responses compared to those receiving standard-dose vaccines (Cowling et al., 2020).

The major issue when deciding whether or not to give an influenza shot to a senior traveler is that the vaccine is usually not available in summer. Hence, two practical possibilities arise for travellers leaving at the end of winter for the opposite hemisphere: 1) vaccinate (or re-vaccinate) with the vaccine of the home hemisphere relying on cross-reactivity, and 2) obtain the vaccine of the destination hemisphere soon after arrival (Vaccines for travelers. M, 2018). There is no formal preference expressed by the Advisory Committee for Immunisation Practices (ACIP) and no delay is allowed, should a specific product be unavailable (Grohskopf et al., 2018).

Pneumococcal Vaccine

Among the routine vaccinations that providers should be recommending and administering to older individuals, perhaps the most important with regard to travel is the pneumococcal vaccine. *Streptococcus pneumoniae* is a ubiquitous pathogen that causes a large spectrum of invasive and non-invasive disease in both children and adults. Pneumococcal pneumonia remains the most common form of invasive pneumococcal disease (IPD), and it disproportionately affects older individuals and those with weakened immune systems, such as patients with human immunodeficiency virus or chronic disease (Berical et al., 2016; Rivero-Calle et al., 2019). Other severe types of disease, such as meningitis or septicaemia may also occur, while less severe presentations such as acute otitis media or sinusitis are common in children. *Streptococcus pneumoniae* is reportedly responsible for 30–38% of community-acquired pneumonia (Johansson et al., 2010; Holter et al., 2015). Hospital-acquired pneumonia is among the most frequent nosocomial infections, and is associated with prolonged hospital stay and high costs (Guidelines for the manage, 2005), and the case fatality rate increases in line with increasing age (Dirmesropian et al., 2019). *Streptococcus pneumoniae* is spread by coughing, sneezing and respiratory secretions, and up to 50% of children may be asymptomatic carriers (Hussain et al., 2005), although carriage rates decrease to around 10% in adulthood (Goldblatt et al., 2005), and even lower in elderly individuals (Ridda et al., 2010). The incidence of IPD in 2013/2014 was reported to be 6.85/100,000 for all adult age groups and 20.58/100,000 among individuals aged >65 years in the United Kingdom (Thomas, 2021), while there were an estimated 192,281 hospital admissions for pneumonia and 6,000 cases of IPD in 2013/14 (Chalmers et al., 2016). Data are sparse regarding the incidence of IPD specifically among travellers, but available data from studies in Hajj pilgrims have reported that *Streptococcus pneumoniae* accounted for six out of nine cases of meningitis from among 808 patients hospitalized in 2 hospitals in 2003 (Madani et al., 2006), 19% (12/64) of isolates in ICU patients with sepsis in two major hospitals in Makkah during the 2004 season (Baharoon et al., 2009), while in 2005, 2.7% (44/1,626) of isolates from patients with sepsis in four Mecca hospitals were due to *S. pneumoniae* (Asghar, 2006).

An increasing body of evidence in favor of drug resistance to a growing list of antibiotics, including penicillin, cephalosporins, macrolides and fluoroquinolones, underline the need for vaccines to be used widely to prevent pneumococcal disease. In elderly individuals who are traveling, often in enclosed spaces such as airplanes, ships or buses with high potential for transmission, vaccination against pneumococcal disease is therefore of paramount importance.

There are two pneumococcal vaccines currently available, namely the 23-valent pneumococcal polysaccharide vaccine (PPSV23), and the 13-valent pneumococcal conjugate vaccine (PCV13). PPSV23 is recommended for all adults aged 65 years and older, while PCV13 is widely recommended in infant vaccination schedules worldwide, and for adults aged 65 years and over. Both pneumococcal vaccines are recommended for adults aged less than 65 in the presence of immune-compromising conditions. The antibody response stimulated by PPSV23 is T-cell independent, leading to waning protection over time (Musher et al., 2010; Aliberti et al., 2014) and there is no induction of immunological memory (Rijkers et al., 2018). Therefore, booster shots should be recommended in line with recommended intervals.

There can be no doubt as to the efficacy of vaccination in preventing invasive and non-invasive pneumococcal disease. A systematic review and meta-analysis of the efficacy of PPSV23 in older adults reported pooled efficacy in 73% against invasive pneumococcal disease caused by any serotype (Falkenhorst et al., 2017). Similarly, in the CAPAMIS study, Ochoa-Gondar et al. reported that PPSV23 was effective in reducing the risk of all-cause and pneumococcal pneumonia (Ochoa-Gondar et al., 2014). For conjugate vaccines, the randomized, double-blind

TABLE 1 | Centre for disease control recommendations for pneumococcal vaccination.

- For immunocompetent adults aged 65 years and older, vaccination with PCV13 is no longer routinely recommended, but may be used after shared clinical decision-making in those not previously vaccinated with PCV13
- For travellers in this age group, healthcare providers should discuss with patients the fact that PCV13 vaccine serotypes may still be circulating in some destination countries with lower vaccination rates
- All immunocompetent adults aged ≥65 years should receive 1 dose of PPSV23 ≥5 years after any previous PPSV23 dose, regardless of previous pneumococcal vaccine history
- No additional doses of PPSV23 should be given following the dose administered at age ≥65 years
- For immunocompetent adults aged 65 years and older, if a decision to give PCV13 has been made, 1 dose of PCV13 should be administered first, followed by 1 dose of PPSV23 ≥12 months after PCV13
- Immunocompetent adults aged 19 through 64 years with chronic medical conditions (e.g., chronic heart disease excluding hypertension; chronic lung or liver disease; diabetes, alcoholism, cigarette smoking) should receive a single dose of PPSV23
- For immunocompetent adults aged ≥65 years with chronic medical conditions (e.g., chronic heart disease excluding hypertension; chronic lung or liver disease; diabetes, alcoholism, cigarette smoking), vaccination with PCV13 is no longer routinely recommended, but may be used after shared clinical decision-making
- Immunocompetent adults aged ≥65 years with chronic medical conditions (e.g., chronic heart disease excluding hypertension; chronic lung or liver disease; diabetes, alcoholism, cigarette smoking) should receive 1 dose of PPSV23. If PCV13 has been given, then give PPSV23 ≥1 year after PCV13 and ≥5 years after any PPSV23 at age <65 years
- PCV13 and PPSV23 should NOT be administered during the same visit
- Pneumococcal vaccine can be administered concomitantly with seasonal influenza vaccine
- Contraindications to pneumococcal vaccine include notably previous hypersensitivity reaction to the vaccine or any component thereof

CAPITA trial including over 84,000 adults reported vaccine efficacy of 75% (95% CI 41.4–90.8%) for the prevention of a first episode of vaccine-type invasive pneumococcal disease with PCV13 (Bonten et al., 2015). A recent review of the literature found no evidence of hyporesponsiveness after repeat vaccination with PPSV23 at an interval of 5 or more years after the previous dose, or after sequential administration after PCV (Cripps et al., 2021).

Consequently, pneumococcal vaccination should be routinely recommended to older individuals, particularly when they plan to travel, and irrespective of whether the travel involves planned participation in mass gatherings. While there is no specific evidence to indicate that pneumococcal vaccination is especially necessary in healthy individuals planning to travel, travel consultations are a useful opportunity to recommend this vaccine, which should be a part of routine healthcare in all individuals over 65 years of age. The CDC recommendations for pneumococcal vaccination are summarized in **Table 1**.

TRAVEL-SPECIFIC VACCINES

Travel-specific vaccines must be discussed in detail on a case-by-case basis, and the risk associated with the vaccine should be carefully weighed against the risk of contracting the disease during travel. Some destination countries may require vaccination against certain diseases before allowing entry, and this must be taken into consideration. Alternative itineraries could be envisaged if the vaccine requirements of the destination are not compatible with the traveler's state of health.

Yellow Fever

Yellow fever (YF) is a vector borne disease that is transmitted by the *Aedes* species of mosquitoes, and mainly a zoonotic disease in monkeys and apes, occasionally infecting humans. The majority of infections are asymptomatic, with a ratio of symptomatic to asymptomatic infection ranging from 1:7 to 1:12 (Vasconcelos and Monath, 2016), or may lead only to mild disease. However, a small proportion of patients will progress to the second phase of disease, characterised, as the name suggests, by haemorrhagic febrile jaundice. The virus is endemic in tropical areas of Africa and Central and South America. The overall estimated risk of acquiring YF for an unvaccinated traveler for a 2 weeks stay to a high-risk area in Africa is 50 cases per 100,000 people, and five cases per 100,000 people in South America (Reno et al., 2020). Between 2016 and 2018, there were several outbreaks of YF in various parts of the world (notably Brazil), resulting in increased numbers YF in travellers from affected areas (Reno et al., 2020).

YF vaccine is a live attenuated 17D virus, in use since the 1950s and grown on fertilized eggs. There is only one vaccine available, and it is known to be highly effective–a single dose of YF vaccine confers sustained immunity within 10 days for over 80% of those vaccinated, and within a month for almost all those vaccinated. It provides life-long protection against the disease. The question of whether or not to give YF vaccine is a dilemma, not only in older adults, but in all travellers. The World Health Organization (WHO) policy on YF vaccine and risk states that vaccination is recommended for all travellers aged 9 months or older traveling to areas where there is evidence of persistent or periodic YF virus transmission. Vaccination is generally not recommended for travellers going to areas where the risk of exposure is low and any risk should be weighed against individual risk factors (e.g., age, immune-status) for vaccine-associated adverse events, but a number of countries require incoming visitors to be vaccinated against YF (World Health Organization, 2019a).

The actual population risk of YF is hard to estimate. A recent study taking account of vaccination coverage estimated the YF burden in Africa for the year 2013 as 130,000 (95% CI 51,000–380,000) cases with fever and jaundice or haemorrhage, including 78,000 (95% CI 19,000–180,000) deaths (Garske et al., 2014). An outbreak of YF occurred in Luanda (Angola) in 2016 that rapidly spread to the Democratic Republic of Congo, and from there, was carried onwards by travellers to Mauritania, Kenya, and even China, the first time in

history that confirmed YF cases have occurred in Asia. The tardy response and shortfall of YF vaccines led some experts to qualify YF as a threat to the entire world (Woodall and Yuill, 2016). These outbreaks, along with outbreaks in Brazil and Nigeria in 2017 prompted the WHO to introduce the Eliminate Yellow Fever (EYE) strategy, to respond to the increased threat of urban YF outbreaks with international spread. In absolute terms, with a reported 677 deaths from the 2 outbreaks in Brazil from 2016 (World Health Organization, 2018a), and 140 deaths for all of Africa in 2016 (Yellow fever in Africa an, 2017), one might consider the absolute risk for a traveler staying only a few days to weeks to be relatively low. The CDC estimates that for a 2-week stay, the estimated risk of infection and death from YF for an unvaccinated traveler visiting an endemic area in West Africa is 50 per 100,000 and 10 per 100,000, respectively; and 5 per 100,000 and 1 per 100,000, respectively in South America (Gershman and Staples, 2018). In practice, the actual risk is highly variable across regions, and even within countries. Guidance from vaccine recommendation maps is helpful, but may be based on historical data that no longer accurately reflect the real-time risk (Fletcher et al., 2020).

It has been shown that the neutralizing antibody response to YF vaccination is not diminished in healthy older adults, with equivalent categorical and quantitative responses across younger and older subjects in clinical trials involving a total of 4,532 subjects (Monath et al., 2005). Roukens et al. found that older subjects (aged 60–81 years) had a delayed antibody response and higher viraemia levels after yellow fever primovaccination compared to their younger counterparts (18–28 years) but there were only 28 subjects in the older group and 30 in the younger group in this study (Roukens et al., 2011).

YF vaccine was long considered to be one of the safest vaccines available, but in recent years, this has been called into question by the occurrence of two major types of adverse events following YF vaccination, namely YF vaccine-associated neurologic disease (YEL-AND) and YF vaccine-associated viscerotropic disease (YEL-AVD). YEL-AND may occur within 2–56 days post-vaccination (Fletcher et al., 2020) and may take the form of various distinct clinical syndromes, such as meningoencephalitis, Guillain-Barre syndrome (GBS), or acute disseminated encephalomyelitis (ADEM) (Lindsey et al., 2016). It is usually self-limiting, neurological sequelae are unusual and deaths are rare (1–2%) (Monath, 2012). Certain acquired risk factors are known to increase susceptibility to YEL-AND and are of relevance to older individuals considering YF vaccination, namely immune suppression and age >60 years. Indeed, immune suppression promotes prolonged viremia, and incurs a higher risk of neuroinvasion. Similarly, the age-related decline in immunity or the frequent presence of comorbidities (e.g., hypertension, diabetes) put older individuals at higher risk (Monath, 2012).

YEL-AVD is a rare, but serious, multisystemic disease with multiorgan failure, that mimics wild-type YF infection and is frequently fatal. It may occur up to 18 days after a first YF vaccination (Fletcher et al., 2020). The frequency of YEL-AVD is estimated at between 0 and 12 cases per 100,000 vaccinees (Seligman, 2014), but rates vary widely between regions, with a 33-fold higher rate in Peru and a 19-fold lower rate in Brazil as compared to estimates from the US Vaccine Adverse Event Reporting System (Seligman, 2014). The reporting rate of YEL-AVD increases with age (Lawrence et al., 2004; Lindsey et al., 2016). In a review of adverse events occurring after YF vaccination between 2007 and 2013, Lindsey et al. reported 6 cases of YEL-AVD after an estimated 2,224,790 doses of YF vaccine, corresponding to a reporting rate of 0.3; of note, 5 of these cases occurred in persons aged over 70 years (reporting rate = 4.0), and all were males (Lindsey et al., 2016). Using a list of cases from the US Centre for Disease Control (CDC), incremented by literature data, Seligman identified potential risk groups for YEL-AVD as older (>56 years) males, women between the ages of 19 and 34, people with various autoimmune diseases, individuals who have been thymectomized because of thymoma, and children under the age of 11 years (Seligman, 2014).

Despite intense research into the mechanisms that underpin these complications, our understanding of host genetics and the host immune response after vaccination remains incomplete. Pulendran et al. described the interaction of YF-17D with the innate immune system and the consequences of these interactions on the stimulation of the adaptive immune response (Pulendran, 2009). The application of systems biology approaches to studying immune responses shows that such systems approaches could be used to identify early signatures of vaccination that predict the immunogenicity and efficacy of vaccines. More recently, Azamor et al. conducted a follow-up study in volunteers immunized with YF 17D, investigating the humoral response, cellular phenotypes, gene expression, and selected single nucleotide polymorphisms, to clarify the role and interaction of these factors in early response after vaccination, and concluded that the early events elicited after YF 17D vaccination are, at least partly, associated with and controlled by human genetic background (Azamor et al., 2021).

Considering the overall absolute number of people at risk, and the low rate of serious vaccine-related adverse events and infection, the risk-benefit ratio of YF vaccination must be weighed carefully in travellers, especially those who are older in age. As a general rule, if the risk of getting YF is greater than the risk of serious adverse events, then vaccination should be mandatory, bearing in mind that YF vaccination is obligatory for access to certain countries. Personal factors, such as overall health status, genetic disposition, age or immune status, also play a role and should be taken into consideration in deciding on YF vaccination. Finally, travel-related factors, such as the destination, and the type of activity the person plans to undertake there, will also be important. Points to consider when deciding on the need for YF vaccination for travel are summarized in **Table 2**. Although it may be challenging to cover so many points in a short travel consultation, the best strategy is to present all the data to the patient and allow them to make their own decision, unless YF vaccine is obligatory for the country they plan to travel to. For persons who cannot get YF vaccine for medical reasons and planning to travel to a country with a YF vaccination entry requirement, the physician may provide a medical waiver letter and complete the medical contraindication section of the International Certificate of Vaccination or Prophylaxis.

TABLE 2 | Points to consider when deciding on the need for yellow fever vaccination for travellers.

- Is YF fever mandatory for entry into the destination country or other countries on the itinerary?
- Has the traveler been previously immunised with YF?
- Current age
- Immunocompromised
- Presence of autoimmune disease
- Patient thymectomized
- Itinerary and activities on site
- Destination YF activity and transmission
- WHO assessment
- Travellers opinion, thoughts and consent on vaccination, based on the above

Hepatitis

Hepatitis A is a very common travel-related infectious disease and one of the most common causes of viral hepatitis worldwide. Evaluation of the risk of a senior of contracting hepatitis A during travel must consider their destination, since the majority of touristic destinations in the world are places where hepatitis A is endemic, and there is likely to be a risk of travellers of any age encountering the virus. In a population-based study of the incidence rate of travel-related hepatitis A from 1997 to 2005, Askling et al. reported that the incidence rate in unprotected travellers to East Asia, North Africa, and the Middle East was 2, 12, and 18 cases/100,000 person-months, respectively, although children aged 0–14 years accounted for 43% of all travel-related cases (Askling et al., 2009).

Seroprevalence of anti-HAV immunoglobulin (Ig) G, a marker of prior infection with HAV, increases with age, and data from the United States collected before widespread implementation of HAV vaccination, indicate that 75% of individuals aged over 70 years had serological evidence of prior HAV infection (Bell et al., 2005), such that pre-vaccination screening for evidence of prior infection may be warranted. Development of anti-HAV IgG antibodies confers lifelong immunity against re-infection (Marcus and Tur-Kaspa, 1997). Older individuals are at greater risk of more severe disease. Indeed, in the elderly, the disease course tends to be prolonged, with more frequent jaundice, coagulopathy, cholestasis, pancreatitis and ascites (Marcus and Tur-Kaspa, 1997; Carrion and Martin, 2012). As a result, hospitalization, morbidity, and mortality rates increase with age (Marcus and Tur-Kaspa, 1997; Carrion and Martin, 2012; Nagaratnam et al., 2017). As for YF, immune status, travel destination, and activities on site should be kept in mind when considering the risk of hepatitis A in older people planning to travel. Indeed, older people have a lower risk of infection, given their higher likelihood of previous exposure, but they are at risk of greater clinical severity if infected compared with their younger counterparts. A review of studies investigating response to hepatitis A vaccination according to age reported lower seroconversion rates, and also that titers of antibody to hepatitis A virus achieved after vaccination were inversely proportional to age (Leder et al., 2001). In an open-label study, D'Acremont et al. compared the immune response of subjects aged 18–45 years old to a cohort over 50 years old and showed that the seroprotection rates were respectively 100 and 65% after the first vaccination, and 100 and 97% after the booster dose (D'Acremont et al., 2006). These findings show that hepatitis A is a well-tolerated and immunogenic vaccine in the older population.

Hepatitis B also has low endemicity in Europe, but as with hepatitis A, there can be some risk of exposure during travel, particularly in relation to sexual behaviors or medical/surgical procedures, notably due to accidents occurring during travel (Zuckerman and Steffen, 2000), and medical emergencies related to underlying chronic health conditions in the traveler. Medical tourism is also associated with an increased risk of infection. A meta-analysis by Anker et al. reported that patients traveling to undergo kidney transplant were more likely to develop hepatitis B virus infection compared to domestic kidney transplant recipients (Anker and Feeley, 2012). The hepatitis B virus (HBV) is highly prevalent, and despite widespread vaccination, a large proportion of the world population remains unprotected (Miglietta et al., 2018a). Universal vaccination programmes have been successful in reducing HBV infections in many countries (Zanetti et al., 2008). This success has been linked to four main criteria, namely initiation of universal vaccination before 1995, implementation of catch-up vaccination, achievement of high coverage rates, and endemicity, whereby countries starting from high endemicity saw a greater impact of vaccination (Miglietta et al., 2018a). A report of the trends in HBV infection over 5 decades in Italy found that sexual transmission and cosmetic treatment with percutaneous exposure such as piercing and tattooing, are the major modes of transmission nowadays (Sagnelli et al., 2014). The WHO Global Hepatitis Report 2017 indicates that the use of childhood HBV vaccination has greatly reduced the incidence of new chronic HBV infections, and most of those now living with such infections are persons who were born before the availability and implementation of childhood vaccination programmes (World Health Organization, 2017a). Steffen reported higher risk for persons working in endemic countries for prolonged periods, as compared to short-term vacationers (Steffen, 1990). In a European study of 9,008 individuals, 6.6–11.2% of travellers were classified as being at high risk of hepatitis B infection, depending on the destination country (Zuckerman and Steffen, 2000). In a pooled analysis of data from healthy adults aged ≥20 years who had been vaccinated with 20 μg HBV vaccine (Engerix™ B, GSK Vaccines, Belgium) in a 0, 1, 6 months schedule in 11 studies performed since 1996, Van Der Meeren et al. found a protection rate of 98.6% in the 20–24 years age group, which decreased to 64.8% in those aged ≥65 years (Van Der Meeren et al., 2015). In a study of 52 healthy, community dwelling elderly adults (65–82 years), seronegative for HBV, enrolled in the SENIEUR protocol, Edelman et al. reported that lower seroprotection rates were achieved in the healthy seniors overall compared to a junior cohort (aged 21–34 years), with delayed response to vaccination (achieving seroprotection only after the third dose), and lower peak titers (Edelman et al., 2020).

Regarding the recommendations for vaccination, a combined vaccine should be proposed if the traveler is at risk or likely to become at risk of both hepatitis A and hepatitis B; otherwise, a single vaccine can be proposed depending on the endemicity of

the virus in the destination country. The eligibility of people with chronic health conditions for vaccination is often questioned, but it should be noted that it is highly recommended for persons with chronic comorbidities to receive hepatitis vaccination.

Meningococcal Meningitis

Invasive meningococcal disease (IMD) is caused by the bacterium *Neisseria meningitidis* and predominantly affects infants and young children (Strifler et al., 2016; Marten et al., 2019). Although present worldwide, incidence of meningococcal disease is highest in the so-called "meningitis belt" in Sub-Saharan Africa, stretching from Senegal and Guinea across to Ethiopia (Pizza et al., 2020). The risk for travellers is highest among people visiting meningitis belt countries who have prolonged contact with local populations during an epidemic (Centers for Disease Contr, 2020a), and cases of meningococcal diseases in Hajj pilgrims to Saudi Arabia have also been reported (Pizza et al., 2020). Data regarding the true risk to travellers are rare, but the risk is generally acknowledged to be low and cases are rare (Memish, 2002), although the severity of the disease means that the danger should not be underestimated (Wilder-Smith, 2007).

The risk of contracting meningococcal meningitis (MM) during an individual travel is low, and it is just as relevant when considering vaccination needs in seniors to consider the person's risk of getting the disease while at home. Over the period 2004–2014, serogroup B caused 74% of all notified cases of IMD in the EU/EEA, and the majority of cases in all age groups, whereas there were decreasing trends in serogroups B and C and an upward trend in serogroup Y (Whittaker et al., 2017). IMD remains rare at less than 1 case per 100,000 population in the EU/EEA (Whittaker et al., 2017), but in the event of an outbreak, disease risk can increase 1000-fold, and outbreaks can last up to 10 years or more (Oviedo-Orta et al., 2015). In Europe, the highest incidence was observed in the Republic of Ireland and the United Kingdom, while incidence has reached historically low levels in the United States in recent years (Parikh et al., 2020). In South America, Argentina, Chile and Brazil have the highest burden of IMD, although this is likely due to improved surveillance and laboratory testing available in these countries (Sáfadi et al., 2015; Parikh et al., 2020). Risk factors for IMD include viral infections, smoking, alcohol, crowded spaces, and health conditions such as immunodeficiency (e.g., HIV), hemoglobinopathy, asplenia or other chronic conditions such as diabetes and renal failure. Personal behaviors were found to be related to risk in a recent study of disease clusters occurring in Italy (Miglietta et al., 2018b).

A number of European countries recommend meningococcal vaccination for newborns and/or young children. Both vaccines should also be offered to high risk individuals, those with chronic conditions, or those engaging in high-risk personal behaviors. Available vaccines include a quadrivalent conjugate vaccine covering serogroups A, C, W, and Y, and a serogroup B meningococcal vaccine. It should be noted that there is waning immunity with meningococcal vaccines (Centers for Disease Contr, 2011). In a phase IIIb, open-label, randomized, controlled study, Dbaibo et al. evaluated the immunogenicity and safety of a meningococcal conjugate vaccine (MenACWY-TT) and a quadrivalent polysaccharide vaccine in adults aged over 55 years (range 56–103 years). They reported that one month after a single dose of vaccine, at least 97.4 and 95.5% of subjects in the conjugate and polysaccharide groups respectively had serum bactericidal antibodies above the cutoff indicative of seroprotection (Dbaibo et al., 2013). Vaccine response rates tended to be lower in subjects who were aged >65 years at the time of vaccination, compared to their younger counterparts (Dbaibo et al., 2013). In a study from Latin America, Stamboulian et al. reported that the quadrivalent meningococcal conjugate vaccine (MenACWY-CRM) was well tolerated and elicited higher immune responses compared to an unconjugated meningococcal polysaccharide vaccine in subjects 56–65 years of age (Stamboulian et al., 2010). Finally, a single dose of MenACWY-CRM was found to induce a robust immune response against all four meningococcal serogroups in a study of 180 Indian subjects, although only one third of the population was aged 19–75 years, and older individuals were not analyzed separately (Lalwani et al., 2015).

Regarding travellers, the ACIP recommends that travellers to African countries in the "meningitis belt" during the dry season (December–June) receive vaccination with a MenACWY vaccine before travel (Mbaeyi et al., 2020). Similarly, advisories recommending vaccination may be issued for travellers to other areas when outbreaks of meningococcal disease caused by vaccine-preventable serogroups are ongoing. The ACIP further recommends that international travellers receive a booster dose of MenACWY if the last dose was administered 3–5 or more years previously. Of note, meningococcal vaccination is required by the Kingdom of Saudi Arabia for all travellers to Mecca during the Hajj and Umrah pilgrimages (Mbaeyi et al., 2020).

Typhoid Fever

Diseases with a fecal-oral route of transmission have a huge impact worldwide, and include typhoid, cholera and poliomyelitis (polio). Typhoid and paratyphoid fevers, together referred to as enteric fever, are caused by systemic infection with *Salmonella enterica* subspecies serovars Typhi and Paratyphi A, B, and C (Typhoid and Paratyphoid, 2019). It is estimated that more than 14 million cases of enteric fever occurred in 2017, with 76.3% of all cases caused by *Salmonella enterica* serotype Typhi. The global case fatality rate is estimated at 0.95%, corresponding to around 135,900 deaths from enteric fever annually, and is higher among older adults, and among those living in lower-income countries. The mortality rate is higher among patients aged over 50 years compared to those under 50 years of age (3.3 vs. 0.4%; $p = 0.009$), although these data date from epidemiological information collected from 1977 to 1979 regarding 901 cases of typhoid reported in the United States (Taylor et al., 1983). The majority of cases occur in South/South-East Asia, and sub-Saharan Africa (World Health Organization, 2018b). In travellers to destinations in South Asia, typhoid fever is the third most commonly contracted disease, after traveler's diarrhea and influenza (Steffen et al., 2015a). In a study from the Swedish database on notifiable communicable diseases,

including all cases recorded of typhoid and paratyphoid fevers from 1997 to 2003, Ekdahl et al. reported an overall risk of enteric fever after travel of 0.42 per 100,000 travellers (Ekdahl et al., 2005). The highest risk was observed in travellers returning from India and neighboring countries (41.7 per 100,000), the Middle East (5.91 per 100,000), and Central Africa (3.33 per 100,000), while the risk was comparatively lower in East Asia (0.24 per 100,000) (Ekdahl et al., 2005).

Currently, there are several typhoid vaccines available, including the unconjugated polysaccharide vaccine, containing purified Vi capsular polysaccharide from the Ty2 *S. Typhi* strain; and the Ty21a vaccine, which is an orally administered live attenuated Ty2 strain of *S. Typhi* modified by chemically-induced mutagenesis (World Health Organization, 2018b). Typhoid conjugate vaccines (TCV) have also been developed, consisting of a Vi polysaccharide antigen linked to various carrier proteins. Three such TCVs have been licensed to date, all in India, and others are in the pipeline (Syed et al., 2020). No TCV is licensed in Europe at present. Studies of typhoid vaccines to date have been performed mainly in countries with high endemicity, and not specifically among travellers, which may represent a drawback to estimating vaccine efficacy for travellers. Nevertheless, the WHO recommends vaccination for travellers from non-endemic to endemic areas, in addition to adherence to precautions on hygiene practices (World Health Organization, 2019b).

The conjugate vaccine is a single-dose, intramuscular injection, with an estimated efficacy of 87% when evaluated in a human challenge study in a population of 112 immunologically naive adult (age 18–60 years) volunteers (Jin et al., 2017). There are no head-to-head comparisons of TCV vs. the live attenuated Ty21a vaccine, and the potential for a herd effect with TCV has never been investigated (World Health Organization, 2018b). Evidence suggests that protection lasts up to 5 years after the first dose (Voysey and Pollard, 2018), but data are lacking regarding the need for booster doses.

The polysaccharide vaccine is administered in a single dose, either intra-muscularly or subcutaneously, and is recommended for adults. Immunity begins to wane after 2 years in vaccinated subjects, and hypo-responsiveness to revaccination has been reported in some studies (Keitel et al., 1994; Overbosch et al., 2005; Zhou et al., 2007; Roggelin et al., 2015), but existing data are not sufficiently robust to suggest a risk of hypo-responsiveness (World Health Organization, 2018b). The increased risk of infection with typhoid fever observed beyond 2 years after vaccination underpins the recommendation to re-vaccinate every 3 years in order to maintain protection.

The live attenuated Ty21a is given on a 3 dose schedule, by the oral route, ingested on days 1, 3 and 5. It stimulates serum and mucosal antibodies, eliciting long-lived cell-mediated immune responses (World Health Organization, 2018b). There is also evidence to indicate that it induces herd protection (Levine et al., 1989), which is useful from the public health perspective. Protective efficacy of up to 67% was reported at 3 years after vaccination, and 62% after 7 years (World Health Organization, 2018b). Revaccination is recommended every 3 years forViPS, and every 3–7 years for Ty21a (World Health Organization, 2019b). The need for revaccination with TCV is currently unclear (World Health Organization, 2019b). The Ty21a vaccine is well tolerated and associated with low rates of adverse events. However, the live attenuated vaccine should not be given in immunocompromised patients, including those taking immunomodulators, calcineurin inhibitors, cytotoxic agents, antimetabolites, and high-dose steroids (Centers for Disease Contr, 2020b).

In view of the persisting high burden of typhoid fever and the emergence of antimicrobial resistant strains of *S. Typhi*, the WHO recommends programmatic use of typhoid vaccines for the control of typhoid fever from a public health perspective, and recommends the TCV as a 0.5 ml single dose in adults up to 45 years in typhoid endemic regions. Concerning travellers from non-endemic to endemic areas, vaccination with any of the available licensed vaccines is recommended, in conjunction with usual water and sanitation hygiene precautions. Certain special populations who may be at increased risk of acquiring or transmitting *S. Typhi* infection should also be targeted for vaccination, such as professional food handlers working in endemic areas, or healthcare and laboratory workers.

Certain precautions should also be noted regarding typhoid vaccination. Firstly, there are currently no data on the interchangeability or sequential use of the different available vaccines. Second, typhoid vaccines are contraindicated for individuals with known hypersensitivity to any component of the vaccine. Third, the polysaccharide vaccine can be co-administered with other vaccines routinely recommended for international travellers, such as YF and hepatitis A vaccines (World Health Organization, 2018b). Certain antimalarials particularly mefloquine, exhibit activity against Ty21a, and may suppress the Ty21a antibody response, and should therefore not be given from 3 days before until 3 days after giving the Ty21a vaccine (World Health Organization, 2018b). Antimicrobial agents may also be active against the typhoid strain in the vaccine, and therefore, the typhoid vaccine should be delayed by at least 72 h after antimicrobial administration. Fourth, *Salmonella paratyphi A* and *B* do not have the Vi antigen, and thus, the polysaccharide vaccine is not effective against these forms of infection (Guzman et al., 2006; Fangtham and Wilde, 2008). Finally, data are sparse regarding seroconversion rates in older individuals after vaccination.

Cholera

Cholera is an acute diarrheal disease in humans caused by the Gram-negative bacillus *Vibrio cholerae*. Cholera can be contracted *via* the fecal-oral route, or from the environment, and is characterized by the abrupt onset of acute watery diarrhea and vomiting. Prompt treatment with rehydration solution and antibiotics is highly effective in treating cholera, but if left untreated, severe cases can rapidly result in death (Ganesan et al., 2020), with death rates up to 70% reported (Ramamurthy et al., 2019), although the majority of infected individuals will only suffer mild diarrhea, or may even be asymptomatic (Jilg, 2020). The estimated incidence is between 1.3 and 4.0 million cases per year, with up to 143,000 deaths (Ganesan et al., 2020). Modern sanitation has led to the virtual

eradication of cholera in industrialized countries, with disease now mainly occurring in low-resource countries with poor infrastructure, especially in terms of sanitation and access to clean drinking water (Rabaan, 2019). A global strategy to control cholera was launched by the WHO in 2017. The mainstay of prevention of both endemic cholera and outbreaks is the provision of safe drinking water and adequate sanitation, while cholera vaccination can be implemented as a complementary measure. Published data about the risk of cholera in travellers is scarce, although a recent systematic review of 91 reports of cholera or diarrhoea among travellers from 1990 to 2018 found only 156 cases of cholera imported as a consequence of travel, and cholera vaccination information was not provided in most of the reports (Connor et al., 2019).

There are currently two types of oral cholera vaccine available internationally for global use, namely WC-rBS, killed whole cell monovalent (O1 subtype) vaccines with a recombinant B subunit of cholera toxin, and second, WC, killed modified whole cell bivalent (O1 and O139) vaccines without the B subunit (World Health Organization, 2018c). In addition, a single-dose oral live attenuated vaccine is approved in the United States only for use among travellers to areas with active cholera transmission, namely CVD 103-HgR (Wong et al., 2017). It was shown to have vaccine efficacy of 90.3% at 10 days, and 79.5% at 3 months in a human cholera challenge model among 197 healthy adult volunteers aged 18–45 years old (Chen et al., 2016). In a summary of opinion dated January 30, 2020, the Committee for Medicinal Products for Human Use of the European Medicines Agency (EMA) recommended marketing authorization for this new cholera vaccine (European Medicines Agency, 2020). Limitations of this vaccine at present include the fact that its effectiveness has not been established in persons living in cholera-affected areas, nor in those who have pre-existing immunity due to previous exposure to *V. cholerae* or receipt of a cholera vaccine. Similarly, CVD 103-HgR has not been shown to protect against disease caused by *V. cholerae* serogroup O139 or other non-O1 serogroups. Reported side effects include tiredness, headache, abdominal pain, nausea/vomiting and loss of appetite (Chen et al., 2016). Finally, it should be remembered that cholera vaccination offers incomplete protection, and must always be considered as a complementary measure on top of standard water, sanitation and hygiene measures.

A common consideration for the use of cholera vaccine that is particularly relevant to older travellers is to prevent traveler's diarrhea. Indeed, traveler's diarrhea, caused by enterotoxigenic *Escherichia coli* (ETEC) frequently occurs in individuals traveling from industrialized countries to less industrialized zones. The whole-cell *V. cholerae* O1 in combination with a recombinant B-subunit of cholera toxin (WC/rBS) vaccine (Dukoral®) has been shown to provide cross-protection against traveler's diarrhea caused by *E. Coli* (Leung et al., 2006; Jelinek and Kollaritsch, 2008; López-Gigosos et al., 2013). A Cochrane systematic review concluded that there was insufficient evidence to support the use of cholera vaccine to prevent traveler's diarrhea (Ahmed et al., 2013), and another recent review concluded that no vaccine offers satisfactory protection (Steffen et al., 2015b). The recent guidelines for the prevention and treatment of traveler's diarrhea from the International Society of Travel Medicine (Riddle et al., 2017) make no mention of vaccination for the prevention of traveler's diarrhea, and stipulate that prophylaxis should only be considered in high risk groups, such as those with underlying health conditions, which may often be the case of older persons. Indeed, diarrheal diseases in older persons are often more serious, and patients with cardiovascular or kidney disease may tolerate dehydration poorly (Jilg, 2020). These considerations should be weighed on a case-by-case basis when considering the need for cholera vaccination in older individuals traveling to areas where cholera is endemic.

Poliomyelitis

Poliomyelitis (polio) is a highly infectious viral disease that mainly affects children under 5 years of age. It invades the nervous system and leads to irreversible paralysis in 0.5% of cases. Polio has been successfully controlled in recent decades, and almost eradicated worldwide, with a decrease of over 99% in wild polio virus cases since 1988. However, wild polio viruses are still endemic in some areas, such as Afghanistan, Nigeria and Pakistan, and an outbreak recently occurred in Papua New Guinea (Hall et al., 2019). Until polio is completely eradicated, all countries remain at risk of imported wild type polio virus cases, and there could be as many as 200,000 new cases every year within 10 years all over the world. This led the WHO to propose the "Polio Endgame Strategy" (World Health Organization, 2019c), a comprehensive approach to working through the final challenges toward complete eradication, and laying the groundwork for a sustainable, polio-free future.

There are two facets to polio eradication, namely the wild polio virus and circulating vaccine-derived poliovirus. Vaccination is key to achieving eradication of polio, since the disease cannot be treated, but only prevented. The WHO goal is therefore to reach every last child with polio vaccination. Oral polio vaccines are the predominant vaccine used in the fight against polio, and contain one, two, or three serotypes of attenuated vaccine. They are inexpensive, safe, highly effective, and orally administered, meaning that no healthcare professionals or sterile equipment is required. Given that wild poliovirus type 2 was officially declared eradicated in 2015, wild type 2 has now been removed from the oral polio vaccine, since it was also responsible for nearly 90% of all cases of circulating vaccine-derived polio. In 2016, all countries switched to the bivalent oral polio vaccine, which contains only type 1 and type 3 components, thus greatly reducing the risk of vaccine-derived cases. The inactivated polio vaccine contains inactivated strains of all three serotypes, and is administered by intramuscular or intradermal injection. It must be administered by a trained healthcare professional. It elicits excellent protective immunity, and is up to five times more expensive than the oral vaccine. Insecure and inaccessible areas represent a major challenge to reaching populations for vaccination while at the same time maintaining front-line worker safety (World Health Organization, 2019c). For the long-term, future use of inactivated polio vaccine after wild-type polio transmission has been stopped will maintain population immunity levels (Bandyopadhyay et al., 2015).

Regarding travellers in particular, the WHO recommends that before traveling abroad, persons residing in countries with active transmission of a wild or vaccine-derived poliovirus should have completed a full course of polio vaccination in compliance with the national schedule, and received one dose of inactivated or bivalent oral polio vaccine within 4 weeks–12 months of travel (World Health Organization, 2017b). Certified polio-free countries may require incoming travellers from polio-infected areas to be vaccinated against polio to obtain entry. Travellers from polio-free to polio-infected areas should be vaccinated in compliance with their national schedule (World Health Organization, 2017b). The latest meeting of the Emergency Committee under the International Health Regulations (2005) (IHR) on the international spread of poliovirus underlined the ongoing frequency of WPV1 international spread and the increased vulnerability in countries where routine immunization and polio prevention activities have been adversely affected by the COVID-19 pandemic (World Health Organization, 2021). The committee issued temporary recommendations for polio-infected states with risk of international spread to require polio vaccination in residents and long-term (>4 weeks) visitors prior to undertaking international travel out of the country (World Health Organization, 2021). Most older individuals living in developed countries will have received polio vaccination during childhood, but one lifetime booster may be administered to previously-vaccinated adults who are at risk of increased exposure to polio virus, e.g., during travel [Prevention (2009). Update, 2009]. Adults who were partially vaccinated may receive the remaining doses of the schedule, regardless of the time interval since the initial doses were given [Prevention (2009). Update, 2009].

Rabies

Rabies is a viral disease that causes fatal encephalitis. It is transmitted to humans *via* the saliva of an infected animal, and 99% of human cases are caused by bites from domestic dogs. Rabies causes an estimated 59,000 deaths per year worldwide (Hampson et al., 2015), and is almost invariably fatal once symptoms appear (Jackson, 2016). Rabies predominantly affects poor rural communities in countries where the disease is endemic (mainly Asia and Africa), and 40% of victims are children aged under 15 years of age (World Health Organization, 2018d). A prospective study of patients presenting to the rabies treatment center in Marseille over a 14-year period (1994–2007) reported a total of 424 injured travellers attending the center, with an increase in the number of patients over time (Gautret et al., 2010). The majority of cases were after travel to from North Africa (41.5%), where bites or scratches from cats and dogs accounted for the highest risk, and Asia (22.2%), where encounters with non-human primates carried the highest risk (Gautret et al., 2010).

With its extremely high case fatality rate, and given that rabies is 100% vaccine-preventable, containing and eradicating human dog-mediated rabies has become a major public health goal. In June 2018, the WHO in collaboration with the Global Alliance for Rabies Control launched the "Zero by 30: Global Strategic Plan" to end human deaths from dog-mediated rabies by the year 2030, an objective that is considered to be feasible with currently available technology and knowledge. To this end, awareness of the disease, how to prevent it, and how to act in case of a bite from a rabid animal, are all key points in achieving a world free of dog-mediated rabies.

The estimated incidence in travellers of a bite requiring post-exposure rabies prophylaxis (PEP) is 0.4 per 1,000 travellers per month (Gautret and Parola, 2012). However, even if the risk of rabies is low, the risk of an animal bite is high. Therefore, prevention is of paramount importance, through simple measures such as avoiding contact with street dogs, monkeys and other mammals. In case of a dog-bite, the wound should be washed immediately with soap and water, or flushed abundantly with water if no soap is available, and medical attention should be sought immediately in the nearest medical centre. This can be problematic for travellers in rural areas where medical help may not be easily available. For this reason, it is advisable to have travel insurance to ensure prompt assistance, especially for unvaccinated persons traveling to areas where rabies is endemic.

The severity of the bite from a suspected rabid animal determines the risk of rabies exposure, and thus, the type of PEP that needs to be implemented. The different categories of exposure are outlined in **Table 3**.

Effective PEP should be provided after rabies exposure, consisting in thorough washing and flushing of the wound, prompt administration of rabies vaccine (if no pre-exposure prophylaxis was possible), and, in case of category III exposure, infiltration of human rabies immunoglobulin (HRIG) in and around the wound (Jackson, 2016). Obtaining HRIG may be difficult in many geographical areas, as the product is not readily available everywhere, is expensive, and needs to be administered rapidly (ideally within the first 2 days after the bite) for maximum efficacy. Recent studies indicate that only 5–20% of travellers who sustained category III exposure and who had an indication for HRIG actually received it in the country of exposure (Gautret et al., 2018). Similarly, in a study of 75 cases of rabies exposure treated with HRIG, Soentjens et al. observed a substantial time delay between exposure and administration of HRIG, in particular when the injury occurred abroad (Soentjens et al., 2019a). Therefore, promoting pre-exposure prophylaxis is a key preventive strategy for avoiding the risk of rabies, especially during travel to endemic areas. Rabies pre-exposure prophylaxis (PrEP) greatly simplifies PEP, by obviating the need for HRIG, and by reducing the number of PEP vaccine doses required (Soentjens et al., 2019b).

In April 2018, the WHO published new guidelines for rabies pre- and post-exposure prophylaxis. The new WHO recommendations for rabies vaccination are summarized in **Table 4**. Regarding PrEP, the WHO now recommends a 2-visit regimen, with the additional possibility of a single-visit shot for last-minute travellers who are leaving at very short notice. This new recommendation aims to increase cost-effectiveness, and to spare both doses and time, while still ensuring safety and clinical effectiveness (World Health Organization, 2018d).

As regards senior travellers in particular, there is a gap in the evidence concerning this population. In one retrospective study of vaccination data from 2,112 Belgian military personnel who received intradermal rabies PrEP with a three-shot regimen during the period 2014–2017, only one failed to achieve seroconversion to a level of

TABLE 3 | Categories of exposure to rabies virus, according to World Health Organization (2018d).

Category	Description	Exposure level
Category I	Touching or feeding animals, animal licks on intact skin	None
Category II	Nibbling of uncovered skin, minor scratches or abrasions without bleeding	Exposure
Category III	Single or multiple transdermal bites or scratches, contamination of mucous membrane or broken skin with saliva from animal licks, exposures due to direct contact with bats	Severe exposure

TABLE 4 | WHO recommendations for pre- and post-exposure rabies prophylaxis.

Pre-exposure prophylaxis

-2-sites ID on days 0 and 7
OR
-1-site IM on days 0 and 7

Pre-exposure prophylaxis under specific circumstances[a]

-2-sites ID on day 0
OR
1-site IM on day 0

Post-exposure prophylaxis

	Category I exposure	Category II exposure	Category III exposure
Immunologically naïve, all ages	No PEP required	Immediate vaccination: -2-sites ID on days 0, 3 and 7 OR -1-site IM on days 0, 3, 7 and between day 14–28 OR -2-sites IM on days 0 and 1-site IM on days 7 and 21 RIG is not indicated	Immediate vaccination: -2-sites ID on days 0, 3 and 7 OR -1-site IM on days 0, 3, 7 and between day 14–28 OR -2-sites IM on days 0 and 1-site IM on days 7 and 21 RIG administration is recommended
Previously immunized, all ages	No PEP required	Immediate vaccination[b]: -1-site ID on days 0 and 3 OR -4-sites ID on day 0 OR -1-site IM on days 0 and 3 RIG is not indicated	Immediate vaccination[b]: -1-site ID on days 0 and 3 OR -4-sites ID on day 0 OR -1-site IM on days 0 and 3 RIG is not indicated

ID, intradermal injection; IM, intramuscular injection; RIG, rabies immunoglobulin.
[a]*When there are time constraints or a single visit to the clinic is the only option, the shortened PrEP course can be given, but the patient should plan to receive a second vaccination as soon as possible to complete PrEP. In case of rabies exposure before the second vaccination, the patient is recommended to receive a full course of PEP, with RIG in cases of severe (Category III) exposure.*
[b]*Immediate vaccination is not recommended if complete PEP already received within <3 months previously.*
Note: Immediate washing of the exposure surface/wound is recommended for all age groups, categories of exposure and types of prophylaxis.

rabies virus neutralizing antibodies (RVNA) >0.5 IU/ml, the threshold that is internationally recognized as safe and capable of being boosted with PEP vaccination (Van Nieuwenhove et al., 2019). However, most of the patients included in the study were younger than 60 years of age, and the oldest participant was 62. Data specifically in elderly patients are lacking. A recent systematic review and dose-response meta-analysis reported that older participants required a longer time to achieve maximum geometric mean titre (GMT) levels (42 days in older participants with a median age of 62 years, vs. 30 days in the group with a median age of 28 years) (Xu et al., 2021). In addition, the maximum level achieved by older participants was lower. In this study, no dataset was identified that contained information on GMT levels after PrEP in adults over 50 years of age (Xu et al., 2021). In another study assessing rabies-specific neutralizing antibody responses in a cohort of rabies vaccine recipients (including 97 subjects aged over 50 years) over a period of 20 years, Mansfield et al. reported that following a primary vaccination course, subject age did not appear to influence antibody response, although subjects in the older age group (>50 years) showed a significant decline in antibody titers over time (Mansfield et al., 2016). Finally, in a study of 835 travellers (37.1% aged >50 years) who received intra-dermal PrEP at a travel medicine clinic in South Australia from 2000 to 2016, Furuya-Kanamori et al. investigated immunogenicity post-primary course and post-booster (Furuya-Kanamori et al., 2020). They reported that among the 145 subjects who were aged 60 years or over, 90.5% (95% CI 83.9–94.5%) and 86.7% (95% CI 57.4–96.9) were seropositive after a primary course consisting of 3 doses of 0.1 ml (days 0, 7 and 21–28) or 2 doses of 0.1 ml (days 0 and 7) respectively. Furthermore, the respective rates of seropositive individuals in the group aged

60 years and over were 96.7% (95% CI 91.5–98.8) and 100% after a booster dose, even when the booster dose was given 3 years or more after the initial course, including in older individuals (Furuya-Kanamori et al., 2020).

In this context, the recommendation for rabies prophylaxis in senior travellers is to apply the same principles and procedures as for younger travellers. As stated in the 2018 WHO guidelines, pre- and post-exposure schedules are recommended for all ages. When considering PrEP, the duration of the stay in endemic areas, the remoteness of the destination, the type of activity planned (outdoor activities, caves etc.) the likelihood of prompt medical assistance (potentially including HRIG) being available, are all factors that should be taken into account.

Japanese Encephalitis

Japanese Encephalitis (JE) is an infectious disease of the brain caused by the Japanese encephalitis virus (JEV), an arbovirus spread to humans by *Culex* mosquitoes. It is the most common viral encephalitis in Asia, occurring more frequently in children under the age of 15, because naïve, and in the elderly, as a possible consequence of diminished protection. However, in travellers arriving from non-endemic countries, it can obviously occur at any age since there is no previous immunity (Yun and Lee, 2014). The risk of JE for visitors to endemic areas is clearly dependent on the travel itinerary and exact destination and activities, and has historically been reported to be low, at less than 1 case per million travellers (Hills et al., 2010). However, more recent analyses have advanced the lower figure of 1/400,000 per visit (Lindquist, 2018). Moreover, the consequences of climate change, the spread of the disease into new areas and to both urban and peri-urban settings have made JE an unpredictable emerging disease (Lindquist, 2018).

There is no specific treatment for JE and therefore, and with roughly 30% of fatal cases and 30–50% of survivors with neurologic sequelae (Hills et al., 2019), besides protection from mosquito bites, vaccination remains the mainstay of prevention. There are different vaccines available worldwide, with varying distribution: the most common are an inactivated Vero cell vaccine (licensed in the United States, Europe, and several Asian countries), a live attenuated vaccine (licensed in all Asian endemic countries) and a chimeric live attenuated vaccine (licensed in Australia and a few other endemic countries). Seroconversion rates in older adults are about 65% with the inactivated vaccine available in Europe, whereas data from Australia show that the chimeric vaccine can be immunogenic in >99% of vaccinated individuals older than 60 years of age (Australian Government Dep, 2010). A single-centre, open label, phase IV trial comparing immune response to primary vaccination with the inactivated, adjuvanted JE vaccine reported that vaccine-specific antibody titres were significantly lower in older participants (mean 69, range 61–78 years, $n = 30$) and 47% of them were non- or low responders after the two doses of the vaccine neo-antigen (Wagner et al., 2018). Among 200 subjects aged 64–83 years, Cramer et al. observed seroconversion in 65% of subjects (95% CI 58.1–71.3%) at 70 days after vaccination with the Vero cell-amplified, purified, inactivated JE vaccine (Cramer et al., 2016). While all vaccines have a high profile of tolerability, live vaccines must be avoided in immune-compromised subjects, severe HIV patients as well as in pregnant and breastfeeding women.

Tick-Borne Encephalitis

Tick-Borne Encephalitis (TBE) is another example of a viral infectious disease of the brain spread to humans through the bite of a vector, specifically hard ticks, most frequently of the genus *Ixodes*. Only very few cases of the disease are acquired through the consumption of unpasteurised dairy products. Affected areas spread from Central-Eastern Europe north and eastwards through Russia and Siberia up to the coastline of the Pacific Ocean. Publications of imported cases of TBE due to travel are scarce, and therefore, the travel-associated risk is difficult to identify, but based on estimates of exposed visitors, Steffen et al. roughly extrapolated the attack rate for an undefined period of time to be 0.5–1.3 per 100,000 (1 per 77,000–200,000) overall in Western/Central Europe endemic areas for the exposed at-risk population (Steffen, 2016). TBE is endemic in many countries of the European Union, including Germany, Austria, Sweden and Switzerland (European Centre for Disea, 2012), yet many travellers may not be aware that such destinations might require specific vaccination. Several European countries recommend TBE vaccination, including Austria, Finland, the Czech Republic, Latvia and Slovenia. Sweden also recommendations vaccination consisting of three doses during the first year (or four if aged 50 or over) followed by a first booster dose after three years, then every 5 years, regardless of age.

Although TBE infection tends to display seasonality, accordingly to the tick life cycle (spring to autumn), global climate change and the milder winters make occurrence of disease possible all year round. The incidence varies greatly between countries and between regions of the same country. Regarding TBE infection among travellers, both the number at risk and the number affected are far from being precise (Steffen, 2016), and therefore only a crude estimate can be presented (Chrdle et al., 2016). As in other similar viral infections, the case-fatality rate is between 0.5 and 10%, but the percentage of survivors suffering from chronic neurologic complications can be as high as 60% (Taba et al., 2017). The clinical course is more severe with increasing age, especially as a consequence of immune senescence (Kunze, 2016).

Similar to what has been described for JE, prevention of TBE is also based on the avoidance of tick bites and more importantly, on vaccination (Chrdle et al., 2016). Four different TBE vaccines are available (two European and two Russian); all are safe and the main contraindications are represented only by severe allergy to eggs or to any specific component. There is no upper age limit to receive the vaccine, especially in consideration of the worse clinical scenarios in older adults. Both European vaccines require three doses for the full primary schedule. Boosters are needed every 3–5 years depending on risk exposure and age (the older the vaccinee, the shorter the boosting interval). A shorter primary schedule is validated for both European vaccines (Haditsch and Kunze, 2013). In a systematic review evaluating the immunogenicity and safety of TBE vaccination, Rampa et al. reported that lower protective antibody titers were found, with a diminishing immune response in individuals aged 60 years and

over, and in studies investigating vaccine failure, most (although not all) failures occurred in individuals aged 50 years or over (Rampa et al., 2020). In one of the studies included in this systematic review, Weinberger et al. investigated antibody titers and booster responses in 79 healthy Austrian volunteers receiving TBE vaccination and aged 50 years or over, in comparison to a control group aged <30 years (Weinberger et al., 2010). They reported that lower antibody concentrations and neutralizing titers were found starting from the age of 50 years, and that booster vaccination even 5–7 years after the previous vaccine administration was successful in inducing adequate protection, even in older individuals (Weinberger et al., 2010).

Dengue

Dengue is the most widespread mosquito-borne disease after malaria, and the leading cause of fever in returning travellers, ahead of malaria (Schwartz et al., 2008). A Swedish study of residents with confirmed dengue after travel between 1995 and 2010 reported an increasing trend over time for most destinations, with the majority of dengue cases acquired in Thailand (492/925 travellers; 53%) (Rocklöv et al., 2014).

Dengue fever is caused by a flavivirus, named dengue virus, transmitted to humans by *Aedes* mosquitoes. This virus exists in 4 serotypes, each able to elicit a serotype-specific sustained immune response. According to the most accepted theory, antibodies arising from a first dengue infection act as an enhancer in case of a new infection with a different serotype, causing the novel virus to enter more rapidly into target cells and multiply faster. This in turn increases the risk of severe disease, especially the so-called dengue haemorrhagic fever (the theory of the "enhanced antibody response") (Halstead and Wilder-Smith, 2019). The substantial climate changes of recent years, the wider distribution of *Aedes* mosquitoes, and the mounting evidence that *Aedes albopictus* (Asian tiger mosquito) is also becoming a better vector for this virus, all make dengue likely the most important arboviral infection on the international scene (Johansson et al., 2019). Although there have been few studies specifically in older people, available data suggest that the disease is of greater severity in older individuals, with worse outcomes compared to their younger counterparts (Lee et al., 2013; Rowe et al., 2014).

A call for a vaccine was launched several years ago, ending with the license of the first tetravalent dengue vaccine in 2015 (Villar et al., 2015). Shortly after its launch, evidence that the number of hospitalisations secondary to vaccination tended to increase in naïve children in the Philippines made the WHO withdraw the campaign of immunisation, and limit the use of this vaccine only to people with proven existing immunity to a previous dengue infection (Wilder-Smith, 2018). A new tetravalent dengue vaccine, presumably free from drawbacks that beset its predecessor, is about to be launched (Biswal et al., 2019), giving new hope for effective prevention of this scourge.

CONCLUSION

In summary, the number of older people who have the financial resources and good health to travel is increasing dramatically, and age in itself is not a contra-indication to travel. However, age-related changes to the immune system and the higher prevalence of chronic disease and frailty create a specific risk profile for older travellers. All older individuals planning to travel should be advised during specific pre-travel consultations, or during routine visits to a healthcare professional, about travel-specific vaccines, and preventive behaviors in view of travel. The destination, duration and purpose of the trip, the activities planned, the type of accommodation and plans for other future travels are all important elements that will help orient advice and information prior to travel, while patient-specific characteristics, such as health status and current medications, must be noted to tailor specific recommendations. Senior travellers should consult a travel medicine specialist long in advance of a planned trip in order to allow time to obtain appropriate travel-specific vaccines. Physicians should allow ample time for consultations with elderly patients envisaging travel, as these patients may need more information, and their health status may require more thorough investigation.

AUTHOR CONTRIBUTIONS

Conception: SM, J-PM, and AR; data curation: FE, NV, and AR; analysis and interpretation: FE, SM, J-PM, NV, and AR; writing of the first draft: FE, SM, J-PM, NV, and AR; critical revision and final approval: FE, SM, J-PM, NV, and AR.

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Inflammasome Activity in Response to Influenza Vaccination Is Maintained in Monocyte-Derived Peripheral Blood Macrophages in Older Adults

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Introduction: Each year, a disproportionate number of the total seasonal influenza-related hospitalizations (90%) and deaths (70%) occur among adults who are >65 years old. Inflammasome activation has been shown to be important for protection against influenza infection in animal models but has not yet been demonstrated in humans. We hypothesized that age-related dysfunction (immunosenescence) of the inflammasome may be associated with poor influenza-vaccine response among older adults.

Methods: A cohort of younger (18–40 years of age) and older (≥65 years of age) adults was recruited prior to the 2014–2015 influenza season. We measured hemagglutination inhibition (HAI) titers in serum before and 28 days after receipt of the seasonal inactivated influenza vaccine. Inflammasome-related gene expression and protein secretion were quantified in monocyte-derived macrophages following stimulation with influenza A/H1N1 virus.

Results: Younger adults exhibited higher HAI titers compared to older adults following vaccination, although inflammasome-related protein secretion in response to influenza stimulation was similar between the age groups. Expression of *P2RX7* following influenza stimulation was lower among older adults. Interestingly, *CFLAR* expression was significantly higher among females ($p = 2.42 \times 10^{-5}$) following influenza stimulation and this gene may play an important role in the development of higher HAI antibody titers among older females.

Conclusion: Inflammasome activation in response to influenza vaccination appears to be maintained in monocyte-derived macrophages from older adults and does not explain the poor influenza vaccine responses generally observed among this age group.

Keywords: inflammasome, influenza, NLRP3, vaccination, aging

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INTRODUCTION

Seasonal influenza A has varying yearly mortality and is estimated to cause 3,000–49,000 deaths annually, resulting in over 250,000 excess hospitalizations and related healthcare costs exceeding $90 billion in the US alone (Poland, 2009; Centers for Disease Control and Prevention, 2010). The 2017–2018 influenza season saw marked increases in morbidity and mortality, with 79,400 reported

deaths and 959,000 influenza-related hospitalizations (Rolfes et al., 2019). According to the Centers for Disease Control and Prevention (CDC), approximately 90% of influenza-related deaths and 70% of reported hospitalizations occurred among adults ≥65 years old (Rolfes et al., 2019). Older adults suffer disproportionately from influenza—and its exacerbation of underlying co-morbidities—as a result of immunosenescence, which is a collective of inherent age-associated changes in immune cell biology that results in weakened B cell and T cell immune responses (Crooke et al., 2019a; Crooke et al., 2019b). Immunosenescence not only limits the immune response against natural infections but also compromises responses to vaccination, thus hindering the primary preventive strategy employed against seasonal influenza. Although vaccine formulations have been specifically designed to improve immune responses in older adults, influenza-specific antibody titers induced by these vaccines are often still lower than those observed in younger adults receiving a standard dose trivalent influenza vaccine (TIV) (Goodwin et al., 2006; Chen et al., 2011).

Immunosenescence is marked by a series of complex biological changes that clearly impact adaptive immunity; however, age-related changes to the innate immune system are far less understood or characterized. A limited number of studies investigating this phenomenon in the context of influenza have implicated dysregulated cytokine production as one of the primary factors associated with poor immune outcomes. Sridharan and others have observed decreased secretion of IFN type I and IFN type III from aged plasmacytoid dendritic cells (pDCs) in response to influenza virus stimulation Sridharan et al. (2011), and correlations between decreased cytokine responses and influenza-specific antibody titer have also been reported (Panda et al., 2010). Significant decreases in IL-6, TNF-α, IL-12p40, and IFN-α production have been observed following Toll-like receptor (TLR) stimulation in myeloid dendritic cells (mDCs) and pDCs from older adults, identifying strong associations between dysregulated TLR function and influenza antibody response (Panda et al., 2010). While these studies highlight important aspects of innate immunity that are impacted during aging, the full extent to which immunosenescence affects innate immunological responses to influenza virus is currently unknown.

The inflammasomes are a class of multimeric complexes comprised of NOD-like receptors (NLRs) that are responsible for the enzymatic processing and maturation of certain innate cytokines (e.g., IL-1β, IL-18) (Schroder and Tschopp, 2010), and studies have found recognition of influenza A by the inflammasome complex to be essential for establishing protective adaptive immunity (Ichinohe et al., 2009). Activation of the inflammasome occurs upon recognition of intracellular pathogens or other cellular stressors through two distinct signaling events. In the case of influenza, recognition of viral RNA by TLR7 results in the NF-κB-mediated expression of inflammatory cytokine proforms (signal 1), while the influenza virus M2 protein or the PB1-F2 polymerase stimulate activation of the NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome complex (signal 2) (Ichinohe et al., 2010; McAuley et al., 2013). Activation of the inflammasome complex leads to increased caspase-1 activity, the processing of inflammatory cytokines into their bioactive forms, and the ultimate release of inflammatory mediators via pyroptosis (Fink and Cookson, 2005).

Multiple animal studies have demonstrated the importance of the inflammasome during influenza infection. Increased mortality, massive cellular infiltration, and elevated NO production have been observed in *Il18*$^{-/-}$ mice infected with influenza A (Liu et al., 2004); with increased susceptibility to influenza reported for *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice (Thomas et al., 2009). Schmitz and others observed decreased neutrophil and CD4^{+} T cell recruitment, diminished inflammatory lung infiltrates, reduced mucosal and serum IgM levels, and decreased CD4^{+} T cell activation following influenza A infection in mice deficient for the IL-1β receptor (Schmitz et al., 2005). A decline in inflammasome function with age has also been observed to contribute to increased mortality in mice following influenza infection (Stout-Delgado et al., 2012). Despite increasing evidence supporting the role of inflammasomes during influenza infection in animal models, studies investigating inflammasome activity in humans are scarce.

In this work, we sought to investigate the contributions of inflammasome activation to vaccine-induced humoral immunity as well as the impact of age and immunosenescence on inflammasome function. To do this, we recruited 147 subjects to receive a standard dose of seasonal TIV balanced by age and sex. We assessed humoral immunity post-vaccination by hemagglutination inhibition (HAI) titers and evaluated inflammasome activity in macrophages derived from peripheral blood monocytes at both the gene and protein level using reverse-transcription (RT)-PCR and cytokine ELISA. To our knowledge, this is the first study to investigate the influence of age on vaccine-induced inflammasome activation in humans.

METHODS

Subject Recruitment

The final study cohort was comprised of 147 adult subjects divided into two subgroups on the basis of age: 18–40 years of age ($n = 75$) and ≥65 years of age ($n = 72$). All subjects in the study were recruited in 2014 from Olmsted County, MN, and the surrounding area. Each subject completed a blood draw prior to receipt of the 2014–2015 standard dose TIV, at 24 h post-vaccination, and at 28 days post-vaccination. Peripheral blood mononuclear cells (PBMCs) and serum samples collected at each timepoint were processed for cryogenic storage using previously standardized protocols (Umlauf et al., 2012). All study procedures were reviewed and approved by the Mayo Clinic Institutional Review Board, and written informed consent was obtained from each study participant.

Hemagglutination Inhibition Titers

HAI assays were performed using a standardized procedure set forth by the Human Immunology Project Consortium (Wang et al., 2008). Briefly, serum samples were treated overnight with receptor-destroying enzyme (RDE) from *Vibrio cholerae*

(MilliporeSigma; Burlington, MA) and subsequently heat-inactivated for 45 min at 56°C. Serial two-fold dilutions of RDE-treated serum (25 µl; beginning at 1:10) were prepared in 1x PBS and incubated with influenza virus A/H1N1/California/7/2009 (25 µl; hemagglutination titer = 8 HA units) in a 96-well V-bottom plate for 30 min at room temperature. A 0.5% solution of turkey red blood cells (Lampire Biological Laboratories; Pipersville, PA) was subsequently added to each sample and incubated for 45 min at 4°C. Serum samples were analyzed in duplicate with a third measurement being conducted when the initial two runs differed. The HAI titer was reported as the reciprocal of the highest dilution where hemagglutination was inhibited.

Macrophage Culture

$CD14^+$ monocytes were isolated from PBMCs and cultured for 2 weeks under conditions supporting differentiation into macrophages. Briefly, $CD14^+$ monocytes were isolated from whole PBMCs through negative selection using the human Monocyte Isolation Kit II (Miltenyi Biotec, Inc.; Auburn, CA). Purified $CD14^+$ monocytes were incubated in serum-free Macrophage-SFM culture media (Thermo Fisher Scientific; Waltham, MA) supplemented with 20 mM HEPES, 10 ng/mL GM-CSF, 1% penicillin/streptomycin, and 1% amphotericin B, for 2 weeks at 37°C. Fresh culture media was added every 48 h. Cells were subsequently washed with 1x PBS, and non-adherent cells were discarded. Differentiated macrophages were detached using 0.25% trypsin-EDTA, harvested, and plated for all subsequent assays. Differentiation status and purity of the macrophage population was determined visually and by flow cytometry, by decreased expression of CD14 and increased expression of CD16 (Fluorochrome-labeled antibodies obtained from BD Biosciences, CA) (Akagawa et al., 2006; Jin and Kruth, 2016).

Inflammatory Cytokine Measurements

$CD14^+$ macrophages were isolated from differentiation cultures and 5×10^4 cells added to each well of a 96-well tissue culture plate (round bottom). Cells were incubated at 37°C with media alone (unstimulated), influenza virus A/H1N1/California/7/2009 (MOI = 0.5), resiquimod (R848, 1 µg/ml; Mabtech, Inc.; Cincinnati, OH), or both influenza virus and R848. After 24 h, cell culture supernatants were collected and assayed for IL-1β, pro-IL-1β, caspase-1, and IL-18 using Quantikine® ELISA kits (R&D Systems, Inc.; Minneapolis, MN) and Human IL-18 Instant ELISA kits (Thermo Fisher Scientific; Waltham, MA) according to the respective manufacturer's protocols. The coefficient of variation (CV) for all cytokine ELISA assays are presented in **Supplementary Table S1**.

Inflammatory Gene Expression

$CD14^+$ macrophages from the cytokine assays detailed above were stored at −80°C in RNAprotect Cell Reagent (Qiagen, Inc.; Valencia, CA) until further use. Cell pellets were subsequently thawed and total RNA was extracted using RNeasy Mini kits (Qiagen, Inc.; Valencia, CA) according to the manufacturer's protocol. Isolated RNA was quantitated, normalized for concentration across all samples, and sent to the Mayo Clinic Medical Genome Facility for quality control and analysis. All RNA samples were analyzed using an inflammasome pathway-focused RT^2 Profiler™ PCR Array (PAHS-097Z, Qiagen, Inc.; Valencia, CA).

Statistical Analysis

Difference between age groups in demographic variables were assessed using Pearson's chi-square test. Correlations between variables are calculated using Spearman's rank correlation, Wilcoxon's rank-sum test was used to test for differences between age groups and sexes for all continuous variables. Wilcoxon's signed-rank test was used to test for differences when the data is paired (e.g. stimulated vs unstimulated values). Gene expression values (ΔCt) were first normalized by subtracting the cycle threshold (Ct) from the corresponding *B2M* housekeeping gene from the Ct value for each gene. Log_2 fold-changes for stimulated vs unstimulated samples were calculated by taking the difference between the ΔCt values for the unstimulated minus the ΔCt from the stimulated samples.

RESULTS

Subject Demographics

The study cohort of 147 subjects ranged from 18.1 to 92.3 years of age and was designed to equally represent both younger (18–40 years of age, $n = 75$) and older (≥65 years of age, $n = 72$) age groups. The study cohort was predominantly White (89%), with the remaining individuals identifying as either Asian (3%), Other (1%), or electing not to disclose their ethnicity (7%). Most of the study participants (89%) identified as non-Hispanic or Latino. The younger and older age subgroups were 58.7 and 59.7% female, respectively. See **Table 1** for a complete summary of subject demographics.

Humoral Response to Influenza Vaccination

As expected, HAI titers against influenza A/H1N1 were significantly higher 28 days after vaccination with TIV (Day 28 median HAI titer = 1:160; $p = 8.6 \times 10^{-10}$) compared to pre-vaccination titers (Baseline median HAI titer = 1:80) in all subjects as well as within both younger ($p = 1.9 \times 10^{-5}$) and older ($p = 8.3 \times 10^{-6}$) age subgroups (**Figure 1A**). Younger subjects exhibited significantly higher median HAI titers compared to older subjects both at Baseline (1:160 vs 1:40; $p = 2.3 \times 10^{-8}$) and Day 28 (1:160 vs 1:80; $p = 2.5 \times 10^{-8}$) (**Figure 1B**). Following vaccination, 7 of 114 (6%) of the subjects were found to seroconvert (as defined by a 4-fold increase in HAI titer), and 94% of the subjects were seropositive, defined as an HAI titer ≥1:40, at Day 28 which is also considered a correlate of protection (Cox, 2013). We compared seroconversion rates in subjects with low HAI titers (<40) before vaccination to subjects with higher HAI titers (≥40) and found: 4/22 (18.2%) versus 3/92 (3.3%%) seroconverted, respectively. When comparing the younger (18–40) and older (≥65) subgroups, we found that 10% of the younger subjects seroconverted and 100% were seropositive at Day 28; while 2% of the older subjects seroconverted and 87%

TABLE 1 | Study cohort demographics.

	Young (N = 75)	Old (N = 72)	Total (N = 147)	*p* value
Age				
Mean (SD)	26.3 (4.03)	73.5 (5.46)	49.5 (24.1)	—
Q1, Q3	24.1, 29.1	69.3, 76.6	26.8, 72.8	—
Range	18.1–39.6	65.2–92.3	18.1–92.3	—
Sex	—	—	—	0.896[1]
Female	44 (58.7%)	43 (59.7%)	87 (59.2%)	—
Male	31 (41.3%)	29 (40.3%)	60 (40.8%)	—
Race	—	—	—	0.019[1]
White	62 (82.7%)	69 (95.8%)	131 (89.1%)	—
Asian	5 (6.7%)	0 (0%)	5 (3.4%)	—
Other	0 (0%)	1 (1.39%)	1 (0.68%)	—
Unknown	8 (10.7%)	2 (2.78%)	10 (6.8%)	—
Ethnicity	—	—	—	—
Choose Not to Disclose	0 (0%)	1 (1.39%)	1 (0.68%)	—
Hispanic or Latino	0 (0%)	1 (1.39%)	1 (0.68%)	—
Not Hispanic or Latino	64 (85.3%)	67 (93.1%)	131 (89.1%)	—
Unknown	11 (14.7%)	3 (4.17%)	14 (9.52%)	0.086[1]

[1]*Pearson's Chi-squared test.*

were seropositive at Day 28. Recognizing that the rates of seroconversion were low, we relaxed the definition to a 2-fold rise in HAI titer and repeated the analysis, revealing a similar seroconversion trend with respect to baseline HAI titer (40.9% of low HAI subjects seroconverted compared to 10.0% of high HAI subjects), and a similar rate of seroconversion for younger and older subjects (16.7% for both).

No significant difference in HAI titer was detected between men and women in the overall cohort; however, sex-based differences were apparent within each age subgroup (**Figure 1C**; **Supplementary Figure S1**). Younger men exhibited slightly higher HAI titers than young women at Baseline (p = 0.048), and this disparity became more apparent following vaccination (p = 0.019). Conversely, women in the older age group exhibited markedly higher HAI titers than men following vaccination (p = 0.0021), despite having similar HAI titers at Baseline (p = 0.38).

Inflammasome-Mediated Protein Production Following Influenza Vaccination

In order to assess inflammasome activity, we measured IL-1β, pro-IL-1β, IL-18, and caspase-1 production by PBMCs in response to *in vitro* stimulation with the following: A/H1N1 influenza virus, resiquimod (R848, a TLR7 agonist), or a combination of A/H1N1 and R848. Stimulation of whole PBMCs resulted in high but variable levels of pro-IL-1β and IL-1β, while production of IL-18 and caspase-1 was much lower but also inconsistent (data not shown). Because monocytes and macrophages have been suggested to play a key role in immune responses to influenza with age (Roberts, 2020), we refocused our analyses on $CD14^+$ peripheral blood monocytes. Monocytes were isolated using negative selection to minimize manipulation of the cells and cultured *in vitro* under conditions to promote macrophage differentiation prior to stimulation (Jin and Kruth, 2016). As expected, differentiated macrophages had an oval, "fried egg morphology" and expressed high levels of CD16 with little to no CD14 (Akagawa et al., 2006).

We first evaluated the extent of inflammasome activity in macrophages at baseline. Levels of inflammasome-related proteins and cytokines are summarized in **Figure 2** and **Supplementary Tables S2–S4**. Influenza stimulation resulted in a significant percent increase in caspase-1 (27.2%, p = 0.007), pro-IL-1β (19.1%, $p = 2.22 \times 10^{-16}$), and IL-1β (22.8%, $p = 2.22 \times 10^{-16}$) secretion relative to unstimulated macrophages. Treatment with R848 induced similar increases in pro-IL-1β (24.5%, $p = 2.22 \times 10^{-16}$) and IL-1β (33.6%, $p = 2.22 \times 10^{-16}$) production, but a slight decrease in caspase-1 levels (-3.1%, p = 0.02). Combined stimulation with influenza and R848 resulted in markedly increased production of pro-IL-1β (153.9%, $p = 2.22 \times 10^{-16}$) and IL-1β (339.8%, $p = 2.22 \times 10^{-16}$), with more moderate but significant increases in caspase-1 (32.0%, p = 0.009) and IL-18 (8.5%, $p = 2 \times 10^{-5}$).

We next evaluated if age or biological sex were associated with baseline inflammasome activity (**Supplementary Tables S3 and S4**). Interestingly, only IL-1β secretion in response to influenza virus at day 1 (33.8 vs 22.4 pg/ml; p = 0.025) and IL-18 secretion in response to R848 at baseline (1.35 vs 0.37 pg/ml; p = 0.021) were significantly associated with age and both were higher in older adults. Regarding sex-based differences, females exhibited significantly higher caspase-1 (21.16 vs 3.16 pg/ml; p = 0.029) and pro-IL-1β levels (50.97 vs 32.9 pg/ml; p = 0.016) in response to influenza stimulation at baseline, whereas males secreted higher levels of pro-IL-1β (49.39 vs 24.9 pg/ml; p = 0.034) and IL-18 (3.06 vs 0.83 pg/ml; p = 0.0093) at Day 1 in response to stimulation with R848 or influenza, respectively.

Influenza vaccination did not appear to have an immediate effect on macrophage inflammasome activity, although IL-1β levels were slightly elevated in unstimulated macrophages 24 h post-vaccination compared to baseline (161.27 vs 152.01 pg/ml, p = 0.01). Stimulation of Day 1 macrophages with influenza virus elicited a significant increase in caspase-1 production (35.7%, $p = 9.8 \times 10^{-5}$), but more moderate increases in IL-18 (4.2%,

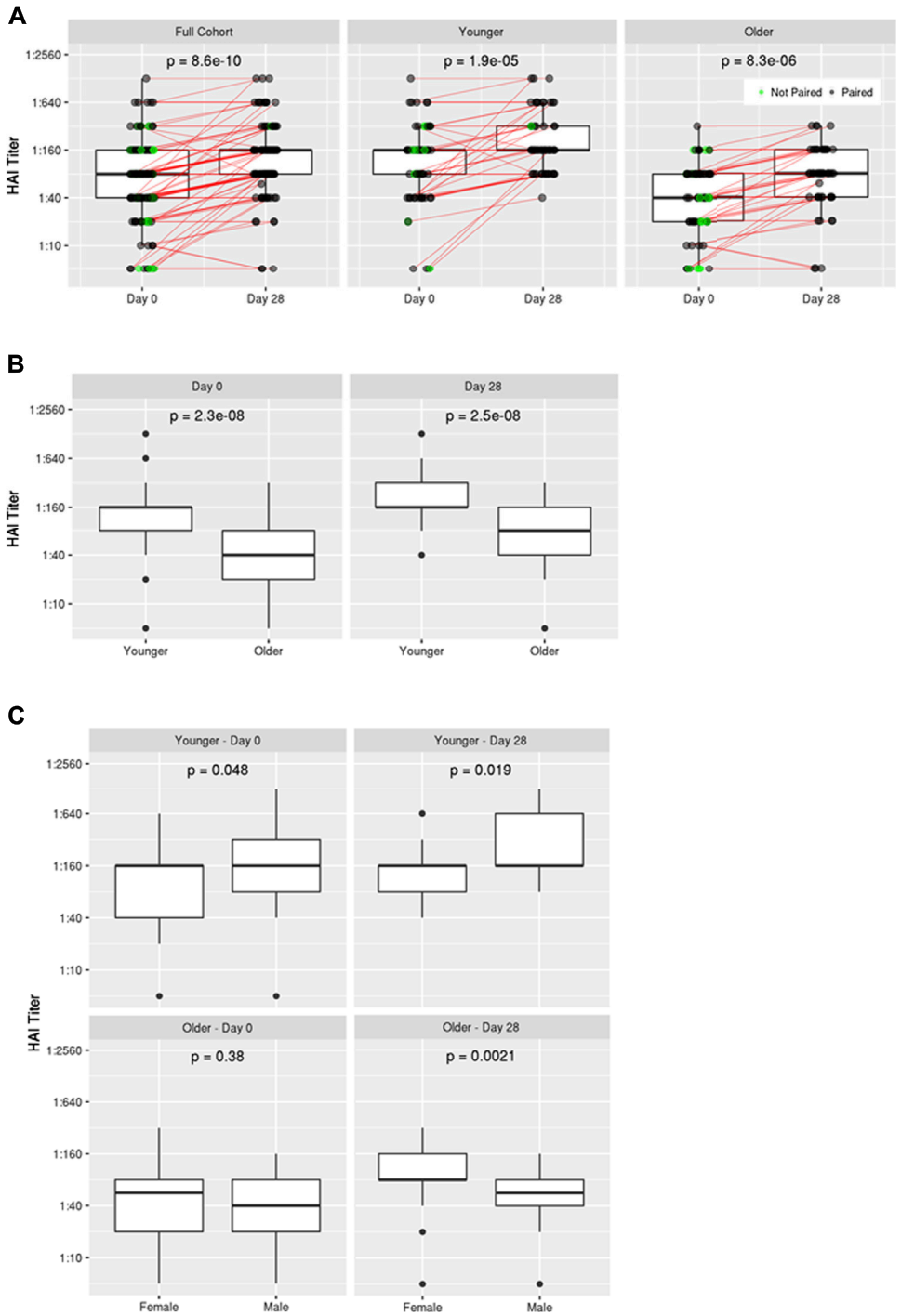

FIGURE 1 | Influenza A/H1N1 HAI titers pre- and post-vaccination. **(A)** Comparison of HAI titers at Baseline (Day 0) and Day 28 for the full cohort as well as younger and older subgroups, **(B)** Comparison of HAI titers between younger and older subgroups at Day 0 and Day 28, **(C)** Comparison of HAI titers in younger and older subgroups at Day 0 and Day 28 on the basis of biological sex.

$p = 0.0013$), IL-1β (15.7%, $p = 2.22 \times 10^{-16}$), and pro-IL-1β (13.4%, $p = 3.43 \times 10^{-14}$) compared to unstimulated. Treatment with R848 induced moderate increases in IL-1β (36.7%, $p = 2.22 \times 10^{-16}$) and pro-IL-1β (15.9%, $p = 4.49 \times 10^{-15}$) production, while combined influenza and R848 stimulation resulted in significant increases for all four proteins (**Figure 2**, **Supplementary Table S2**). Interestingly, the magnitude of caspase-1 ($p = 0.012$) and IL-18 ($p = 0.024$) production in response to influenza + R848 was greater at Day 1 compared to baseline, while the opposite was true for IL-1β ($p = 0.031$) and pro-IL-1β ($p = 0.024$)

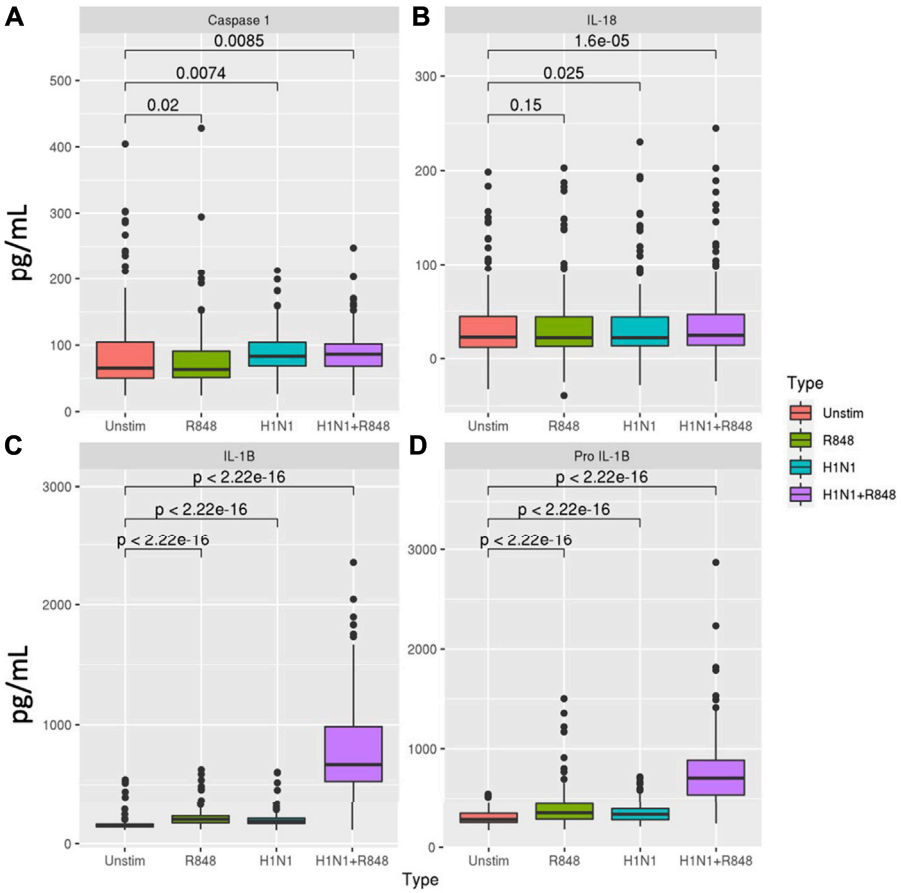

FIGURE 2 | Inflammasome-related caspase and cytokine secretion profiles in macrophages. Comparison of **(A)** caspase-1, **(B)** IL-18, **(C)** IL-1β, and **(D)** pro-IL-1β secretion in response to either R848 (a TLR7 agonist), influenza A/H1N1 virus, or a combination of both stimuli for subjects with sufficient cells for evaluation (n = 138). p-values derived from Wilcoxon's signed-rank test.

secretion in response to influenza alone. We did not observe any associations between inflammasome-mediated cytokine production and change in HAI titer after vaccination (R ranged from -0.057 to 0.031 and $p > 0.56$ for all 4 outcomes). Correlations with inflammasome activity were also weak to non-existent for baseline and Day 28 HAI titer.While we believe that signaling *via* TLR7 plays an important role (as evidenced by our data from cells stimulated with R848 alone), but we do not think a TLR7-related mechanism/pathway solely explains our observations. Stimulation of cells with A/H1N1 alone often resulted in similar levels of cytokine production as R848 alone (see **Supplementary Table S2**). Only when both signals were provided to the cells did we note a synergistic effect on cytokine/protein production–which is presumably due to complete inflammasome activation.

Expression of Inflammasome-Related Genes Following Influenza Vaccination

To further characterize the contributions of inflammasome activation to vaccine-induced influenza immunity, we isolated RNA from cultured macrophages at Baseline and evaluated the expression levels of 84 inflammasome-related genes using a custom RT-PCR array. Baseline unstimulated gene expression was associated with age in a single gene (*P2RX7*) which had a significantly lower expression in older subjects ($p = 0.006$). Comparing baseline unstimulated gene expression between sexes, eight genes (i.e., *CFLAR, NFKBIB, NFKBIA, CCL7, CHUK, HSP90AB1, RIPK2, PYCARD*) were identified with significantly higher expression levels ($p < 0.01$) among women. There were fewer differences observed between old and young individuals, suggesting that macrophages from both age groups had a similar ability to activate inflammasome pathways upon stimulation. Older subjects had greater expression of *CASP5* ($p = 0.013$) and lower expression of *CCL2* (0.043) and *CFLAR* (0.006). Following stimulation of baseline macrophages with A/H1N1 and resiquimod, we identified 51 genes with significantly altered expression levels (28 upregulated, 23 downregulated; $p < 0.01$) (**Figure 3**, **Table 2**). When evaluating the effects of both age and sex on gene expression in response to influenza stimulation, only *CFLAR* expression was statistically significant for age ($p = 0.006$), but not with sex ($p = 0.057$).

We detected several correlations between changes in gene expression at baseline and the secretion of inflammasome-related proteins. Caspase-1 secretion at baseline was moderately

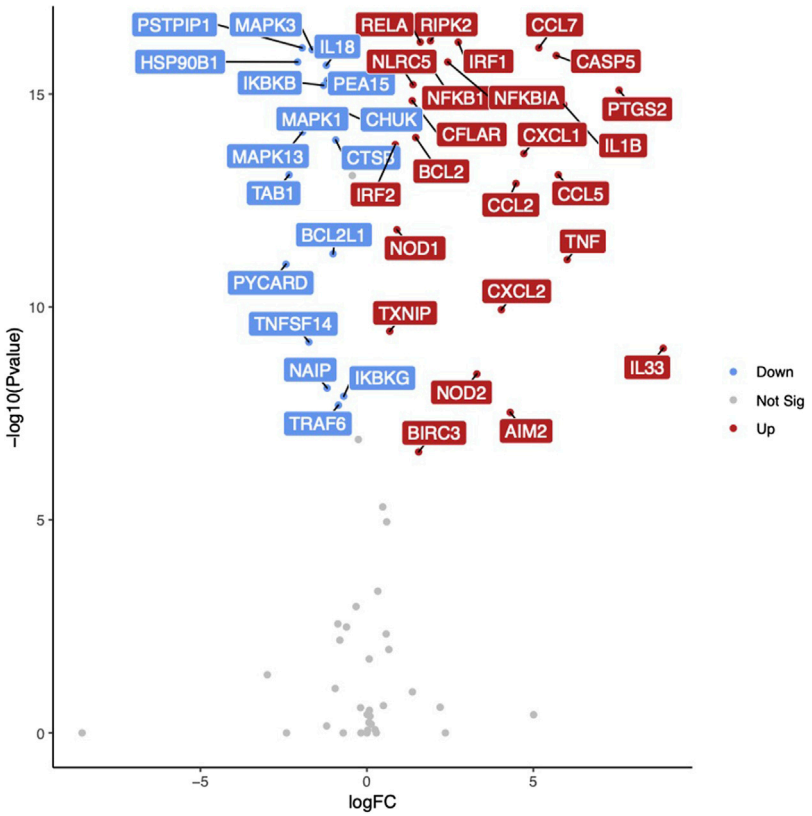

FIGURE 3 | Inflammasome-related gene expression in response to influenza stimulation. Volcano plot illustrating differentially expressed genes in monocyte-derived macrophages at Baseline in response to influenza A/H1N1 + R848 stimulation. Gene names highlighted for 40 genes with lowest *p*-values. Red indicates increased gene expression, blue indicates decreased gene expression.

associated with *TXNIP* expression ($r = 0.278$, $p = 0.01$), while IL-18 secretion at Day 1 was positively associated with *CASP1* expression ($r = 0.344$, $p = 0.002$) and negatively associated with *TNF* expression ($r = -0.372$, $p = 0.007$). Secreted levels of pro-IL-1β were positively associated with the expression of several inflammasome-related genes at baseline (*PTGS2*: $r = 0.421$, $p < 0.005$; *RELA*: $r = 0.318$, $p = 0.002$; *RIPK2*: $r = 0.265$, $p = 0.01$) and 24 h post-vaccination (*CCL2*: $r = 0.385$, $p = 0.002$; *HSP90AB1*: $r = 0.311$, $p = 0.006$). IL-1β secretion at baseline was positively associated with *CXCL1*($r = 0.437$, $p < 0.005$) and *PTGS2* ($r = 0.423$, $p < 0.005$) expression but negatively associated with *NAIP* ($r = -0.423$, $p = 0.004$), *PEA15* ($r = -0.297$, $p = 0.004$), and *MAPK3* expression ($r = -0.272$, $p = 0.009$).

Interestingly, we did not detect any robust correlations between the expression of inflammasome-related genes in response to influenza stimulation at baseline and HAI titers at either timepoint.

DISCUSSION

In the current study, we evaluated the effect of age on inflammasome activation in response to the influenza vaccine among a cohort comprised equally of younger ($n = 75$) and older ($n = 72$) healthy adults. We observed lower than expected seroconversion rates in our cohort (≥4 fold rise in HAI titer). This may have been due to the considerable pre-existing immunity present in the cohort, as 75% of the cohort had titers of 1:40 at baseline. We and others have previously observed a negative correlation baseline HAI titer and seroconversion following another dose of influenza vaccine in older adults Ehrlich et al. (2012), Jacobson et al. (2015), as well as in children and adolescents (Ehrlich et al., 2012). As expected, younger adults exhibited higher median HAI titers than older adults both before and 28 days following influenza vaccination, although the majority of subjects (94%) achieved HAI titers ≥1:40 (the presumptive correlate of protection for influenza) (Cox, 2013). This is consistent with studies by our group and others that have consistently observed lower antibody responses among older adults following influenza vaccination (Hobson et al., 1972). Notably, inflammasome activation in monocyte-derived macrophages was similar between both age groups, as assessed by the production of inflammasome-associated proteins in response to influenza stimulation. In fact, IL-1β secretion in response to influenza stimulation at 24 h post-vaccination was higher in older adults. These findings provide evidence that inflammasome function in humans is not impaired with age (at least in peripheral

TABLE 2 | Inflammasome-related gene expression in response to influenza stimulation.

Genes	N	Median log_2 FC	Median FC[a]	*p*-value[b]
RIPK2	93	1.909	3.755	5.66E-17
IRF1	93	2.746	6.709	6.04E-17
RELA	93	1.604	3.041	6.04E-17
CCL7	92	5.166	35.91	8.28E-17
PSTPIP1	92	−1.942	0.26	8.28E-17
MAPK3	92	−1.642	0.32	9.14E-17
NFKB1	91	1.691	3.23	1.21E-16
CASP5	91	5.69	51.634	1.25E-16
HSP90B1	90	−2.079	0.237	1.77E-16
NFKBIA	90	2.429	5.386	1.77E-16
IL18	93	−1.217	0.43	2.12E-16
PEA15	90	−1.19	0.438	4.83E-16
NLRC5	89	1.385	2.611	6.04E-16
IKBKB	90	−1.3	0.406	6.29E-16
PTGS2	86	7.576	190.837	8.13E-16
CFLAR	90	1.361	2.568	1.43E-15
IL1B	86	5.916	60.379	1.76E-15
CHUK	93	−0.714	0.609	2.53E-15
MAPK1	92	−1.184	0.44	2.82E-15
MAPK13	82	−1.933	0.262	7.78E-15
BCL2	90	1.467	2.764	1.05E-14
CTSB	89	−0.935	0.523	1.20E-14
IRF2	91	0.854	1.808	1.54E-14
CXCL1	77	4.715	26.258	2.51E-14
CCL5	74	5.75	53.814	7.89E-14
TAB1	74	−2.337	0.198	7.89E-14
HSP90AB1	91	−0.437	0.739	8.23E-14
CCL2	73	4.479	22.294	1.26E-13
NOD1	88	0.903	1.87	1.53E-12
BCL2L1	90	−1.015	0.495	5.64E-12
TNF	62	6.016	64.724	7.77E-12
PYCARD	62	−2.43	0.186	9.91E-12
CXCL2	34	4.04	16.451	1.16E-10
TXNIP	86	0.687	1.61	3.72E-10
TNFSF14	74	−1.741	0.299	6.67E-10
IL33	31	8.903	478.796	9.31E-10
NOD2	29	3.305	9.886	3.73E-09
NAIP	45	−1.194	0.437	8.03E-09
IKBKG	76	−0.695	0.618	1.25E-08
TRAF6	77	−0.854	0.553	2.02E-08
AIM2	26	4.305	19.767	2.98E-08
HSP90AA1	93	−0.255	0.838	1.29E-07
BIRC3	36	1.555	2.938	2.53E-07
PPC	93	0.477	1.392	4.94E-06
RTC	90	0.596	1.512	1.11E-05
CASP1	90	0.329	1.256	0.000471
P2RX7	86	−0.324	0.799	0.00108
MAPK9	29	−0.871	0.547	0.00276
SUGT1	58	−0.613	0.654	0.00326
PANX1	18	0.579	1.494	0.00475
MAP3K7	35	−0.811	0.57	0.00665
FADD	17	0.66	1.58	0.011
CASP8	92	0.071	1.05	0.0184
TNFSF4	18	−2.99	0.127	0.0432
CARD6	14	−0.952	0.544	0.0906
NLRP3	7	1.368	2.582	0.109
BIRC2	18	0.498	1.413	0.229
IL6	3	2.201	4.599	0.25
MAPK8	76	−0.189	0.877	0.256
NFKBIB	89	0.076	1.054	0.295
TAB2	77	0.01	1.007	0.369
CIITA	4	5.008	55.928	0.375
TIRAP	24	0.094	1.067	0.406
IRAK1	36	0.064	1.05	0.571
XIAP	19	0.128	1.093	0.623
IL12A	6	−1.208	0.862	0.688
NLRC4	6	0.247	1.187	0.844
MYD88	89	0.023	1.016	0.867
CARD18	1	−2.414	0.188	1
IFNG	1	−8.555	0.003	1
MAPK11	1	−0.71	0.611	1
NLRP1	1	2.358	5.128	1
NLRP9	1	−0.179	0.883	1
NLRX1	1	0.283	1.216	1
RAGE	1	0.005	1.003	1
CD40LG	0	—	—	—
IFNB1	0	—	—	—
IL12B	0	—	—	—
MAPK12	0	—	—	—
MEFV	0	—	—	—
NLRP12	0	—	—	—
NLRP4	0	—	—	—
NLRP5	0	—	—	—
NLRP6	0	—	—	—
PYDC1	0	—	—	—
TNFSF11	0	—	—	—

(Continued in next column)

TABLE 2 | (*Continued*) Inflammasome-related gene expression in response to influenza stimulation.

[a]*Represents the fold-change in ΔΔCT values for Baseline samples stimulated with influenza A/H1N1+R848.*

[b]p < *0.01 was considered statistically significant.* p-*values derived from Wilcoxon's signed-rank test.*

monocytes), suggesting that other biological mechanisms are responsible for the decline in influenza vaccine response among older adults. An alternate explanation is that macrophages from older adults have diminished inflammasome activity but are more susceptible to influenza infection and the greater number of infected macrophages in our assay compensated for the reduced activity. This is an interesting possibility that is currently under investigation. Another possibility is that the 2-weeks *in vitro* culture to differentiate monocytes into macrophages obscured age-related differential inflammasome activation that might have been seen directly *ex vivo*. If this is true, our results would then indicate that monocytes derived from young and old individuals have similar differentiation capabilities.

While the expression of several genes was correlated with inflammasome-related cytokine production, we did not pursue this analysis further as there were few significant differences in the secreted levels of cytokines and proteins between age groups in our cohort. Notably, *P2XR7* gene expression was significantly lower in monocyte derived macrophages from older adults compared to those from younger adults following influenza stimulation. *P2XR7* encodes the purinergic receptor P2X7, which senses extracellular concentrations of adenosine triphosphate and has been identified as one of the major drivers of inflammasome activation (Perregaux and Gabel, 1998; Perregaux et al., 2000; Ferrari et al., 2006). Although lower expression of *P2XR7* would be expected to result in decreased inflammasome activation, we observed no such decline in the production of inflammasome-associated protein

markers. This suggests that other factors compensate for the decreased expression of *P2XR7* or that an alternative mechanism unaffected by age is responsible for stimulating inflammasome activation following influenza vaccination.

We were also interested in evaluating sex-based differences in inflammasome activation and antibody response following vaccination. We and others have previously reported that females exhibit more robust humoral immune responses following vaccination (Kennedy et al., 2009; Lorenzo et al., 2011; Fink et al., 2018; Fink and Klein, 2018; Voigt et al., 2019). While this was true among the older age group, younger men exhibited significantly higher HAI titers compared to younger women following receipt of the influenza vaccine. The reasons for this sex-based disparity in HAI titer between age groups in our cohort are unclear. The cohort was a convenience sample recruited from the local population and it is possible that a sex-based recruitment bias among the younger half of the cohort occurred (e.g., differential rates of vaccination in the young men vs the young women).

Several genes associated with NF-κB regulation (e.g., *NFKBIB*, *NFKBIA*, *CHUK*, *RIPK2*) were more highly expressed among females compared to males for the entire cohort, suggestive of a greater degree of immune cell activation. Notably, *CFLAR* expression was significantly higher among females ($p = 2.42 \times 10^{-5}$) following influenza stimulation. The *CFLAR* gene encodes a caspase-8 regulatory protein that has been shown to play a role in governing the activation of the NLRP3 inflammasome and alternative mechanisms for inflammatory cytokine production (Wu et al., 2014). Interestingly, a model evaluating the effects of both age and sex on gene expression found that *CFLAR* was negatively associated with age ($p = 0.006$) and no longer exhibited a significant association with biological sex ($p = 0.057$). While this indicates that age has a more significant impact on *CFLAR* expression, collectively this data suggests that *CFLAR* may play a significant role in the development of higher HAI antibody titers among older females. Females have been reported to have more robust humoral immune responses to a number of vaccines, therefore these results may represent a potential mechanism for those findings. Further studies are warranted to assess the functional role of *CFLAR* in inflammasome activation with respect to both age and biological sex.

The strengths of our study include the relatively large cohort that was well-balanced and stratified with respect to both age and sex - allowing for analyses with both of these variables, the clear chronologic separation between young and old, the uniform assay measurements using established protocols, and the ability to evaluate both inflammasome-related gene expression and cytokine/caspase secretion. The limitations of our study include that the study cohort was predominantly Caucasian (89.1%), and we therefore lacked sufficient statistical power to evaluate any biological associations with race (**Table 1**). This may also limit the applicability of our results, as the demographics of our cohort do not reflect those of the general population. While the cohort was powered for analysis of age or sex, the group sizes became smaller when stratified by age and sex. We were also limited by the need to isolate monocytes and further differentiate them into macrophages for our measurements of inflammasome activation. We found that direct stimulation of PBMCs *ex vivo* produced very little evidence of inflammasome activity, prompting us to isolate and differentiate the monocytes. However, this *ex vivo* manipulation of monocytes may have introduced into our data artifacts that confounded the measurable effects of aging on the inflammasome activity of cells stimulated directly *ex vivo*. Nevertheless, to our knowledge, this is the first study to investigate the effect of age on inflammasome activation following influenza vaccination in humans and represents a key step toward understanding the age-associated decline in influenza vaccine response.

While the inflammasome has been implicated as a critical component for protective immunity against influenza in several animal studies, there have been no prior studies investigating this phenomenon or how it relates to vaccine response in human populations. The study reported here has extended our understanding of age-associated dysregulation of the immune response to influenza vaccination by comparing inflammasome function in older vs younger adults. Importantly, we provide evidence that macrophages from both older and younger adults are equally capable of inflammasome activation. We also demonstrate that the inflammasome response in macrophages after influenza vaccination is not significantly altered with age in humans; however, further study of this phenomenon is warranted. Additional human studies are desirable to validate our observations in this study, including a direct investigation of inflammasome activation in peripheral monocytes without differentiation into macrophages, and an examination of inflammasome function/activation in additional cell types (e.g., dendritic cells). Our results also suggest that sex-based and age-based differences in *CFLAR* expression may play a role in influenza vaccine response, and replication of this finding with further studies are warranted in order to understand the complex influence of biological sex and age on the immune response.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Mayo Clinic IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RK, IO and SC contributed to the conception and design of the study. KG performed the statistical analysis. SC wrote the first

draft of the manuscript. KG, IO and RK wrote sections of the manuscript. RK obtained funding for and supervised the project. All authors approved the final submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fragi.2021.719103/full#supplementary-material

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Diminished Pneumococcal-Specific CD4$^+$ T-Cell Response is Associated With Increased Regulatory T Cells at Older Age

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Respiratory infection caused by *Streptococcus pneumoniae* is a leading cause of morbidity and mortality in older adults. Acquired CD4$^+$ T cell mechanism are essential for the protection against colonization and subsequent development of infections by *S. pneumoniae*. In this study, we hypothesized that age-related changes within the CD4$^+$ T-cell population compromise CD4$^+$ T-cell specific responses to *S. pneumoniae*, thereby contributing to increased susceptibility at older age. To this end, we interrogated the CD4$^+$ T-cell response against the immunogenic pneumococcal protein AliB, part of the unique oligopeptide ABC transporter system responsible for the uptake of nutrients for the bacterium and crucial for the development of pneumococcal meningitis, in healthy young and older adults. Specifically, proliferation of CD4$^+$ T cells as well as concomitant cytokine profiles and phenotypic markers implied in immunosenescence were studied. Older adults showed decreased AliB-induced CD4$^+$ T-cell proliferation that is associated with an increased frequency of regulatory T cells and lower levels of active CD25$^+$CD127$^+$CTLA-4$^-$TIGIT$^-$CD4$^+$T cells. Additionally, levels of pro-inflammatory cytokines IFNy and IL-17F were decreased at older age. Our findings indicate that key features of a pneumococcal-specific CD4$^+$ T-cell immune response are altered at older age, which may contribute to enhanced susceptibility for pneumococcal infections.

Keywords: ***Streptococcus pneumoniae*, CD4$^+$ T cells, aging, tregs, infection, immunosenescence, pneumococcal proteins, proinflammatory cytokines**

INTRODUCTION

Streptococcus pneumoniae is a leading cause of respiratory infections in adults above 65 years of age, which is associated with substantial morbidity and mortality (Vila-Corcoles and Ochoa-Gondar, 2013; Drijkoningen and Rohde, 2014). The Gram-positive bacterium resides as a commensal in the human nasopharynx, with highest carriage rates in children under 5 years old, but can become invasive depending on microbial, environmental and host factors (Hussain et al., 2005; Abdullahi et al., 2012; Weiser et al., 2018).

The natural incidence of invasive pneumococcal disease (IPD) describes a typical U-shape throughout life, with reduced rates as children become older and a sharp increase at older age (Jansen

et al., 2009). These shifts in susceptibility are most likely explained by alterations in the acquired host immune mechanisms with age.

Pneumococcal protein-specific $CD4^+$ T cells are considered key players in the control of pneumococcal infections. Stimulation of $CD4^+$ T cells with pneumococcal strains induces proliferation and cytokine secretion by Th1, Th2 and Th17 memory $CD4^+$ T cells, with predominant pro-inflammatory Th1 and Th17 responses (Engen et al., 2014). IL-17, in particular, was shown to enhance the recruitment of phagocytes to the site of invasion and boost their phagocytic abilities, leading to rapid and effective clearance of *S. pneumoniae* via opsonophagocytosis (Lu et al., 2008; Zhang et al., 2009; Wright et al., 2013). The induced effector $CD4^+$ T-cell response is also modulated by pneumococcal-specific regulatory T cells (Tregs) to prevent excessive tissue damage and subsequent severe infection due to compromised tissue integrity and barriers (Pido-Lopez et al., 2011; Neill et al., 2012). Both low $CD4^+$ T-cell effector responses and increased numbers of Tregs are associated with positive pneumococcal carriage in children and young adults (Zhang et al., 2007; Zhang et al., 2011; Jiang et al., 2015; Mubarak et al., 2016), highlighting the importance of these responses in regulating colonization, a pre-requisite towards disease. However, less is known about alterations in pneumococcal-specific $CD4^+$ T cell immunity at older age.

Deterioration of immune responses with age is a well-known phenomenon, termed immunosenescence, that reduces the ability of older adults to respond to infections and vaccines (Ginaldi et al., 2001). Decreased $CD4^+$ T-cell proliferation is one of the hallmarks of immunosenescence that affects the efficiency of the $CD4^+$ T-cell response, as observed in aged mice and humans (Wikby et al., 1998; Aiello et al., 2019; Pieren et al., 2019). In addition, constitutive expression of inhibitory co-receptors including Programmed cell-death (PD-1) and Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) on T cells (Leng et al., 2002; Channappanavar et al., 2009) as well as accumulation of Tregs, observed in aged mice (Nishioka et al., 2006; Sharma et al., 2006; Lages et al., 2008), could counteract and imbalance the stimulatory signal of T cells upon activation, thereby resulting in reduced $CD4^+$ T cell response at older age. To explore whether human anti-pneumococcal $CD4^+$ T cell immunity is negatively impacted at older age, we examined the presence of these immunosenescent features on $CD4^+$ T cells of young and older adults using an important class of pneumococcal proteins.

AmiA, AliA, AliB, AliC and AliD are substrate binding lipoproteins, showing approximately 60% sequence homology (Alloing et al., 1994; Hathaway et al., 2004). AmiA, AliA and AliB are known to induce antibody responses in humans (Croucher et al., 2017). The proteins are part of the unique Ami-AliA/B oligopeptide ABC transporter system of *S. pneumoniae* and are essential for the uptake of nutrients and environmental signals by the bacteria (Nasher et al., 2017; Nasher et al., 2018). Furthermore, AmiA and AliA are involved in the colonization of the nasopharynx (Kerr et al., 2004), while AliB is essential for the development of pneumococcal meningitis (Schmidt et al., 2019). AliC and AliD are AliB-like homologues only expressed by non-encapsulated *S. pneumoniae* (NESP) (Hathaway et al., 2004; Keller et al., 2016).

In our study of the $CD4^+$ T-cell response against the broadly recognized AliB protein, evidence was provided suggesting that older adults experience diminished pneumococcal-specific $CD4^+$ T-cell responses associated with increased Treg-mediated regulation. This could contribute to increased susceptibility for IPD at older age.

MATERIALS AND METHODS

Ethics Statement and Study Population

Buffy coats were obtained from healthy adult blood donors in the age range of 20–75 years old with written consent in accordance with local protocols for blood products destined "not for transfusions" (Sanquin Blood Supply, Netherlands). In total, thirty-six healthy adults (20 females and 16 males) were included in this study. Twenty-four donors were sub-selected in two age cohorts of 20–35 year old (young adults, $n = 12$) and 60–75 year old (older adults, $n = 12$), respectively, to study aging of pneumococcal-specific $CD4^+$ T-cell responses in detail.

PBMC Isolation

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from the buffy coats via Lymphoprep (Axis-shield) density gradient centrifugation using standard procedures and frozen in 10% DMSO. Samples were kept at −80°C overnight and then stored at −135°C until use.

Pneumococcal Antigens

Pneumococcal protein AliB with N-terminal His_6-tag was recombinantly produced and purified as previously described (Schmidt et al., 2019). The other pneumococcal proteins AmiA, AliA, AliC and AliD were generated by cloning amplified target genes *amiA* (sp_1891, nt 122-2032), *aliA* (spd_0334, nt 85-1980), *aliC* (nt 73-1965), and *aliD* (nt 82-1965), without their signal sequences, into the pTP1 expression vector (Saleh et al., 2013). PCR reactions were performed using *S. pneumoniae* TIGR4 (*amiA*), D39 (*aliA*), or MNZ41 (*aliC*, *aliD*) chromosomal DNA as a template and the primer pairs listed in **Table 1**. The primers contained restriction sites (*Nhe*I/*Sac*I, *Spe*I/*Hin*dIII), which were used to ligate the fragments into the similarly digested expression vector. Resulting plasmids were transformed into competent *E. coli* BL21 (DE3) for heterologous protein expression. Protein expression and purification of N-terminal His_6-tag fusion proteins were performed as previously described (Saleh et al., 2013). Purity of the proteins was subsequently analyzed by SDS-PAGE followed by silver staining or immunoblotting with anti-Penta-His-tag mouse antibody (Qiagen) (Voss et al., 2018).

Thymidine Incorporation Assay

PBMCs from donors were seeded at a density of 1.5×10^5 cells/well in AIM-V medium (Gibco, Thermo Fisher Scientific) containing 2% human AB serum (Sigma-Aldrich) in 96-well U-bottom plates. Cells were stimulated with medium control

TABLE 1 | Primer list.

Primer name	Primer No.	Restriction site	Sequence (5′-3′)
AmiA_F	1562	*Nhe*I	GCGCGCGCTAGCAGTTCTTCAAAATCATCTGATTC
AmiA_R	1563	*Sac*I	GCGCGCGAGCTCTTACTTCACATGACTTGCCAATTC
AliA_F	1931	*Nhe*I	AATTGCTAGCTCTGGATCAGGTTCAAGC
AliA_R	1932	*Sac*I	GCGCGAGCTCATTTCACATGTTTTGC
AliC_F	1921	*Nhe*I	GCGCGCGCTAGCAAAAGTGAAAAGAATGC
AliC_R	1922	*Sac*I	GCGCGCGAGCTCATTTTATGTGCTTTTC
AliD_F	1923	*Spe*I	GCGCGCACTAGTTCAGATACAAAAACTTAC
AliD_R	1924	*Hin*dIII	GCGCGCAAGCTTATTTAACATGTTTTTCTGC

(mock), AmiA, AliA, AliB, AliC or AliD at 1 μg/ml, in replicate wells per condition for 6 days at 37°C with 5% CO_2 in the absence or presence of anti-MHC class II blocking antibodies consisting of a cocktail of anti-HLA-DR (B8.11-2; in-house produced), anti-HLA-DQ (SPV-L3; in-house produced) and anti-HLA-DP (B7/21; Leinco Technologies) monoclonal antibodies, as indicated. Tritium thymidine (18 kBq/well) was added to the wells on day six before overnight incubation at 37°C with 5% CO_2. Cells were then harvested on a filter and incorporated label was determined as counts per minute (cpm) using a MicroBeta Counter (Perkin Elmer). Tritium thymidine based stimulation indices (SI) were calculated by dividing the mean cpm of the replicate stimulated wells, by the mean cpm of the quadruplicate medium control wells. SI's > 1.7 were considered positive (van de Garde et al., 2019).

CellTrace-Based CD4$^+$ T-Cell Proliferation Assay

PBMCs from donors were rapidly thawed at 37°C and labeled with CellTrace™ Violet proliferation dye according to manufacturer's protocol (Gibco, Thermo Fisher Scientific). CellTrace™ Violet-labelled cells were seeded at a density of 1×10^6 cells/well in a 48-well flat-bottom plate in AIM-V medium with 2% AB serum and stimulated with either mock, AmiA, AliA, AliB, AliC or AliD at 1 μg/ml, individually, in replicate wells per condition at 37°C with 5% CO_2 for 5 and 7 days, in separate plates per timepoint. Cells were harvested from both day 5 and day 7 cultures, after which replicates were pooled, washed and used for flow-cytometric analysis. Based on optimization assays, supernatants for cytokine analysis were collected from the day seven cultures, pooled per stimulus and stored at −80°C until use.

Flow-Cytometric Analysis

Before and after *in vitro* culturing, CellTrace™ Violet-labeled cells were stained with the following fluorochrome-labeled antibodies at 4°C: CD3(SK7)-Alexa700, CD4(OKT-04)-BV711, CD8 (RPA-T8)-BV785, CD45RO(UCHL1)-PerCP-Cy5.5, FoxP3 (295D)-Alexa647, PD-1 (EH12.2H7)-BV605, CD127 (A019D5)-BV650, CTLA-4 (L3D10)-PE, Helios (22F6)-PE-Cy7, TIGIT (MBSA4)-PE/efluor-610 (ThermoFisher), CD27 (L128)-BUV395 (BD), CD25 (2A3)-BUV737 (BD) and Zombie NIR™ Fixable Viability kit (APC-Cy7, Biolegend). All antibodies are from Biolegend, unless stated otherwise. Acquisition was performed on BD LSR Fortessa X-20. Data was analyzed via FlowJo (Treestar). Frequencies of proliferated antigen-specific CD4$^+$ T cells were determined by analyzing sequential halving of the Cell trace Violet fluorescence intensity within the CD4$^+$ T cell gate (gating strategy in **Supplementary Figure S1**).

Dimensionality Reduced Analysis

Dimensionality reduction (viSNE) analysis of flow cytometry data was performed in Cytobank (www.Cytobank.org) (Amir et al., 2013). All events within pre-gated CD4$^+$ T-cell or proliferated CD4$^+$ T-cell gates were concatenated in Flowjo for each age group per culture condition and uploaded to Cytobank. viSNE clustering was performed on equal samples of randomly selected 5×10^5 cells from each age group based on expression of FoxP3, Helios, TIGIT, CTLA-4, PD-1, CD25 and CD127 expressions. Marker expression was represented as ArcSinh5-transformed medians within heatmaps generated via viSNE analysis.

Cytokine Measurement

Levels of Th cell cytokines were measured in day 7 culture supernatants, with Bead-based LEGENDplex™ Human TH cytokines (13-plex) kit (Biolegend) according to the manufacturer's instructions. Acquisition was performed on BD FACSCANTO™ II and data were analyzed with LEGENDplex™ Data Analysis Software. Levels of cytokines are expressed in pg/ml.

Statistical Analyses

Statistical analysis was performed using GraphPad Prism 7 software (La Jolla, CA, United States). Significant differences between young and older adults were determined by non-parametric Mann-Whitney *U* test or parametric Two-way ANOVA. Correlations were tested with Spearman's rank correlation coefficient. For all analyses, p-values < 0.05 were considered statistically significant.

RESULTS

Pneumococcal Substrate-Binding Lipoproteins Induce CD4$^+$ T-Cell Proliferation

Whilst AmiA, AliA and AliB are shown to be able to induce sustained antibody responses in humans (Croucher et al., 2017), it is not yet known whether this class of substrate-binding lipoproteins, including AliC and AliD, is able to elicit memory CD4$^+$ T-cell responses. To investigate this, we first screened PBMCs from randomly selected healthy blood donors for recognition of AmiA, AliA, AliB, AliC and AliD in a

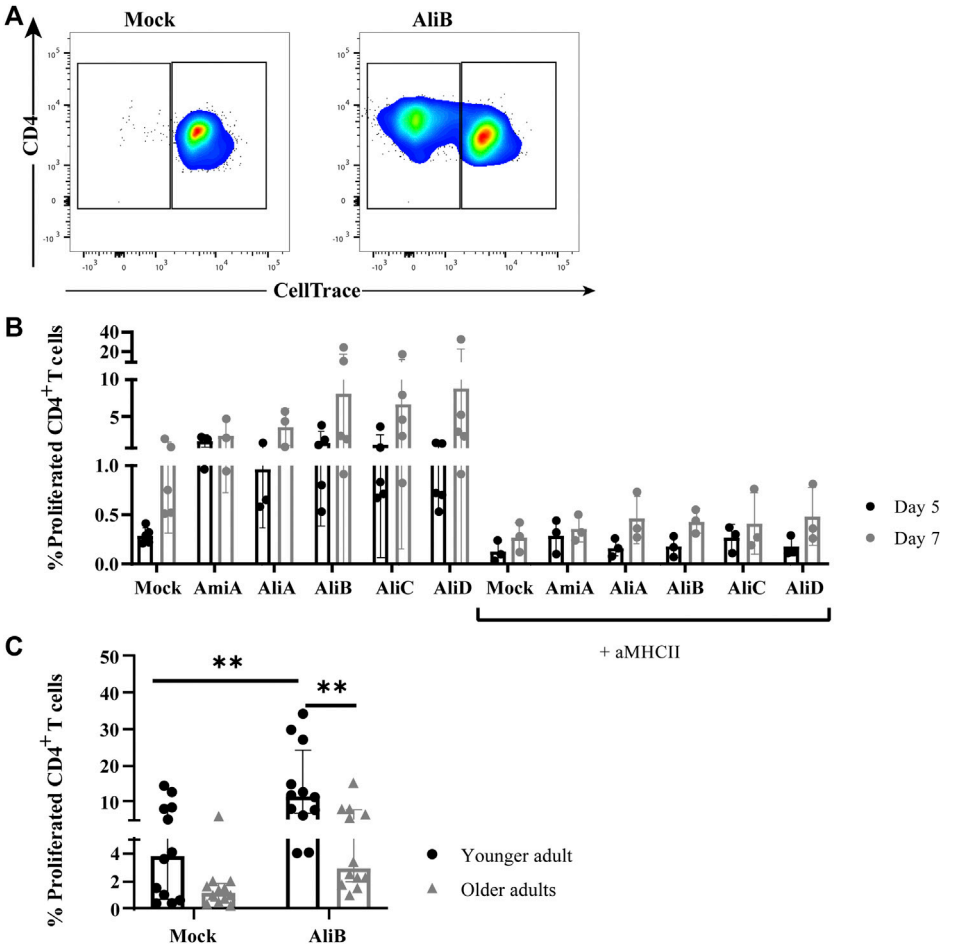

FIGURE 1 | $CD4^+$ T-cell immunogenicity of pneumococcal substrate-binding lipoproteins. **(A)** Example of gating strategy for proliferated ($CellTrace^{dim}$) $CD4^+$ T cells post 7 days stimulation with mock or AliB protein, as indicated (see general gating strategy of $CD4^+$ T cells in **Supplementary Figure S1**). **(B)** Proliferation of PBMCs (n = 5 donors) stimulated with mock or five pneumococcal substrate-binding lipoproteins for five (black circles) and 7 days (grey circles) in the absence or presence of anti-MCH class II (aMHCII) blocking antibodies on both timepoints, as indicated. Bars represent mean with SD. **(C)** Frequency of proliferated $CD4^+$ T cells in young (20–35 years old n = 12, black circles) and older adults (60–75 years old, n = 12, grey triangles) after CellTrace labeling and 7 days AliB stimulation of PBMCs. Bars represent median with interquartile range. Statistical significance is calculated with Mann Whitney U-test (** = $p < 0.01$).

thymidine incorporation assay. Results indicated that all five proteins were capable of inducing proliferative responses in PBMCs from healthy donors of random age, based on the calculated SI (**Supplementary Figure S2**).

To investigate whether the observed proliferation was $CD4^+$ T-cell mediated, PBMCs from additional donors were labeled with CellTrace Violet and subsequently stimulated with all five pneumococcal proteins or mock. CellTrace diminution of $CD4^+$ T cells was measured after five- and seven-day protein exposure, respectively, in the absence and presence of anti-MHC class II blocking antibodies. Depending on the donor, background CellTrace diminution of $CD4^+$ T cells in mock-stimulated conditions could be observed. All five substrate-binding lipoproteins were $CD4^+$ T-cell immunogenic as shown by the generation of a $CellTrace^{dim}$ $CD4^+$ T-cell population after protein stimulation (dot plot shown for AliB, **Figure 1A**) on day five that further expanded to day seven (**Figure 1B**). The presence of anti-MHC class II blocking antibodies fully inhibited the protein-specific responses to base-line levels of mock-stimulated cells on day five and day seven. This inhibition supports the notion that proliferation of the pneumococcal substrate-binding lipoprotein-specific $CD4^+$ T cells involves recognition of cognate epitopes generated after protein processing and presentation in the context of MHC class II molecules by antigen presenting cells. Together these data establish that $CD4^+$ T cell memory responses to AmiA, AliA, AliB, AliC and AliD are being acquired in adult donors, likely through pneumococcal carriage episodes throughout life, which are known to be immunizing (Malley et al., 2005; Ferreira et al., 2013; Wright et al., 2013; Wilson et al., 2015).

No Phenotypical Differences in the *ex vivo* Naïve and Memory $CD4^+$ T-Cell Subsets at Older Age

Next we selected a panel of young (age 20–35 years old, n = 12) and older adult donors (age 60–75 years old, n = 12) to study

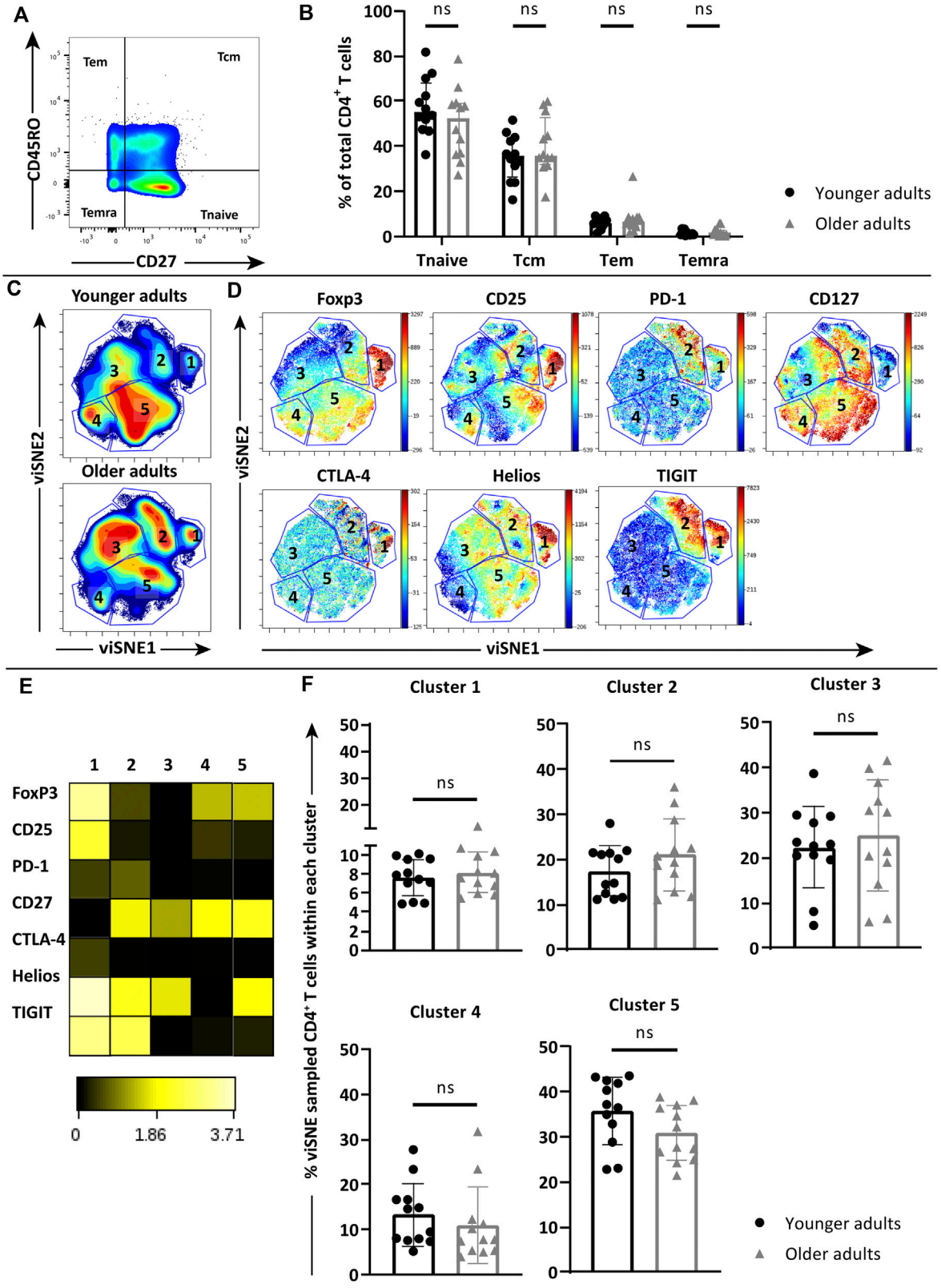

FIGURE 2 | Phenotype analysis of *ex vivo* naïve and memory CD4$^+$ T-cell subsets in young and older cohorts. **(A)** Gating of naïve (Tnaive), central memory (Tcm), effector memory (Tem) and terminally differentiated (TEMRA) CD4$^+$ T cells based on CD45RO and CD27 expression. **(B)** Frequency of naive and memory subsets within

(*Continued*)

FIGURE 2 | ex vivo *CD4$^+$ T-cell pools of young (n = 12, black circles) and older adults (n = 12, grey triangles).* ***(C)*** *Density plots of pooled ex vivo CD4$^+$ T cells of young (n = 12) and older adults (n = 12) clustered by viSNE based on the expression of FoxP3, CD25, PD-1, CD127, CTLA-4, Helios, and TIGIT. Five clusters were manually identified.* ***(D)*** *Fingerprint color dot plots showing the location and intensities of each marker within the identified clusters.* ***(E)*** *Heatmap depicts the ArcSinh5-transformed median expression of markers within each cluster.* ***(F)*** *Frequency of identified clusters within the total viSNE for each individual young (black circles) and older (grey triangles) donor. Bars represent median with interquartile range. Statistics is calculated with Two-way ANOVA in panel B and Mann Whitney U-test for panel F, ns = not significant.*

whether features of immunosenescence negatively impact the observed anti-pneumococcal CD4$^+$ T cell responses at older age. We first assessed whether age-dependent differences existed between the major CD4$^+$ T-cell subsets of the young and older cohorts at baseline. Distribution of CD4$^+$ T-cell memory subsets was compared between *ex vivo* PBMCs from the young (age 20–35 years old, $n = 12$) and older adults (age 60–75 years old, $n = 12$) based on expression of CD45RO and CD27 (**Figure 2A**). Comparable levels of naïve (Tnaive, CD45RO$^-$CD27$^+$), central memory (Tcm, CD45RO$^+$CD27$^+$), effector memory (Tem, CD45RO$^+$CD27$^-$) and terminally differentiated (Temra, CD45RO$^-$CD27$^-$) CD4$^+$ T cells were found between the two age groups (**Figure 2B**).

We then investigated the *ex vivo* expression of co-inhibitory receptors PD-1 and CTLA-4, Treg-associated markers FoxP3, TIGIT and Helios, implied in immunosenescence of CD4$^+$ T-cell responses, as well as activation marker CD25 (IL-2α receptor) and CD127 (IL-7α receptor) on the overall CD4$^+$ T cell pools of young and older adults. Expression data were pooled per age group and subsequently clustered based on Median Fluorescence Intensity (MFI) of each marker by viSNE analysis. Five major clusters were manually identified in the pooled CD4$^+$ T cells from the two age groups based on the densities (**Figure 2C**). Fingerprint expression plots (**Figure 2D**) showed clear clustering of FoxP3-expressing CD4$^+$ T cells within cluster 1, which together with cluster 2 also contained CD4$^+$ T cells with relatively higher expression of PD-1 and CTLA-4. Further examination of combined marker expression patterns within each cluster via heatmap (**Figure 2E**) showed that the CD4$^+$ T cells in cluster 1 also expressed relatively high CD25, Helios and TIGIT with low expression of CD127, which together with the expression of FoxP3 suggest that cluster 1 consist of activated Tregs. However, comparison of the percentages of CD4$^+$ T cells in each of the five clusters within the total viSNE of individual young and aged donors revealed no statistically significant differences (**Figure 2F**), indicating that the studied CD4$^+$ T-cell phenotypes were similar at baseline between our age cohorts.

Older Adults Show Altered CD4$^+$ T-Cell Functional Responses to AliB Stimulation

To elucidate whether functional CD4$^+$ T cell responses against the pneumococcal protein were impacted by age, we selected AliB as the prototype antigen as it evoked high CD4$^+$ T-cell proliferative responses (**Figure 1B**) and was implied in pneumococcal disease (Schmidt et al., 2019).

CD4$^+$ T-cell proliferation in the younger and older adult cohorts were compared after 7 days stimulation with AliB via CellTrace. Significantly decreased percentages of CellTrace^{dim} CD4$^+$ T cells were observed in older adults compared to the younger cohort (median of 3.18 vs. 9.66%, respectively, $p = 0.0024$) (**Figure 1C**).

Next, we investigated whether reduced CD4$^+$ T-cell proliferation in older adults was accompanied by differential Th-and Treg cytokine profiles compared to younger adults. Levels of IL-2, IL-9, Th1 (IFNy and TNFα), Th2 (IL-4, IL-5 and IL-13), Th17 (IL-17A, IL-17F, IL-21 and IL-22) and Treg-associated cytokines (IL-10) were measured in the supernatant after 7 days AliB stimulation. The levels of IFNy was significantly lower in older adults, whereas no differences in TNFα, IL-4, IL-5, IL-13, IL-10 and IL-9 were observed (**Figure 3** + **Supplementary Figure S3**). Older adult PBMCs also showed significant lower levels of IL-17F and demonstrate a trend towards lower IL-17A and IL-22 production (**Figure 3**).

Interestingly, we also detected significantly increased levels of IL-2 in older adults after 7 days AliB stimulation. To investigate whether this was due to less efficient IL-2 consumption, expression of IL-2 receptor α-subunit, CD25, on proliferated CD4$^+$ T cells was compared between young and older adults. Frequencies and MFI of CD25 were comparable between the two age cohorts (**Supplementary Figure S4**), indicating that higher IL-2 supernatant levels at older age may not be explained by altered CD25 expression.

Together, the decreased AliB-specific CD4$^+$ T cell proliferation and Th1-Th17 cytokine profiles indicate an impaired functional response towards pneumococcal protein stimulation at older age.

Altered Cluster Composition of Proliferated CD4$^+$ T Cells at Older Age

Next, we addressed the phenotypes within proliferated CD4$^+$ T cells to see whether age-related differences were present after 7 days AliB stimulation that may explain the reduced proliferation and altered cytokine profile at older age. The proliferated CD4$^+$ T cell populations from young and older adults did not differ in their distribution of Tnaïve, Tcm, Tem and Temra CD4$^+$ T cell memory subsets, and both mainly comprised of Tcm and Tem cells (**Figure 4A**). To explore whether functional differences between the AliB-responding CD4$^+$ T cells of young and older adults could be related to a differential expression of functionality markers, expression of PD-1, CTLA-4, FoxP3, TIGIT, Helios, CD25 and CD127 on pooled proliferated CD4$^+$ T cells from each age group were analysed via viSNE. A total of 5×10^5 proliferated CD4$^+$ T cells were randomly sampled by viSNE from the pooled proliferated CD4$^+$ T cells of young ($n = 12$) and older adults ($n = 11$, one donor excluded due to very low numbers of proliferated CD4$^+$ T cells). After viSNE clustering, six major clusters were manually identified based on the density plots (**Figure 4B**). Both expression plots of individual markers

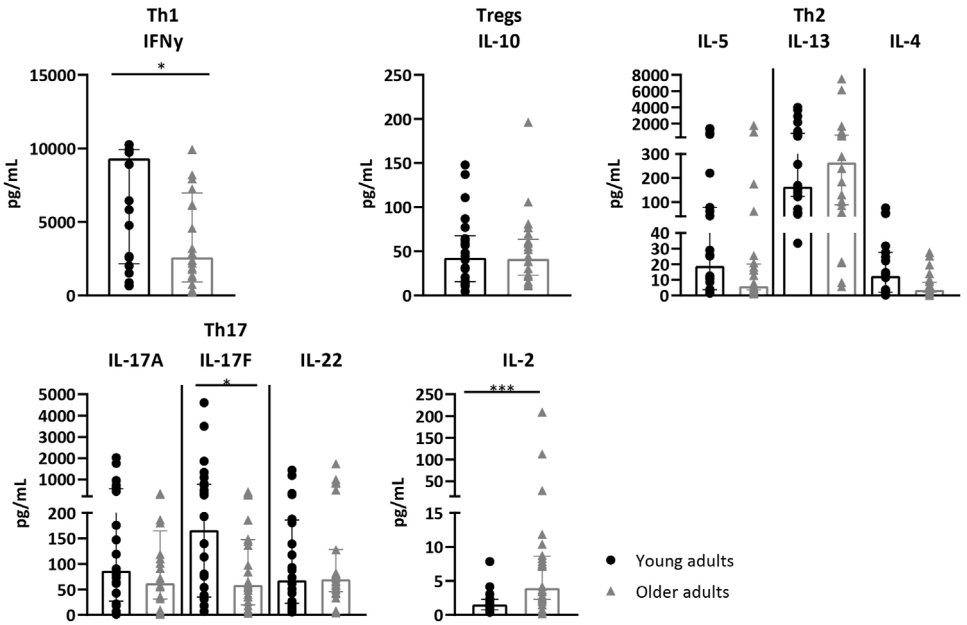

FIGURE 3 | Cytokine profiles after AliB stimulation in young and older adults. Bar graphs show the levels of Th1, Th17, Th2, IL-10 and IL-2 cytokines, as indicated, measured in the supernatant of PBMCs of young (n = 12, black circles) and older (n = 12, grey triangles) adults after 7 days culture with AliB. Bars represent the median with interquartile range per age group. Statistical significance is calculated with Mann Whitney U-test (* = $p < 0.05$, *** = $p < 0.001$).

(**Figure 4C**) and heatmap (**Figure 4D**) showed that FoxP3 expression is mainly restricted to cluster 5, whilst CD127 seemed to be associated with clusters 2, 4 and 6. Comparison of cluster frequencies in individual donors further showed that cluster 5 was significantly increased in older adults (median of 16.86 vs. 4.76%, respectively; p = 0.0007) (**Figure 4E**), whilst cluster 6 was significantly decreased compared to the younger age group (median of 3.36 vs. 7.25%; p = 0.012) (**Figure 4E**).

Higher Frequencies of Tregs Associated With Decreased CD4$^+$ T-Cell Proliferation in Older Adults After AliB Stimulation

Further examination of marker expression within cluster 5, via expression plot (**Figure 4C**) and heatmap (**Figure 4D**), revealed that the high FoxP3-expressing CD4$^+$ T cells within this cluster also had relatively high expression of TIGIT and Helios, indicating that this enhanced cluster at older age consisted of activated Tregs (**Figure 4D**).

To assess whether the observed difference was applicable to the total proliferated CD4$^+$ T-cell pool of each individual donor, we manually gated on FoxP3$^+$ cells within proliferated CD4$^+$ T cells and quantified the frequencies in all individual stimulated PBMCs. Similar to the viSNE result, a significant increase in Tregs amongst proliferated CD4$^+$ T cells was observed in older adults (median of 19.80 vs. 5.27% in younger adults; $p < 0.0001$) (**Figure 5A**, gating strategy in **Supplementary Figure S5**). Importantly, increased frequencies of Tregs in the proliferated CD4$^+$ T-cell population correlated with low CD4$^+$ T-cell proliferative responses ($p < 0.0001$, $r = -0.72$) after AliB stimulation (**Figure 5B**). Further delineation of this Treg fraction showed no differences for individual CD25, TIGIT, Helios, and PD-1 marker expression between young and older adults (**Supplementary Figure S6**), whilst a significantly higher expression of CTLA-4 on proliferated Tregs (MFI 1627 vs. 1318 in younger adults; p = 0.0068) and a lower frequency of proliferated Tregs expressing CD127 (median of 4.44 vs. 7.77% in younger adults; p = 0.04) were observed in older adults (**Supplementary Figure S6**).

Lower Levels of Active CD25$^+$CD127$^+$CD4$^+$ T-Cells in Older Adults After AliB Stimulation Associated With Decreased CD4$^+$ T-Cell Proliferation

Comparison of viSNE clusters also showed a significant decrease of cluster 6 in older adults compared to the younger age group (**Figure 4E**). Expression plots showed that cluster 6 contained CD4$^+$ T cells with high expression of CD127 (**Figure 4C**). Further comparison of relative marker expression intensities between the identified clusters revealed that CD4$^+$ T cells within cluster 6 also expressed relatively high CD25 and lower levels of inhibitory markers PD-1, CTLA-4 and TIGIT (**Figure 4D**). Quantification of manually gated CD25$^+$CD127$^+$CTLA-4$^-$TIGIT$^-$CD4$^+$ T cells showed a similar decrease within the total proliferated CD4$^+$ T cells of each individual at older age (median of 2.64 vs. 6.83% in younger adults; p = 0.008) (**Figure 5C**, **Supplementary Figure S5**). Moreover, decreased frequencies of these cells correlated with lower proliferative CD4$^+$ T cell responses (p = 0.0094, r = 0.51) (**Figure 5D**).

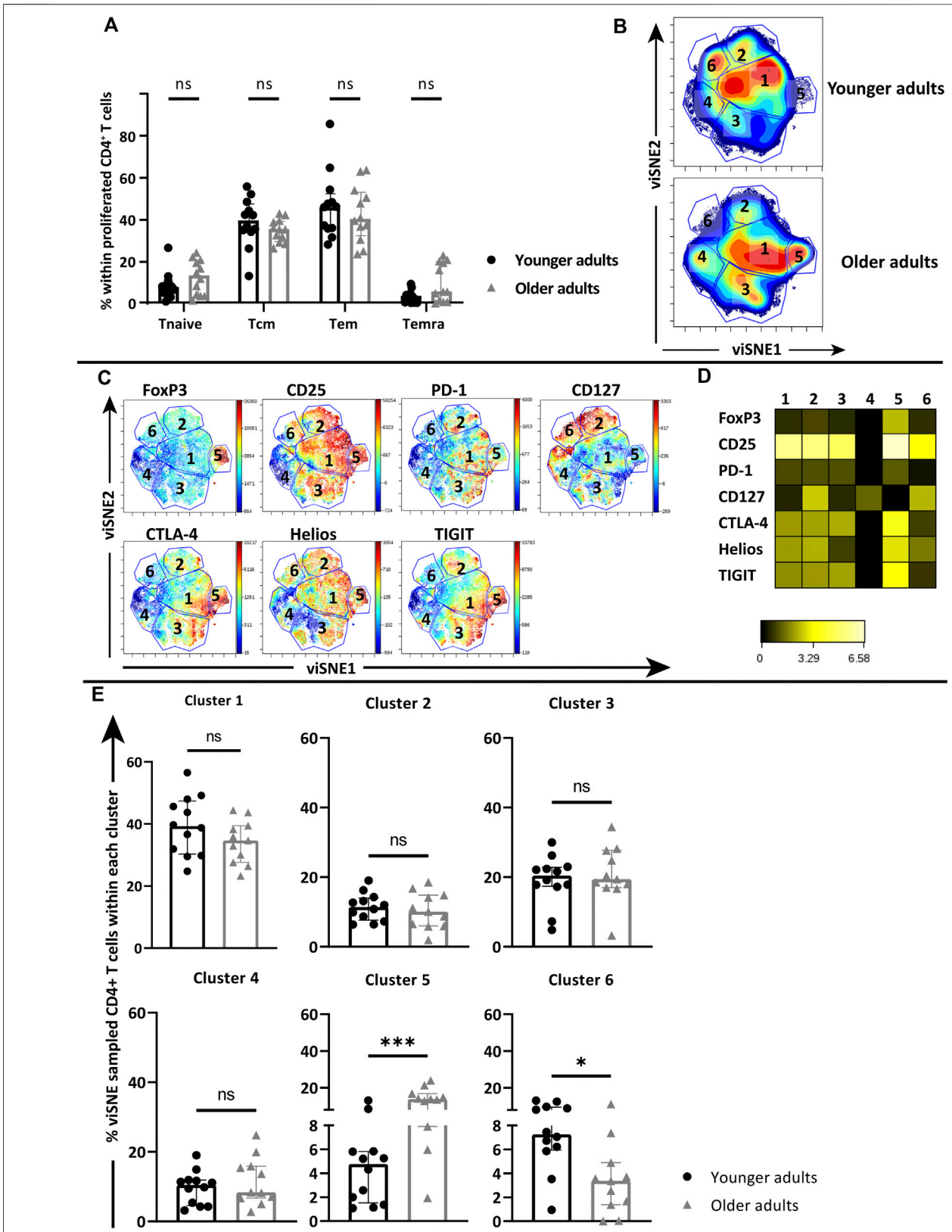

FIGURE 4 | Cluster composition of proliferated CD4$^+$ T cells in young and older cohorts. PBMCs of young and older adults were stimulated with AliB for 7 days. **(A)** Frequency of naive and memory subsets within proliferated CD4$^+$ T-cell pools of young (n = 12, black circles) and older (n = 12, grey triangles) adults post stimulation. **(B)** (*Continued*)

FIGURE 4 | Density plots of pooled proliferated CD4$^+$ T cells from young and older adults clustered by viSNE based on the expression of FoxP3, CD25, PD-1, CD127, CTLA-4, Helios and TIGIT. Six main clusters were manually identified. **(C)** Fingerprint color dot plots show the location and intensities of each marker within the identified clusters. **(D)** Heatmap depicts the ArcSinh5-transformed median expression of markers within each cluster. **(F)** Bar graphs show the frequency of identified clusters within the total viSNE for each individual young (black circles) and older (grey triangles) donor. Bars represent median with interquartile range per age group. Statistical significance is calculated with Mann Whitney *U*-test (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

DISCUSSION

Adults above 65 years of age become increasingly susceptible to pneumococcal infections associated with substantial morbidity and mortality. This could relate to immunosenescence of many components of the innate and adaptive immune system, including loss of effectivity of acquired specific immunity. Here, to our knowledge, for the first time evidence is presented for features of immunosenescence in CD4$^+$ T cell responses to pneumococcal proteins that could contribute to susceptibility to pneumococcal infections at older age.

Two major observations were made in our study. First, we demonstrated that substrate-binding lipoproteins AmiA, AliA, AliB, AliC and AliD, are capable of inducing memory CD4$^+$ T-cell responses in humans. Interestingly, despite the restricted expression of AliC and AliD to exclusively NESPs, we were able to detect substantial CD4$^+$ T-cell responses against the two proteins. Both AliC and AliD share up to 60% sequence homology with AmiA, AliA and AliB (Alloing et al., 1994; Hathaway et al., 2004), with several conserved regions that could potentially harbour cross-reactive CD4$^+$ T cell epitopes. Therefore, CD4$^+$ T cells targeting these conserved regions might recognise pneumococci irrespective of their capsule status and contribute to protection against non-encapsulated strains that are on the rise due to the selective pressure mediated by current capsular polysaccharide-based vaccines (Bradshaw et al., 2018).

Second, and importantly, older adults showed lower proliferative CD4$^+$ T-cell responses when stimulated with the immunodominant AliB protein compared to young adults, correlating with increased frequencies of proliferated Tregs and lower levels of an activated CD25$^+$CD127$^+$CTLA-4$^-$TIGIT$^-$CD4$^+$ T-cell subset. Moreover, stimulation of PBMCs

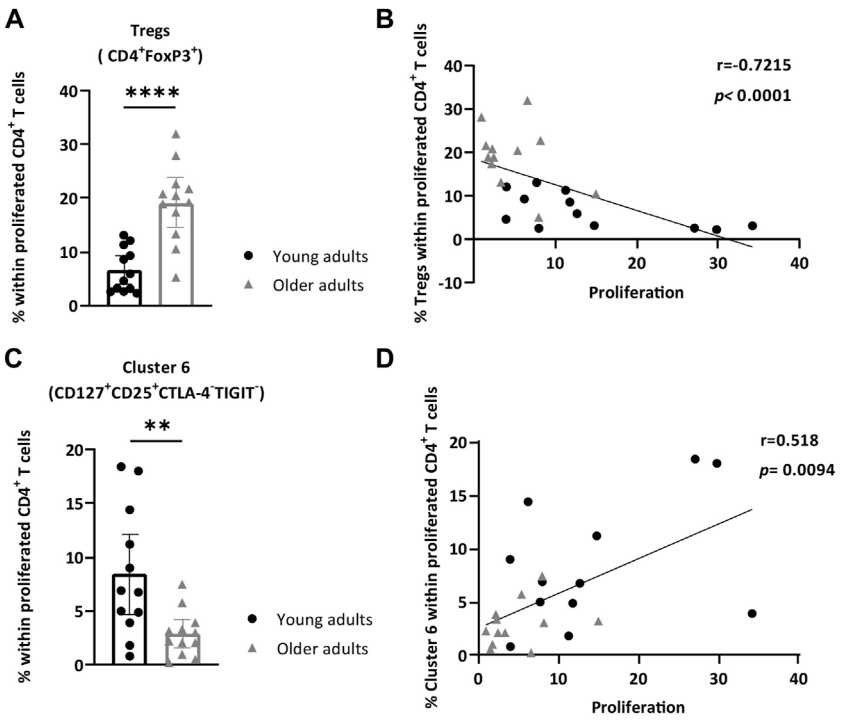

FIGURE 5 | Increased frequency of Tregs and lowered levels of CD25$^+$CD127$^+$CTLA-4$^-$TIGIT$^-$ cells within responding CD4$^+$ T cells are associated with low CD4$^+$ T cell proliferation. PBMCs of young and older adults were stimulated with AliB for 7 days. **(A)** Bar graph depicts the frequencies of manually gated Tregs (FoxP3$^+$CD4$^+$ T cells) within proliferated CD4$^+$ T cells in young (n = 12, black circles) and older (n = 12, grey triangles) adults. **(B)** Relationship between the frequency of Tregs in the responding CD4$^+$ T cell fraction and frequency of proliferated CD4$^+$ T cells (CellTrace^{dim}) (p and r) was assessed by Spearman's test. **(C)** Bar graph depicts the frequency of manually gated cluster 6 (CD25$^+$CD127$^+$CTLA-4$^-$TIGIT$^-$) within proliferated CD4$^+$ T cells young (n = 12, black circles) and older (n = 12, grey triangles) adults. **(D)** Relationship between the frequency of cluster 6 in the responding CD4$^+$ T cell fraction and proliferated CD4$^+$ T cells is assessed by Spearman's test (p and r). Bars represent median with interquartile range per age group. Statistical significance is calculated with Mann Whitney *U*-test (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

from older adults with AliB revealed decreased levels of pro-inflammatory cytokines IFNy and IL-17F and increased levels of IL-2.

Reduced $CD4^+$ T-cell proliferation, which is a key feature of immunosenescence, was not associated with shifts in the $CD4^+$ T-cell memory subsets in our aged cohort, similar to what others have shown (Czesnikiewicz-Guzik et al., 2008; Aiello et al., 2019; Pieren et al., 2019; Callender et al., 2020). This is in stark contrast with the human $CD8^+$ T-cell compartment, known to undergo significant changes in Tnaïve, Tcm and Temra subset distribution with age (Czesnikiewicz-Guzik et al., 2008; Callender et al., 2020).

Interestingly, a previous study found low $CD4^+$ T-cell proliferation in 2 years old children after *in vitro* challenge with detoxified pneumolysin, which progressively increased until the age of 20 years followed by a plateau until the age of 40 (Pido-Lopez et al., 2011). The findings on the memory $CD4^+$ T-cell responses to AliB from our aged cohort shed light on the other end of the age spectrum, and together these studies may indicate an inversed relationship of $CD4^+$ T-cell proliferation with IPD incidences throughout life. In addition, low specific $CD4^+$ T-cell proliferation early in life is associated with positive carriage of *S. pneumoniae* (Zhang et al., 2007). Altogether, this suggests that the reduced pneumococcal-specific $CD4^+$ T-cell proliferation observed in young children as well as older adults could play a role in the increased susceptibility to pneumococcal infections in these age groups.

Notably, our study indicated that low $CD4^+$ T-cell proliferative responsiveness in older adults was associated with an increased frequency of proliferated Tregs and accompanied by significantly decreased levels of pro-inflammatory cytokines IFNy and IL-17F after AliB stimulation. Earlier studies on Tregs and cytokine effector functions of pneumococcal-specific $CD4^+$ T cells in healthy younger individuals showed that both proliferation and production of IFNy and IL-17 cytokines are subjected to Treg-mediated suppression (Pido-Lopez et al., 2011; Mubarak et al., 2016). The combination of higher levels of responding Tregs and decreased inflammatory cytokines in our older cohort suggests that the $CD4^+$ effector/Treg balance is tipped towards stronger regulation of pneumococcal-specific $CD4^+$ T-cell response at older age. This is further supported by the comparable expression of markers classically associated with the suppressive function (FoxP3, TIGIT, Helios) on proliferated Tregs between young and older adults, suggesting that the Tregs were equally suppressive in both age groups, which is in line with findings in other studies (Gregg et al., 2005; Nishioka et al., 2006; Lages et al., 2008; Hwang et al., 2009). Moreover, increased Treg frequencies were associated with positive carriage of *S. pneumoniae* in children (Zhang et al., 2011). Based on this we hypothesize that the increased frequencies of specific Tregs seen at older age contribute to a less efficient $CD4^+$ T-cell response against *S. pneumoniae* and thereby lead to increased susceptibility to colonization and subsequent development of IPD in the elderly population.

Interestingly, while expression of Treg-associated markers (FoxP3, TIGIT and Helios) did not differ between young and older individuals, we detected a decreased frequency of Tregs expressing CD127 (IL-7Rα) at older age. Low expression or absence of CD127 is considered as classic Treg characteristic. However, recent studies discovered that expression of CD127 on Tregs were beneficial for the development, survival and homeostasis of Tregs in *in vitro* and murine models (Mazzucchelli et al., 2008; Simonetta et al., 2010; Schmaler et al., 2015). To further clarify the role of decreased CD127 expression of Tregs in pneumococcal $CD4^+$ T cell responses at older age, more in depth studies on this cell subset would be needed.

Tregs exert their suppression via several mechanisms including the secretion of IL-10 and cell-cell contact (Schmidt et al., 2012). Despite the increased frequency of Tregs in older adults, no difference was observed in produced IL-10 levels between younger and older adults. Instead, the Treg population in older adults expressed elevated levels of CTLA-4. This suggests that the Tregs in older adults may mediate their suppressive function mainly via cell-cell contact and other functions such as the secretion of TGF-β and IL-35 (Schmidt et al., 2012). This would need further research to be confirmed.

Another key finding of this study is the association between the low proliferative response in older adults and the reduced frequency of $CD25^+CD127^+CTLA\text{-}4^-TIGIT^-$ cells in the proliferating $CD4^+$ T cell fraction after stimulation. While this subset of $CD4^+$ T cells is relatively unknown, one study found that it predominantly consists of Th1, Th2, and to a lesser extent Th17 $CD4^+$ T cells that actively proliferate and secrete cytokines upon polyclonal stimulation (Narsale et al., 2018). Decreased presence of $CD25^+CD127^+CTLA\text{-}4^-TIGIT^-$ $CD4^+$ T cells could therefore contribute to the decreased levels of IFNy and IL-17F observed within our older age cohort. IFNy is essential for the protection against pathogens, as mice with non-functional IFNy or IFNy receptor were demonstrated to be extremely susceptible to microbial pathogens (Shtrichman and Samuel, 2001). IFNy polarizes macrophages towards a pro-inflammatory phenotype with opsonophagocytic properties (Herbst et al., 2011), whilst IL-17 is shown to be important in neutrophil and macrophage attraction and potentiation in pneumococcal immunity (Lu et al., 2008; Zhang et al., 2009; Wright et al., 2013). It would therefore be of interest to investigate if the decreased presence of $CD25^+CD127^+CTLA\text{-}4^-TIGIT^-$ proliferated $CD4^+$ T cells in older adults is causally related to the decreased levels of IFNy and IL-17F and could thereby result in less efficient clearance of *S. pneumoniae* via opsonophagocytosis by phagocytes.

IL-2 plays an essential role in promoting T cell proliferation (Pol et al., 2020). We detected significantly elevated levels of IL-2 in older adults, which is in striking contrast to the findings of other studies showing reduced IL-2 levels at older age (Whisler et al., 1996; Pieren et al., 2019). This may be explained by the difference in polyclonal versus antigen-specific stimulus between the studies that result in different kinetics of the response. Elevated levels of IL-2 might indicate less efficient IL-2 consumption by $CD4^+$ T cells at older age and contribute to lowered proliferative response. Whilst no difference was detected in the expression of CD25 between the two age cohorts within our study, further studies on the expression of the other two IL-2

receptor subunits, CD122 (IL2R-β and IL15-Rβ) and CD132 (IL2R-γ) is required in order to fully assess the impact of age on the consumption of IL-2 by antigen-responding T cells.

We acknowledge that the age-related changes in $CD4^+$ T-cell response observed in this study are limited to a certain class of pneumococcal proteins and serve as proof of concept. It remains to be determined whether the observed age-related changes can also be detected for other pneumococcal protein classes reported to induce $CD4^+$ T-cell responses, such as choline-binding proteins and cytolysins (Zhang et al., 2007; Pido-Lopez et al., 2011; van de Garde et al., 2019). In addition, while antigen-specific proliferation of $CD4^+$ T cells well exceeded background proliferation in our 7 days flow-based CellTrace diminution assay, the sensitivity and specificity of the assay may need further optimization to distinguish between specific and bystander activated CellTrace^{dim} $CD4^+$ T cells. Finally, whilst $CD4^+$ T cells are the main producers of the cytokines investigated in this study, it should also be noted that other cells within the PBMC are capable of secreting IFNy or IL-17F as well. Therefore, the age-related differences observed for these cytokines should not be solely attributed to immunosenescence of $CD4^+$ T cells, but also to other innate and adaptive cell types as well.

Collectively, our data showed for the first time that this class of pneumococcal substrate-binding lipoproteins was able to induce $CD4^+$ T-cell response in humans and that the pneumococcal-specific $CD4^+$ T-cell immune response is altered in older adults. Excessive regulation by Tregs exerted on the pneumococcal-specific $CD4^+$ T-cell immune response at older age may result in diminished inflammatory response and ineffective clearance of *S. pneumoniae*, which could lead to increased chances of developing IPD at older age.

Immunosenescence is not limited to $CD4^+$ T cells, but has an impact on all aspects of the immune system. Considering that protection against *S. pneumoniae* also requires antibodies and the innate immune response (Zhang et al., 2009; Wright et al., 2013; Ramos-Sevillano et al., 2019), wide-scale immunological studies are needed to fully understand the impact of aging on innate and acquired pneumococcal-specific immunity that causes the enhanced susceptibility at older age.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Sanquin Blood Supply, Amsterdam. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SHe, MvdG and CvE designed the study. FV, MA, SHa produced and purified the pneumococcal proteins. SHe, MvdG, and MP performed experiments. SHe and MvdG analyzed the data. SHe, MP, DP, and CvE interpreted the data. SHe, DP, and CvE wrote the manuscript, MvdG, FV, MA, and SHa edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fragi.2021.746295/full#supplementary-material

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REVIEW
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Patho-Physiology of Aging and Immune-Senescence: Possible Correlates With Comorbidity and Mortality in Middle-Aged and Old COVID-19 Patients

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During the last 2 years, the entire world has been severely devastated by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic (COVID-19) as it resulted in several million deaths across the globe. While the virus infects people indiscriminately, the casualty risk is higher mainly in old, and middle-aged COVID-19 patients. The incidences of COVID-19 associated co-morbidity and mortality have a great deal of correlation with the weakened and malfunctioning immune systems of elderly people. Presumably, due to the physiological changes associated with aging and because of possible comorbidities such as diabetes, hypertension, obesity, cardiovascular, and lung diseases, which are more common in elderly people, may be considered as the reason making the elderly vulnerable to the infection on one hand, and COVID-19 associated complications on the other. The accretion of senescent immune cells not only contributes to the deterioration of host defense, but also results in elevated inflammatory phenotype persuaded immune dysfunction. In the present review, we envisage to correlate functioning of the immune defense of older COVID-19 patients with secondary/super infection, increased susceptibility or aggravation against already existing cancer, infectious, autoimmune, and other chronic inflammatory diseases. Moreover, we have discussed how age-linked modulations in the immune system affect therapeutic response against administered drugs as well as immunological response to various prophylactic measures including vaccination in the elderly host. The present review also provides an insight into the intricate pathophysiology of the aging and the overall immune response of the host to SARS-CoV-2 infection. A better understanding of age-related immune dysfunction is likely to help us in the development of targeted preemptive strategies for deadly COVID-19 in elderly patients.

Keywords: immunosenescence, inflammaging, comorbidities, SARS-CoV-2, COVID-19, innate and adaptive immune system

INTRODUCTION

The pandemic outbreak of a novel coronavirus–severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has tumulted the whole world population into chaos since the first quarter of the year 2020. Over the course of subsequent few months, millions of coronavirus disease-19 (COVID-19) cases had been registered across the world (Mok et al., 2020). The SARS-CoV-2 infection has manifested a wide range of clinical consequences. In spite of the fact that the majority of infected people remain either asymptomatic (estimated to be between 17.9 to 78%) or have moderate disease, around ~20% of the COVID-19 patients contract serious illness, with life-threatening pneumonia and acute respiratory distress syndrome (ARDS) (Yang X et al., 2020; Yin et al., 2021). A substantial subpopulation of COVID-19 patients exhibits systemic symptoms like secondary sepsis, cardiovascular and cardiac complexities, thrombo-embolism, coagulopathy, and as well as multi-organ malfunction (Huang et al., 2020; Yang R et al., 2020). Age is a significant risk factor for severe SARS-CoV-2 infestation. COVID-19 patients who are older or have pre-existing comorbidities are at higher risk of mortality (Elezkurtaj et al., 2021). In addition, elderly patients are more likely to have chronic comorbidities like diabetes mellitus, hypertension, cancer, obesity, and cardiovascular disorders, etc; all of which make them more susceptible to COVID-19 as well as associated complications (Huang et al., 2020; Zhang L et al., 2020; Zheng et al., 2020; Zhang X et al., 2020; Zhou F et al., 2020). The above specified comorbidities, however, are inadequate to explain why age is a risk factor in relenting to COVID-19, in its own right. Besides the elevated risk of older people succumbing to SARS-CoV-2 infection, there are several studies that suggest a great degree of difference in the sickness outcome between younger and older SARS-CoV-2 patients (Yang R et al., 2020; Zhang X et al., 2020; Lennon et al., 2020; Jung et al., 2020; Liu Yet al., 2020). In fact, despite comparable viral loads, younger COVID-19 patients are likely to be asymptomatic than older patients, according to a cross-sectional analysis of residents, and employees in nursing homes and assisted living facilities (Lennon et al., 2020). In Shanghai, a systematic analysis of clinical, genetic, and immunological data from 326 confirmed COVID-19 cases revealed that, among other factors, age was substantially associated with poor clinical outcomes (Zhang X et al., 2020).

The enveloped SARS- CoV-2 possesses a large (27.9–31 kb) positive sense single-stranded RNA as its core genome. The virus enters the human host generally via the respiratory tract. Once inside the host, the contagion employs its spike (S) protein to recognize the angiotensin-converting enzyme 2 (ACE2) receptor on the alveolar epithelial type II pneumocytes in the lung (Zhang H et al., 2020). Next, TMPRSS2 (host transmembrane protease serine 2) facilitates S protein activation and viral entry into the cell (Hoffmann et al., 2020). Inside the cell, it multiplies, and eventually the cell burst causes the release of new virus particles that infect adjacent cells, and also wander in the extracellular spaces to find susceptible cell targets in the host. Besides the lung, the ACE2 receptor is profusely expressed on epithelial cells of the gastrointestinal tract, kidneys, vascular endothelial cells, and adipose tissues, etc (Hamming et al., 2004; Al Heialy et al., 2020; Al-Benna, 2020; Albini et al., 2020; Gu et al., 2020; Li B et al., 2020; Thum, 2020). The expression of ACE2 receptors has also been reported on the gingiva, salivary gland, and taste cells of the oral cavity (Doyle et al., 2021; Huang et al., 2021). It is suggested that SARS-CoV-2 affects olfactory sensory neuronal cells indirectly, as the ACE2 receptor is not directly expressed on olfactory cells rather expressed on supporting cells (Brann et al., 2020). The stay of the SARS-CoV-2 inside the host causes progressing tissue damage that ensues in various related clinical signs and symptoms. The vast array, or distribution of the susceptible target cells in the inflicted host, may lead to varying degrees of clinical presentation as well as indications, involving respiratory symptoms like cough, shortness of breath, and fever being the most common. The digestive system associated symptoms like diarrhoea, nausea, and vomiting are also equally manifested. Thromboembolism and strokes have also been linked to clinical symptoms of vascular endothelial dysfunction and pathological coagulopathy-related manifestations (Oxley et al., 2020). Loss of taste and sense of smell are the commonly observed symptoms of COVID-19 (Brann et al., 2020; Huang et al., 2021).

The post COVID-19 complications may involve pneumonia and acute respiratory distress syndrome (ARDS), which require artificial ventilation and, if not managed appropriately, may be fatal. ARDS is clinically characterized by acute respiratory failure, hypoxemia, bilateral lung infiltrates on chest imaging that is not entirely explained by effusions, collapse, and/or nodules, and a lack of cardiogenic-related edema (Papazian et al., 2016). Patients with severe COVID-19 had been reported to have elevated levels of circulating cytokines and chemokines (cytokine storm), which increases their chance of developing pneumonia, ARDS, sepsis, and coagulopathy, some of the serious complications associated with COVID-19 (Huang et al., 2021; Iba et al., 2019). Alongwith ACE2 receptors, dipeptidyl peptidase 4 (DPP4) also known as CD26 may be a binding target for SARS-CoV-2. In fact, the CD26 molecule (DPP4) has been described as a binding receptor for MERS-CoV, another prominent member of the CoV family (Valencia et al., 2020). The protein docking studies, based on crystal structure, revealed the high affinity between human dipeptidyl peptidase 4 (DPP4), and the spike (S) receptor-binding domain of SARS-CoV-2 (Li et al., 2020a). The DPP4 expression is more common in senescent cells. This may form the basis for the high occurrence of COVID-19 in elderly population. Moreover, enhanced expression of DPP4 in the obese individuals or type 2 diabetic patients correlates with the higher proliferation of human smooth muscle cells and increased expression of pro-inflammatory cytokines like MCP-1, IL-6, and IL-8 via NF-κB activation leading to damaging consequences of SARS-CoV-2 in the inflicted patients (cf. older as well as diabetic patients) (Valencia et al., 2020). Hence, inhibitors of the DPP4 might prove as strategic therapeutic agents as they reduce the level of IL-6, which plays a key role in the inflammatory response in SARS-CoV-2 patients (Valencia et al., 2020; Solerte et al., 2020). Both the innate and the adaptive immune systems are highly involved in the regular immune response to viral infections, including coronaviruses. The innate immune response comprises toll-like

receptors (TLR), which are membrane-bound pattern-recognition receptors (PRRs), expressed by dendritic cells (DC) and macrophages, recognize viral nucleic acids, which is followed by intracellular signaling and synthesis of antiviral type I interferon (IFN) in antiviral defense mechanisms (Kawasaki and Kawai, 2014). TLR7 and TLR8, which sense single-stranded RNA, and may be TLR3, which senses double-stranded RNA intermediates, and are expected to be the first receptors to detect SARS-CoV-2 in the host cells (Khanmohammadi et al., 2021). An interactive association between Myd88 and specific TLRs (TLR1, TLR2, TLR4, TLR5, TLR8, and TLR9) has been suggestively linked with disease progression in COVID-19 patients. In K18-ACE2 transgenic mice, TLR2 has been reported to play important role in sensing the SARS-CoV-2 envelope protein that eventually ensues in profuse inflammatory cytokine production (Zheng et al., 2021).

In general, natural killer (NK) cells play a significant role in imparting antiviral defense. Furthermore, the expression of proinflammatory cytokines such as IL-6, IL-1β, tumour necrosis factor (TNF-α), and chemokines by the active immune cells helps in accumulation of neutrophils and other inflammatory immune cells to the infection site (Bajaj et al., 2021). An adaptive immune response begins when viral antigen is presented to virus-specific CD4 helper T cells and CD8 cytotoxic T cells. Antigen-specific CD8 T cells are essential for destroying virally infected cells, whereas cytokines such as IFN-γ are important for the activation of antigen-presenting cells including macrophages (Bajaj et al., 2021). CD4 helper T (Th) cells are critical for supplying cognate assistance to B cells, which undergo class switching and somatic hypermutation to switch antibody synthesis in favor of IgG isotype, which is more specific, precise, and display a high affinity for neutralization (Bajaj et al., 2021). Various active immune cell populations, including neutrophils, and NK cells, have a great deal of potential to inhibit invading viruses in the circumstances when expression of MHC-I molecules had been downmodulated to a significant level (Bajaj et al., 2021). Markedly, the immune response is a two-edged sword: on the one hand, it mediates defensive immunity and is necessary; on the other hand, excessive and improper development of inflammatory cytokines may contribute to cytokine storm syndrome, which causes organ immunopathology and death of the infected host (Zhou Y et al., 2020).

Normal aging is followed by progressive biological alterations in various systems of the host. Some of these contribute to the weakening of the immune system's functions (Bajaj et al., 2021). The elderly subjects are more susceptible as well as vulnerable to respiratory infections like influenza and novel coronaviruses. Age-related immune-inflammation, or inflammaging, and associated inflammatory diseases are more common in the elderly population (Franceschi et al., 2000; Shaw et al., 2013; Fulop et al., 2018). These alterations, in combination with comorbidities, make elderly people more prone to latent or new infections, resulting in elevated COVID-19 morbidity, and death.

A superior repertoire of naive immune cells in younger people endows them with the potential to fight new infections more profoundly. Young people can withstand pathogen onslaught more successfully. As a consequence, exposure to the virulent pathogen may result in milder or even asymptomatic infection (Bajaj et al., 2021). The majority of younger people who test positive for the novel coronavirus, however, do not show disease symptoms. Exposure to the whole pathogen or any of their antigen components may cause upregulation of MHC molecules, costimulatory surface structures on pathogen-specific T and B cells of healthy individuals. This results in a bigger and more robust pool of effector molecules and their precursors that help in the recognition of a broader spectrum of foreign entities and eventually defend against new infectious diseases such as the COVID-19 pandemic (Bajaj et al., 2021). Interestingly, several studies have demonstrated the effect of aging on host immune responses against viral infections. Immunosenescence, for example, relates to diminished vaccination effectiveness and increased vulnerability to infectious diseases in the elderly. Vaccines for infections like influenza and shingles have been in practice for a long time; though, the poor efficacy and efficiency in the elderly reflect an aging-related drop in vaccination-induced immunogenicity (Oh et al., 2019).

In this review, we will discuss the concept of immunosenescence and inflammaging, as the correlates associated with physiological changes in the immune system of the elderly population, and how they impact the host immunological response to viral infections, mainly SARS-CoV-2. Further, we will present an overview of current studies on senescence-induced dysregulation of the immune system, with an emphasis on functional analysis of innate and adaptive immune cells, as well as its potential influence on viral immune responses, and such as COVID-19.

IMMUNOSENESCENCE AND INFLAMMAGING

Immunosenescence is a term used to describe the degeneration of the immune system. An age-related change, in both the innate and adaptive immune systems, contributes to the development of a chronic inflammatory state, and thereby results in a decreased ability to combat against the new infection (Stahl and Brown, 2015) (**Figure 1**). The deterioration in the immune robustness makes the host susceptible to infectious diseases. This may also result in the inadequate immune response to vaccination or increased susceptibility to cancer and autoimmune diseases. Immunosenescence reflects a relatively complex shift in the activity across many immunological responses, rather than just a loss of immune cells in terms of number. Because of a decline in peripheral naive T and B cells, the immune system's capacity to respond effectively to novel antigens is weakened (Stahl and Brown, 2015). Immunosenescence is also linked to the accumulation of memory T cells (Aiello et al., 2019). Antigen load over time, alongwith reduced lymphopoiesis, causes a decline in naive T cell populations and accretion of memory cell subsets (Aiello et al., 2019). Aging also comprises the loss of expression of the co-stimulatory surface protein CD28 from both

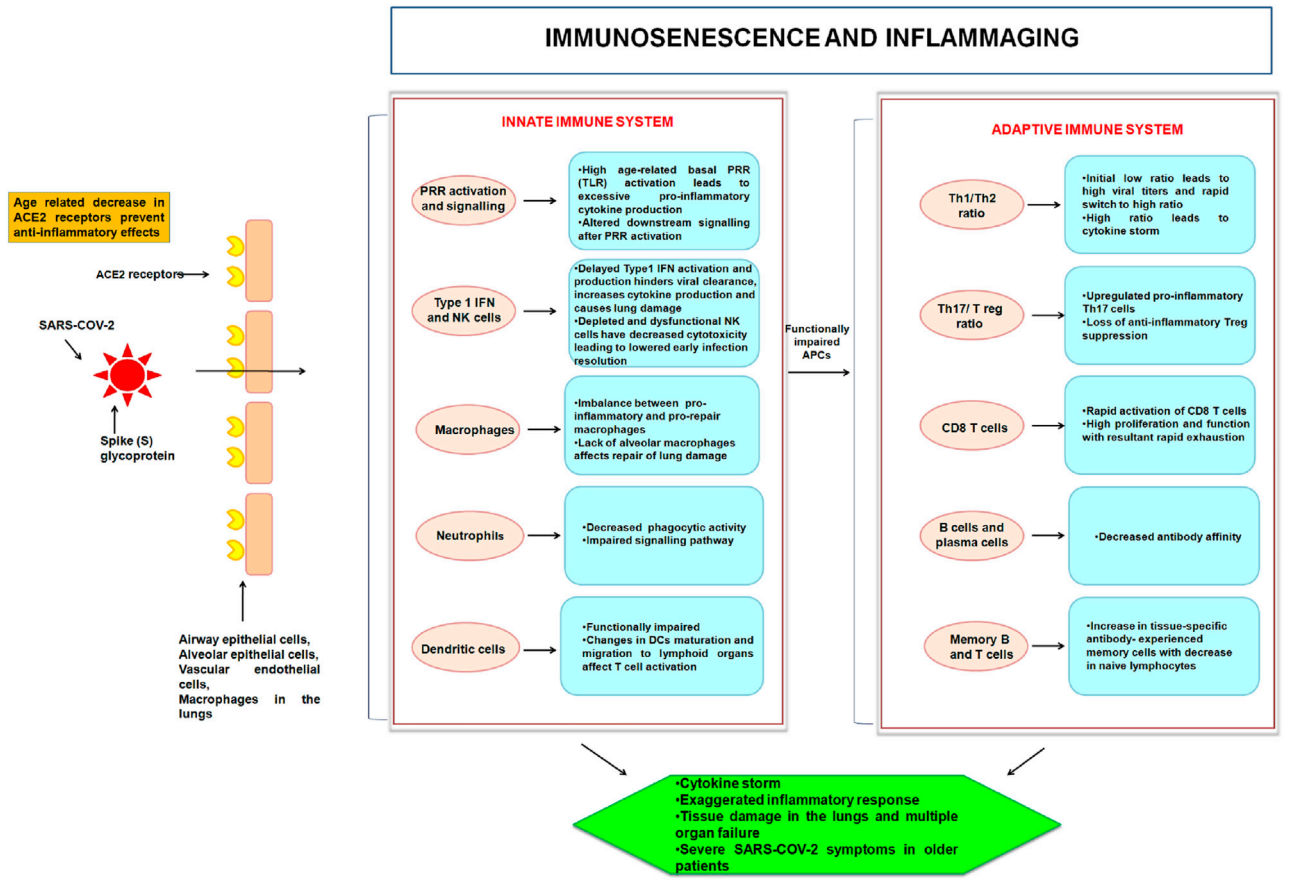

FIGURE 1 | Age-linked modifications in the innate and adaptive immune systems with relevance to COVID-19 are depicted in this illustration. SARS-CoV-2 mediated infliction of parenchymal cells (through ACE2 receptor) ensues in activation of PRR of the innate immune system of the host. With age, cellular activities and signaling become deranged. This hampers the innate activation of an already diminished adaptive immune system. The abnormalities result in "inflammaging" from proinflammatory innate immune responses and adaptive immunological immunosenescence.

CD4 and CD8 T cell populations. The less copy number of costimulatory CD28 molecule is a major hallmark of senescent T cells (Weyand and Goronzy, 2016). Loss of expression of CD27, another costimulatory molecule, and has also been associated with T cell senescence (Larbi and Fulop, 2014). The loss of CD28 and/or CD27 co-stimulatory molecules in T cells can be recognized as a sign of short telomeres and reduced telomerase activity, an indicator of replicative senescence (Larbi and Fulop, 2014). A variety of stress and aging-related illnesses are linked to reduced telomere length in leukocytes (de Punder et al., 2019). The expression of CD57 and KLRG-1 (killer-cell lectin like receptor G1, a co-inhibitory receptor) on T-cells is the marker of senescent T cells, as they are considered to be inhibitors of proliferation, shows replicative senescence (Larbi and Fulop, 2014). $CD57^{+}$ cells have short telomeres, poor telomerase activity, reduced expression of cell-cycle genes, and restricted proliferative potential (Focosi et al., 2010). Senescent T cells persist and accumulate during aging, and secrete high levels of proinflammatory cytokines, chemokines, growth factors, and proteases. These factors represent a typical hallmark of senescence and therefore they have been defined as senescence-associated secretory phenotype (SASP) (Bartleson et al., 2021). Senescent cells frequently modify their gene expression, rendering themselves less prone to apoptosis (Alves and Bueno, 2019).

An imbalance of regulatory and stimulatory mediators, such as cytokines and acute phase reactants, also exemplifies the immune system's aging. This leads to a sub-clinical chronic proinflammatory condition known as inflammaging. The age-related rise in the number of senescent cells, together with the immune system's reduced capacity to eliminate these cells, creates an environment rich in pro-inflammatory cytokines and reactive oxygen species (ROS), which pushes surrounding cells toward senescence (Stahl and Brown, 2015). Chronic activation of the innate immune system and increased release of inflammatory mediators may have a direct correlation with inflammaging that is generally accompanied by increased proinflammatory NF-κβ activation (Salminen et al., 2018). The unregulated inflammatory response to SARS-CoV-2 results in both local and systemic tissue damage. Most of the patients with severe COVID-19 have significantly increased levels of pro-inflammatory cytokines and chemokines in the serum, including IL-6, IL-2, IL-1β, IL-8, IL-17,

granulocyte (G)-colony-stimulating factor (CSF), granulocyte-macrophage (GM)-CSF, C–X–C motif chemokine ligand (CXCL)10, C–C motif chemokine ligand (CCL)2, CCL3, and TNF (Xu et al., 2020; Qin et al., 2020). In a study involving aging mice, it was found that similar cytokines, such as IL-6, IL-1α, IL-1β, TNF-α, and the chemokine CCL2 were also associatedwith the aged host response to SARS-CoV-2 (Dinnon et al., 2020; Leist et al., 2020). Inflammaging causes a rise in several of these cytokines in the elderly. For example, increased level of IL-6 has been considered as a predictor of poor outcomes in COVID-19 patients, because of the fact that IL-6 is indeed one of the most accurate aging indicators (Johnson, 2006). The expression of IL-6 cytokine is a clinical sign of NF-κB (a transcription factor) activation in the vascular system. NF-κB regulates many pro-inflammatory genes of innate immune cells, in fact, there is a great deal of correlation between aging, and NF-κB signaling and inflammation (Salminen et al., 2008; Brasier, 2010).

REMODELLING OF THE INNATE IMMUNE SYSTEM WITH AGING

Toll-like Receptors

The innate immune system uses germline-encoded pattern-recognition receptors (PRRs) in the initial identification of microbes (Kawasaki and Kawai, 2014). PRRs are mainly expressed by dendritic cells, macrophages, neutrophils, monocytes, and epithelial cells. They perceive microbe-specific molecular signatures known as pathogen-associated molecular patterns (PAMPs) as well as self-derived molecules derived from damaged cells known as damage-associated molecular patterns (DAMPs) (Kawasaki and Kawai, 2014). Toll-like receptors (TLRs) are the membrane-bound PRRs that are highly conserved, capable of detecting a wide range of stimuli such as viral DNA/RNA, bacterial lipopolysaccharides, lipoproteins, and nucleic acid, *etc*; which serve as PAMPs (Kawasaki and Kawai, 2014). TLRs are important regulators of innate immunity against microbial infections in the innate immune system. Recent studies have added to the current insights in animal models by elucidating the effects of aging on TLR function in human cohorts. With increasing age, TLR expression and activity in monocytes (Van Duin et al., 2007), DCs (Panda et al., 2010), and neutrophils (Montgomery and Shaw, 2015) decreases. The splenic and peritoneal macrophages of elderly female C57BL/6 mice (18–24 months old), as well as older humans (≥65 years), had been reported to possess lower levels of TLR expression (Renshaw et al., 2002). While most TLRs are upregulated upon interaction with specific ligands in the macrophages of the young, the TLR expression, in the macrophages of elderly mice, is weakly upregulated, and in particular, expression of TLR3 is poorly identified (Renshaw et al., 2002).

Surprisingly, not all different types of TLRs were found to be downregulated in the elderly subjects. TLR1 surface expression, for example, declines with age, and but TLR2 surface expression remains constant (Van Duin et al., 2007). Low TLR1 surface expression and impairment in TLR1/2 signaling in the elderly resulted in reduced MAPK signaling, which in turn reduces the production of TNF-α and IL-6 in the host monocytes. After activation with TLR ligands, such as TLR1/2, TLR2/6, TLR3, TLR4, TLR5, and TLR9 agonists, macrophages from aged mice produce reduced amounts of IL-6 and TNF-α (Renshaw et al., 2002).

In contrast, upon interaction with specific ligands, monocytes from elderly human beings had upregulated expression of TLR5 molecule. This in turn resulted in enhanced production of TLR5-induced cytokines (Qian et al., 2012). TLR5 signaling is effectively maintained during aging, according to Lim et al., who reported that *in vitro* overexpression of caveolin-1 increased TLR5 mRNA via the MAPK pathway and prolonged TLR5 half-life via direct contact (Lim et al., 2015). Principally, TLR expression declines with age, with the exception of TLR5. TLR mislocalization can lead to changes in the expression of cytokine and chemokine thereby affecting the functioning of the immune system of the host (Qian et al., 2012).

TLR-7 and TLR-8, expressed in the endosomal compartments of respiratory epithelial cells, are the two TLRs that play a key role in sensing ssRNA of invading SARS-CoV-2 (Moreno-Eutimio et al., 2020; Onofrio et al., 2020; Sallenave and Guillot, 2020). The production of both IFN regulated cytokines, as well as pro-inflammatory cytokines, were induced by TLR7, and TLR8 respectively (Moreno-Eutimio et al., 2020; Onofrio et al., 2020). Aging impairs the migration of innate immune cells and signaling events following PRRs activation resulting in increased production and dysregulation of cytokines, commonly known as cytokine storm (Shaw et al., 2013; Onofrio et al., 2020). Innate immune cells with age-related changes in TLR expression can still produce cytokines. In human monocytes, for example, reduced TLR1 surface expression combined with lower TLR1/TLR2 might cause cytokine production. The observed post-translational aberration has been reported to worsen with age (Shaw et al., 2013; Onofrio et al., 2020). The production of a plethora of cytokines in the host, post SARS-CoV-2 infestation implies, high basal TLR activation, which cannot be further stimulated in response to a pathogen origin ligand, and may result in innate immune system failure (Shaw et al., 2013; Onofrio et al., 2020).

Monocytes and Macrophages

Even though there are no significant variations in the number of total monocyte subsets between the young (21–40 years) and the elderly (≥65 years) subjects, a worldwide study of circulating monocytes in various age groups reveals substantial age-related alterations (Metcalf et al., 2015). Non-classical $CD14^{+}CD16^{+}$ monocyte counts, for example, increased dramatically with age, however, exhibited underexpression of HLA-DR, and chemokine receptor CX (3)CR1 in the elderly. On the other hand, classical $CD14^{+}CD16^{-}$ monocyte numbers, did not change with age, despite the fact that serum MCP-1 levels elevate with age, but not MIP-1α, MIP-1β, and or fractalkine (CX3CL1) (Seidler et al., 2010). Human monocyte subsets responded differently to TLR agonists depending on their age, resulting in changes in surface molecule expression and lower production of interferons, and cytokines such as IL-1β (Metcalf et al., 2017). Monocytes from elderly people show reduced phagocytosis yet

have shorter telomeres and considerably greater intracellular levels of TNF-α both at the basal level and after TLR4 activation, suggesting that the elderly have dysfunctional monocytes (Hearps et al., 2012).

In general, the functioning of macrophages is impaired by aging, along with the reduction in monocytic activity. TLR expression on macrophages is decreased in elderly humans and mice, as previously documented (Renshaw et al., 2002; Shaw et al., 2011). Macrophages are significantly prevalent as a pro-inflammatory M1 phenotype in healthy old adipose and hepatic tissue, and as an immunosuppressive M2 phenotype in old lymphoid tissues, lung, and muscle (Jackman et al., 2017). In aged people, the buildup of immunological complexes, cytokines, hormones, free fatty acids, oxidized low-density lipoproteins, and immunoglobulins readily activate macrophages (Acharya et al., 2020). This generally ensues in low-grade inflammation, which in turn compromises the overall efficacy of the immune response (Acharya et al., 2020). The expression of IFN family cytokines results in the upregulation of C- reactive protein in the infected host. Due to decreased IFN production, there is an imbalance between proinflammatory, and pro-repair airway macrophages in patients with SARS-CoV-2 (Acharya et al., 2020). Chelvarajan *et. al*. described decreased TNF-α and IL-6 production and enhanced IL-10 production in elderly (22–24 months old) BALB/c and C57BL/6 mice after activation with TLR ligands (Chelvarajan et al., 2005). Also, the CD14 and TLR4-expressing cells had been reported to downfall resulting in a decrease in cytokines including IL-6, TNF-α, IL-1β, and IL-12 in the host (Chelvarajan et al., 2005). Inflammaging can lead to the buildup of alternatively activated (M2-like) macrophages in tissues, which remain pro-inflammatory, and exhibit senescence markers. It can be inferred that aging affects a variety of activities in macrophages, including TLR signaling, polarisation, phagocytosis, and wound healing, *etc.* (Van Beek et al., 2019).

Dendritic Cells (DCs)

Several studies have found that while the numbers of myeloid DCs (mDCs) do not change with age, the number and function of plasmacytoid DCs do decline (Shodell and Siegal, 2002; Della Bella et al., 2007; Jing et al., 2009). It is worth noting that the number of specialized DCs in the epidermis and mucosal tissues, such as Langerhans cells, decreases as people get older (Schwartz et al., 1983; Sprecher et al., 1990). DCs from the aged people have considerably decreased capacity to phagocytose antigens on a functional level (Pietschmann et al., 2000). When exposed to LPS, ssRNA, or self-DNA, aged mDCs produce more IL-6 and TNF-α than young mDCs. This induction had been correlated to age-related changes in signaling pathways that lead to the altered PI3K, NF-κB, or type I IFN responses. DCs function can be impacted by age-related alterations in signaling pathways, which can result in defective cytokine production in response to pathogens or self-DNA, as well as decreased phagocytosis, and migratory capacities (Agrawal et al., 2007; Agrawal et al., 2009). In SARS-CoV-2 patients, changes in the lung microenvironment also impact DCs maturation and migration to lymphoid organs, influencing T cell activation (Tay et al., 2020).

Neutrophils

Neutrophils, major phagocytic cells that specialize in imparting protection against invading pathogens in the early stages of infection (Hong, 2017). While the neutrophil cell count does not vary as people get older, their phagocytic and chemotactic capacities do (Wenisch et al., 2000). Nevertheless, the recruitment of neutrophils to the site of infection is affected by aging, as evidenced by studies that have found decreased neutrophil-mediated chemotaxis in aged hosts (Niwa et al., 1989; Wenisch et al., 2000). Neutrophils require a high level of energy to carry out phagocytosis, aging downgrades hexose transport and raises intracellular calcium levels, preventing the uptake of energy, and as a result, phagocytosis. Age-linked abnormalities in neutrophils are mostly caused by defective signal transduction pathways such as poor anti-apoptotic responses regulated by the JAK-STAT tyrosine kinase and PI3K-AKT pathways (Shaw et al., 2013; Jackman et al., 2017). The phosphorylated form of the PI3K regulates phagocytosis, degranulation, and chemotaxis of the neutrophil population. In the elderly population, the elevated activity of PI3K causes neutrophils to migrate imperfectly and secrete proteases in the surroundings that harm normal tissue rather than aberrant tissue at the site of inflammation or infection (Sapey et al., 2014). It has been demonstrated that like other viruses, SARS-CoV-2 can also stimulate neutrophil extracellular traps (NETs) in a process called NETosis in human neutrophils (Arcanjo et al., 2020). However, aged neutrophils generate reduced neutrophil extracellular traps (NET), which are consisted of nuclear components and granule proteins, and are capable of binding and trapping extracellular pathogens to protect the body from infection (Tseng et al., 2012; Hazeldine et al., 2014). As a result, a decrease in NET production with age might cause delayed wound healing and a greater vulnerability to invading pathogens (Tseng et al., 2012).

Type 1 IFN and NK Cells

PAMPs and DAMPs activate PRRs, which trigger a signaling cascade that results in the production of type I IFN and other inflammatory mediators during a pathogenic invasion (Acharya et al., 2020). During the antiviral defense, early activation and generation of type I interferon are beneficial. Type I IFN inhibits viral replication and dissemination by acting as an early antiviral response through autocrine and paracrine type I IFN receptor (IFNAR) signaling (Nikolich-Žugich, 2018; Acharya et al., 2020). Delayed production of IFN might cause tissue injury and inflammatory cytokine storm. The time-lapse in the production of type I IFN increases with age and has also been observed in the SARS-CoV-2 patients in general (Nikolich-Zugich et al., 2020; Acharya et al., 2020). Furthermore, the SARS-CoV-2 virus adapts immune evasion mechanisms to preclude the early production of type I IFN. It has been reported that SARS-CoV-2 type I IFN is very less in individuals with serious SARS-CoV-2 symptoms, in addition to delayed local IFN responses (Aiello et al., 2019; Acharya et al., 2020). The altered production of IFN led to an unbalanced state between proinflammatory macrophages and reparative airway macrophages (Aiello et al., 2019; Acharya et al., 2020). Host susceptibility to severe COVID-19 disease

can be increased by either genetic or acquired abnormalities in type I interferon signaling. Bastard *et al.* employed sensitive immunoassays and neutralization assays to determine the existence of autoantibodies against α, β, and ω type I interferons in plasma samples from a large cohort of COVID-19 patients and uninfected controls (Bastard et al., 2021). Furthermore, it has been discovered that nearly 10% of patients with severe COVID-19 pneumonia had significant titers of neutralizing autoantibodies against type I IFN-2α and IFN-ω (Bastard et al., 2020). Infected patients, who were asymptomatic or had milder symptoms, as well as healthy people, did not have these autoantibodies. In the control group, the incidence of neutralizing autoantibodies to type I interferon increased with age, peaking at the age of 70. These data suggest that autoantibodies against type I IFNs are a prevalent form of acquired immunodeficiency that accounts for around 20% of all COVID-19 deaths (Bastard et al., 2021). During coronavirus infections, IFN modulates other immune cells such as NK cells, and which are not only down numbered rather become dysfunctional in individuals with advanced-stage SARS-CoV-2 infection (Aiello et al., 2019; Acharya et al., 2020). In elderly people, alteration in the activity of NK cells is linked to increased severity of infection that is accompanied by death (Shaw et al., 2013). There are reports which suggest that type I IFN downmodulates NK cell functioning in elderly mice (Plett and Murasko, 2000). Furthermore, in response to the virus, the synthesis of IFN by plasmacytoid DCs diminishes as people get older (Agrawal, 2013). As a result, the reduced and delayed production of type I IFN, as well as the altered production of NK cells in elderly people, affect the first-line antiviral response to SARS-CoV-2 invasion, and reduce the possibilities of infection resolution at an earlier stage than in younger people (Shaw et al., 2013; Aiello et al., 2019; Acharya et al., 2020).

COMORBIDITIES AND INNATE IMMUNE RESPONSE

SARS-CoV-2 symptoms are more evident in older patients (>60 years) and individuals with a history of comorbidities, owing to hyperactive chronic innate inflammatory responses as well as structural and functional alterations in organs (Frasca et al., 2017). Obesity boosts the difficulties linked with the virus and aging, in part, owing to structural/functional abnormalities and also due to visceral fat that can act as a viral repository and exacerbate inflammatory responses (Iacobellis, 2020; Malavazos et al., 2020; Ryan and Caplice, 2020). In both older (>60 years) as well as younger (under the age of 40) COVID-19 patients with severe disease compared to those with mild disease, Computed Tomography (CT) imaging revealed increased fatty liver and epicardial adipose tissues, implying obesity as a potential predictor of COVID-19 severity (Iacobellis et al., 2020b; Deng et al., 2020). Additionally, adipose tissues function as endocrine organs, producing hormones such as leptin and adiponectin, as well as inflammatory cytokines like IL-6 and TNF (Banerjee et al., 2020). Since obese and diabetic patients have more adipose tissue, they have more ACE2 expressing adipocytes, thus increasing their susceptibility to viral invasion in the adipose tissue, which might lead to greater severity of SARS-CoV-2 infection (Banerjee et al., 2020; Fajnzylber et al., 2020). In obese people, innate immune cells like NK cells, macrophages, and neutrophils, as well as adaptive immune cells, enter the adipose tissue and trigger inflammatory responses through the release of pro-inflammatory cytokines, metabolic alterations, and phenotypic and functional abnormalities (Frasca et al., 2017). Obesity is also a potential risk factor for endothelial dysfunction, which, when coupled with SARS-CoV-2 caused endothelial cell damage, ensuing in increased endothelial dysfunction that eventually worsens associated problems (Engin, 2017; Amraei and Rahimi, 2020). The combination of age-related defective patterns in many components of the innate immune inflammatory responses prevents the virus elimination from the host (Frasca et al., 2017). It explicitly implies that persons over the age of 60 and those with co-morbidities are at a greater risk of SARS-CoV-2 severity because they are more susceptible to induce a defective immune response and have relatively high rates of morbidity and mortality.

MODULATION OF THE ADAPTIVE IMMUNE SYSTEM WITH AGE

B Cells

B lymphocytes are key regulators of the adaptive immune system, and play role in various processes including antibody synthesis to cytokine release (Ponnappan and Ponnappan, 2011; Nikolich-Zugich et al., 2020). Invading pathogens as a strategy to counter host immune system onslaught on one hand and host-related other factors (comorbidities and age-related) may deform the functioning of the host B cells (Ponnappan and Ponnappan, 2011; Nikolich-Zugich et al., 2020). Deformity in the quality of secreted antibodies has been investigated more extensively in the context of aging than any of these two roles (Ponnappan and Ponnappan, 2011; Nikolich-Zugich et al., 2020). Since B cells must produce a variety of specific antibodies to identify a wide range of threatening antigens, heterogeneity is important in the B cell repository for an effective immune response (Anolik et al., 2009). Aged people often have a constrained B cell repository, which could make them more susceptible to infectious diseases, less able to react to vaccinations, and more likely to produce autoreactive Abs (Ponnappan and Ponnappan, 2011). According to a previous study, aging can affect the selection procedure during B cell affinity maturation (Banerjee et al., 2002), and as people get older, the B cell repertory becomes less diversified, with indications of non-pathogenic clonal expansions (Gibson et al., 2009). Poor vaccination responses against various infectious diseases are believed to be linked to the host immune system that loses its potential to generate differentiated antibody pools with expanded and diverse recognition (Goronzy and Weyand, 2013). Aging lowers the number of naive B cells and plasma cells whereas increasing the numbers of $CD27^{+}$ memory B cells in mice (Fecteau et al., 2006). Though B cell lymphopoiesis is active throughout life, there is a reduction in B cell synthesis in elderly

populations as bone marrow is degraded, and also the percentages and quantities of human peripheral B cells significantly reduced with age (Zharhary, 1988; Rossi et al., 2003; Frasca et al., 2008).

Alterations in the B cell population can be linked to Ab production, in which B cells reduce their capacity to generate an adequate Ab response against naive as well as known antigens, as they get older. Furthermore, in the aged host, the Ab response is accompanied by poor isotype switching and a brief time of activation (Nikolich-Žugich, 2018). Also, reduced affinity is not just related to lowered production of B cells, rather related to decreased rates of isotype switching, and less somatic hypermutation in the aged immune system (Henry et al., 2019). The class switch recombination and somatic hypermutation are the two cellular mechanisms that produce newer classes of Abs. The process is mainly regulated by activation-induced cytidine deaminase (AID). In older people, impairment in MAPK signaling as well as the reduction in mRNA stability and DNA binding affinity of transcriptional factor E47 (encoded by E2A), ensues in the downregulated expression of AID gene (Oh et al., 2019). As a result of these internal changes in B cells, Ab diversity is eventually reduced. The microRNA of the B lymphocytes such as miRNA-155 and miRNA-16 had been described as major contributors to the molecular changes linked with B cell senescence mediated by the downregulation of AID and E47 (Frasca et al., 2015). Downregulation of E2A caused by aging promotes abnormalities in class switch recombination of IgM memory B cells, and reduced expression of AID gene in B cells has been found to cause downmodulation of class switch recombination in the elderly subjects (Van der Put et al., 2004; Frasca et al., 2004). Thus, reduction in the expression levels of AID and E47 in the elderly population is expected to diminish the size and the number of germinal centers, in which mechanisms of Ab affinity maturation, including somatic hypermutation take place, therefore, decrease Ab affinity maturation, and the frequency of circulating Abs from plasma cells (Frasca, 2018).

The levels of IgG antibody specific for viral spike (S) protein in rhesus macaques are lower in elder macaques compared to younger ones during the early stages of acute SARS-CoV-2 infection which indicates a strong correlation of aging with impeded production of class-switched antibody titers required to eliminate the viral infection (Singh et al., 2021). The upregulation of age-linked attributes or features has been reported in B cells of aging animals as well as human beings (Cancro, 2020). TLR7 involvement is also important, considering the fact that it is a significant PRR for SARS CoV-2 mediated stimulation of host B cells (Cancro, 2020). These cells, populated in the late memory fraction (IgD^{-} $CD27^{-}$, also known as double-negative B cells), release inflammatory molecules like TNF-α, IL-8, and IL-6, have been linked to autoimmune illness, chronic viral disease, and COVID-19 (Frasca et al., 2017; Cancro, 2020).

T Cells

The T lymphocytes play a major role in the containment of virus-based infections. The role of T cells during aging is being emphasized due to their impact on overall immunological responses. Some of the impacts of aging include a gradual drop in the formation of freshly naive T cells, a more limited TCR repertoire, and ineffective activation of T cells (Salam et al., 2013). T cell output drops by 90% at puberty due to the involution of the thymus, which transforms into adipose tissue. Around the age of 40–50, T cells production declines again, and the remainder of the thymus degenerates (Nikolich-Zugich et al., 2020). The generation of naive T lymphocytes is reduced to just 1% of what it was before primary involution due to secondary atrophy. Furthermore, as people become older, lymph nodes become less capable of storing, and maintaining naive T cells (Nikolich-Zugich et al., 2020). Except in the extremely old, overall T cell counts do not drop with age, and this is owing to a compensatory increase in peripheral T cell proliferation, known as homeostatic proliferation, which is followed by the procurement of activated/memory cell phenotypes (Sprent and Surh, 2011; Ponnappan and Ponnappan, 2011). This indicates that, whereas the elderly have similar lymphocyte counts to younger people, their capacity to develop an adaptive response to a novel antigen is considerably reduced as a result of the exchange of naive T cells with antigen-independent experienced cells (Ponnappan and Ponnappan, 2011). Insignificant counts of naive T cells were linked to severe outcomes of COVID-19 (Moderbacher et al., 2020). This study might point to a link between the reduced naive TCR repertoire of the elderly before SARS-CoV-2 infection and poor disease outcomes.

Aging causes complex changes in T cells, affecting a variety of T-cell subsets such as naive, memory, and effector T-cells, and T_{EMRA} cells (Dixit, 2012). However, IL-7, a critical maintenance component of T cell homeostasis, does not vary with age, it usually reduces the number of naive T cells, and increases the number of senescent T cells (Nikolich-Žugich, 2018). Elderly T cells go through specific alterations that restrict antigenic specificity and decrease TCR expression, resulting in an age-related shift in gene expression which is induced by TCR in human $CD4^{+}$ T cells (Bektas et al., 2013). TCR diversity was shown to be reduced with age utilizing the high-throughput illumina sequencing technology, revealing a substantial drop in the number of naive T cells, and TCR-beta diversity by the age of 40 (Tseng et al., 2012). Furthermore, thymus involution and changes in transcriptional factor expression levels result in dysfunctional T cells, which cause inflammaging and a greater risk for infection by lowering vaccination effectiveness (Britanova et al., 2014).

The rise of poor T cell activity over time was linked partly due to the age-related decrease of immunity or immunosenescene. Rather than becoming inefficient, senescent T cells, particularly those in the CD8 subpopulation, lose major elements of their TCR signaling machinery while gaining Natural Killer (NK cell) features. This suggests that T cells gain a new functional viewpoint when they develop towards a proliferative end-stage, irrespective of their antigen specificity (Abbas and Akbar, 2021). As cytokines are important regulators of the immunological response mediated by T cells, it has been suggested that altered cytokine production may cause age-linked defects in T cells. It has been found that elderly T cells mainly have a Th2-like phenotype and indicated by a shift in cytokine profiling (Mansfield et al., 2012). Th17 cells protect the body against

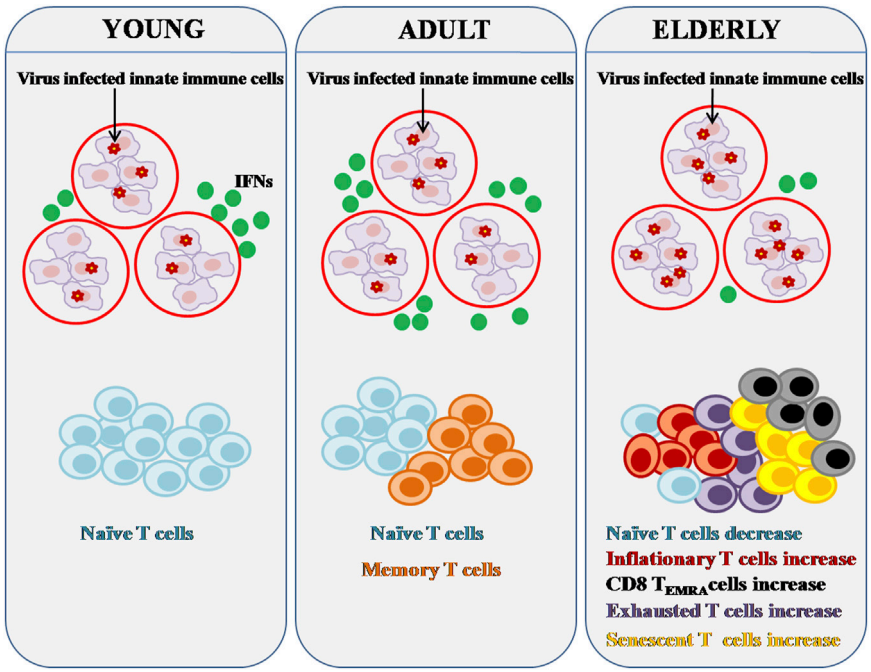

FIGURE 2 | Effect of Immunosenescence on chronic viral infection and immunity. Major components of both the innate and adaptive immune systems alter as people age. Innate immune cells can activate IFN pathways in response to viral infection in order to clear virus-infected cells. Reduced IFN production can be caused by age-related abnormalities in innate immune cells. Persistent viral infection can lead to significant changes in adaptive immunity, particularly T cell composition and function. There are fewer naive T cells in the aged, but there are more senescent, inflationary, and or exhausted T cells that are functionally inert or dormant.

extracellular pathogens and have been linked to the onset of autoimmune and chronic inflammatory disorders in individuals (Sandquist and Kolls, 2018). In the elderly, the ratio between Th17 and Treg seems to rise and it has been speculated that this change in the ratio might explain why the elderly are more prone to autoimmune diseases and have weakened immune responses to pathogens (Schmitt et al., 2013).

The influence of immunosenescence on T cells engaged in persistent viral infections is seen in **Figure 2**. Aging causes a drop in naive $CD8^+$ T cells, a decrease in the heterogeneity of TCR repertoire, and an increase in senescent, exhausted, terminally differentiated $T_{EMRA,}$ and inflationary T cells. It has been described that $PD\text{-}1^+/CD153^+$ memory phenotype $CD4^+$ T cells as the senescence-associated T (SA-T) cell phenotype (Tahir et al., 2015). Both the TCR-mediated proliferation as well as T cell cytokine generation are impaired in SA-T cells, indicating cellular senescence. These cells release pro-inflammatory cytokines, which can build up and cause chronic inflammation in tissues (Fukushima et al., 2018).

Along with a rise in senescent T cells, the elderly experience a gradual and sustained growth of inflationary T cells (Kim et al., 2015). TCR repertoire is restricted in inflationary T cells, and so are the lymph node homing signals that cause accumulation in non-lymphoid peripheral organs and co-stimulatory receptors, as well as large amounts of inhibitory receptors (Snyder et al., 2008). Memory T cells produced by the elderly population may be poorly maintained in the peripheral repertoire, possibly due to niche competition caused by memory inflation (Klenerman and Oxenius, 2016). COVID-19 severity has been connected to the prevalence of cytomegalovirus (CMV), a major pathogen related to memory inflation, but additional research is required to determine how CMV, as well as other latent viruses, influence the production of new memory T cells, function, and longevity in the elderly population (Shrock et al., 2020).

Senescent T cells from both mice and humans lose CD28 expression while gaining CD57 and KLRG-1 expression, similar to inflationary T cells (Akbar et al., 2016). T cell exhaustion, which occurs as a result of persistent viral infections or malignancies, is also more common among the elderly. T cells from the elderly specifically expressed higher levels of CTLA-4 (cytotoxic T-lymphocyte-associated antigen-4), PD-1 (programmed death-1), and TIM-3 (T cell immunoglobulin and mucin domain-containing protein 3) (Leng et al., 2002; Shimada et al., 2009; Lee et al., 2016). CTLA-4 and PD-1 are negative co-stimulatory/inhibitory receptors of T cells and both have been reported to be upregulated in the aged humans and mice T cells (Lee et al., 2016). TIM-3, an exhaustion marker of T cells, together with other inhibitory receptor PD-1 mediates $CD8^+$ T cells exhaustion in terms of proliferative potential and cytokine production such as IL-2, IFN-γ, and TNF-α (Lee et al., 2016). The severity of COVID-19 is linked to lymphopenia, a condition characterized by remarkably low numbers of lymphocytes. The link between poor prognosis and a decrease in the total number of peripheral T lymphocytes in the blood has been reported (Huang and Pranata, 2020). Immunosenescence contributes to weaker T cell responses, which might be worsened

by a drop in T cell counts, and making lymphopenia shown in severe COVID-19 even more dangerous for the aged individuals. A cohort study of 522 COVID-19 patients found a substantial link between lymphopenia and age, with patients >60 years having a minimum number of total T cells in their blood (Diao et al., 2020).

TISSUE-SPECIFIC IMMUNITY

Bone Marrow

All hematopoietic cells are produced in bone marrow, which also serves as the site of B cell maturation where precursor cells acquire surface immunoglobulin (Zhao et al., 2012). Antigen-experienced adaptive immune cells thrive in the bone marrow (BM), which provides a favourable environment for their long-term survival. Effector/memory T cells and plasma cell precursors move to the BM after coming into contact with antigens, where they may live in survival niches without being exposed to antigens (Naismith et al., 2019). In addition, adaptive immune cells phenotype varies with age, as do BM niches, resulting in poor long-term immunological memory maintenance in the elderly. This process appears to be influenced by oxidative stress, age-related inflammation (inflammaging), and cellular senescence (Naismith et al., 2019). In the human BM, the impact of aging on the synthesis of certain survival factors for effector/memory T cells and long-lived plasma cells has been reported (Pangrazzi et al., 2017). Particularly, the levels of IL-7, a cytokine that promotes memory T cell survival, were decreased in BM in old age. According to Stephan *et al*, BM stromal cells taken from elderly donors expressed less IL-7 than stromal elements derived from young BM donors (Stephan et al., 1998). In the BM, IL-15 was shown to be elevated in old age, mainly favouring the maintenance of highly differentiated, senescent-like T cells (Herndler-Brandstetter et al., 2011; Pangrazzi et al., 2017). Furthermore, with aging in the BM, the plasma cell survival factor april (a proliferation-inducing ligand) was reduced whereas the IL-6 level was elevated. The chemokine CXCL-12, which aids in the recruitment of plasma cells into niches was unaffected by age (Hargreaves et al., 2001). As a result, while aging may affect adaptive immune cell survival in the BM, the migration of these cells to niches may not be compromised. In humans and mice, IL-15 accumulation in the BM is thought to be advantageous because it promotes the activation and proliferation of effector/memory $CD8^+$ T cells. (Becker et al., 2005; Herndler-Brandstetter et al., 2011). Despite this, it is crucial to keep in mind that both IL-15 and IL-6 are not only involved in the maintenance of adaptive immune cells, but they are also pro-inflammatory cytokines, hence, their levels should be maintained. It has been hypothesized that inflammation may be accountable, or at least contribute, to age-related deficiencies in the maintenance of immunological memory in the elderly, and because these molecules were over-expressed in the aged BM (Naismith et al., 2019). As the frequency of $CD8^+CD28^-$ T cells in the BM increased with age, it has been observed that IL-15 may play a key role in the survival of this fraction of highly differentiated T cells (Pangrazzi et al., 2017). Furthermore, $CD8^+CD28^-$ T cells in the BM expressed a significant amount of IFN-γ and TNF; thus, BM niches essential for the long-term maintenance of memory T cells seem to become pro-inflammatory in old age, with $CD8^+CD28^-$ T cells supporting this process. Indeed, $CD8^+CD28^-$ T cells produce IFN-γ and TNF after being recruited to the BM, which may operate on BM cells to stimulate the production of IL-15 and IL-6, hence encouraging the attraction of new $CD8^+CD28^-$ T cells. As a result, it is believed that in the aged BM, a vicious cycle of inflammation may occur, and compromising the maintenance of immunological memory (Naismith et al., 2019). In addition, a reduction in cellularity in the bone marrow is the most obvious alteration with age. Adipocytes, which are found in bone marrow, are beneficial for the development of young individuals and also provide a protective role (Ma and Fang, 2013). Hematopoietic tissue takes up 40–60% of marrow space in young individuals, 20–40% in elderly persons, whereas adipocytes take up the rest. By the time a person reaches the age of 30, these adipocytes have nearly completely replaced the bone marrow (Veldhuis-Vlug and Rosen, 2018). When bone marrow cells were irradiated in tissue with a higher proportion of marrow adipose tissue, the replenishment of blood cells was hindered (Ambrosi et al., 2017) as the study has indicated a link between marrow adipose tissue and hematopoiesis impairment. It's conceivable that the negative link between marrow adipose tissue and blood cell growth contributes to the elderly's weakened immune response.

Thymus

The thymus is the principal organ for the maturation of T cells with a wide range of antigen-recognition capabilities. T cells mature in the thymus after originating in the bone marrow (Thapa and Farber, 2019). T cells proliferate in the thymus and differentiate into helper T cells, regulatory T cells, or cytotoxic T cells, and as well as memory T cells. They are subsequently delivered to peripheral tissues or circulated in the bloodstream or lymphatic system (Thapa and Farber, 2019). The thymus starts to shrink around puberty, a condition known as thymic involution that is evolutionarily and conservatively maintained in vertebrates (Gui et al., 2012). The capacity of an individual to fight novel pathogens is reduced as a result of this condition. Thymic involution is characterized by a gradual reduction in thymus size as well as diminished thymic structure as people become older. Thymopoiesis is reduced when the number of thymic compartments decreases (Gui et al., 2012). There are several plausible explanations which shows that why this thymic involution process takes place with age. First, since it produces huge numbers of cells, the thymus is highly metabolically active, and the body has a tendency to downregulate metabolic processes in order to preserve energy, as one gets older (Gui et al., 2012). Second, this is the age when a person has most certainly come into contact with the vast number of pathogens in their local surroundings. As a result, it is physiologically advantageous to reduce energy consumption on the creation of new T cells instead depending on memory cells that have the ability to sense previously experienced pathogens (Gui et al., 2012). Thymic involution could be one reason why the older have worse outcomes when

TABLE 1 | Age related changes in the immune system are summarized below.

Cells	Immune response	Aging associated impairment
Monocytes/macrophages	Innate	Less phagocytic activity Downregulated MHC II expression Decreased ROS and cytokine production Reduced TLR expression (except for TLR5)
Dendritic cells	Innate	Decreased maturation and Ag presentation Reduced TLR expression and signaling Impaired Ag uptake Reduced CD80 and CD86 expression
Neutrophils	Innate	Reduced chemotaxis Downregulated MHC II expression Decreased ROS and cytokine production Altered TLR expression Decreased NET formation
NK cells	Innate	Decreased perforin degranulation Reduced cytotoxicity
B cells	Adaptive	Limited diversity in B cell receptor (BCR) repertoire Decreased numbers of naive or circulating B cells Reduced antibody affinity Diminished memory B cells homeostasis
T cells	Adaptive	Limited diversity in Tcell receptor (TCR) repertoire Relative decrease in naive T cells Relative increase in memory T cells Increased number of senescent T cells Increase of CD8 T_{EMRA} cells Expansion of inflationary CD8 T^{+} cells caused by chronic viral infections Reduced effector T cell response to novel Ag

they get infected with new infections. For combating viruses, CD8 T cells and CD4 Th1 cells are especially important. Because SARS-CoV-2 employs several methods to circumvent the innate immune response (De Wilde et al., 2017), such as masking PAMPs and inhibiting IFN signaling, the adaptive immune response becomes even more crucial. If the new T cell receptors (TCR) are not produced, the individual will be unable to generate an effective immune response against any novel pathogens.

Spleens

The spleen performs an essential function in fighting off infections in the bloodstream and triggering an adequate immune response to them (Bronte and Pittet, 2013). Usually, B cells in the marginal zone (MZ) of the spleen grab antigen and immune complexes from the blood and shuttle them to follicular dendritic cells in B-cell follicles, subsequently conducting reaction in the germinal center to T-independent antigens (Cerutti et al., 2013). It has been studied that antigen capture by MZ B cells in the spleens of elderly mice was decreased, and so their tendency to move between the MZ and the follicles (Turner and Mabbott, 2017a). This impairment in the movement between the two locations in elderly mice's spleen is due to the heightened response to the chemo-attractant sphingosine-1-phosphate (S1P), which tends to keep them in the marginal zone (Turner and Mabbott, 2017b). This may not be significant to the aged response to SARS-CoV-2, since it is a T-dependent antigen, although it is an important factor to be considered (Bajaj et al., 2021).

Lymph Nodes

Lymph nodes are secondary lymphoid organs that are found throughout the lymphatic system. They work to keep the organism healthy by filtering harmful particles out of the lymphatic fluid (Turner and Mabbott, 2017a). Antigen recognition, B and T cell activation, and proliferation are all key processes that occur in lymph nodes. Lymph nodes in aged mice and humans exhibit reduced interactions between B and T cells, lower numbers of T cells, abnormal lymphocyte migration, and a reduction in the number and size of germinal centers (Turner and Mabbott, 2017b). These alterations have a detrimental influence on the establishment of an adaptive immune response, in addition to the loss of follicular areas and germinal centers. A variety of age-related changes are likely to blame for the loss of follicular structure. First, there is a deterioration of follicular dendritic cell network (FDCs) with age. These FDCs play a critical role in the maintenance of the reaction occurring in the germinal centers (Bajaj et al., 2021). Immune complexes are kept by FDCs for presentation to B cells leading to stimulate proliferation in the germinal center and isotype switching in order to establish an adequate immune response. In addition, CXCL13 (selectively chemotactic for B cells) expression in the follicle is lower in the aged lymph nodes. CXCL13 is expressed by both follicular dendritic cells (Wang et al., 2011) and germinal centers follicular helper T cells (Tfh cells) (Rasheed et al., 2006) in the B-cell follicles. This chemokine is required, together with its receptor CXCR5, for the homing of naive B lymphocytes into

a lymph node's follicle, where they might recognize antigens and activated with T cells prior to clonal expansion in the germinal centers. The fibroblastic reticular cells especially play a critical role in the maintenance of naive T lymphocytes and lymph node development. Furthermore, a study revealed that the number of fibroblastic reticular cells and lymphoid endothelial cells had reduced in aged lymph nodes (Thompson et al., 2019). This study revealed that the outer capsule of aged lymph nodes in mice and rhesus macaques was thickened, the stroma was infiltrated with collagen, and the total size of the lymph node was reduced. Thus, these microscopic alterations may explain why the elderly lymph node is unable to maintain naive T cells that have recently moved into it (Thompson et al., 2019). For the novel T-dependent antigens like SARS-CoV-2, the retention and migration of naive T cells into the follicle is required for B cells activation, proliferation, and the appropriate immune response for the elimination of the virus (Bajaj et al., 2021).

COMORBIDITIES, AGING, AND DISEASE SEVERITY IN COVID-19

The severity of COVID-19 disease is linked with numerous comorbidities and patients with old age (>60 years) (Callender et al., 2020). It has been speculated that when infected with SARS-CoV-2, individuals with comorbidities experience more serious clinical outcomes when compared to patients with no pre-existing comorbidities (Wang et al., 2020). The most common pre-existing comorbidity in COVID-19 patients has been reported as hypertension (Yang J et al., 2020; Zuin et al., 2020). According to a retrospective study, people with hypertension are at a higher risk of serious infection and death (Zuin et al., 2020; Lippi et al., 2020). Cardiovascular disease has also been found to be associated with several worse clinical outcomes with the elevated death rates in COVID-19 patients (Guan et al., 2020). COVID-19 severity was also shown to be higher in patients with type 2 diabetes, as it is the third most prevalent underlying symptomatology in COVID-19 patients (Zhu et al., 2020; Fadini et al., 2020). Diabetics are more susceptible to infection, and they are more likely to have several comorbidities, such as cardio-vascular disease (Guo et al., 2020). According to a recent report on COVID-19, diabetics were more susceptible to acquire pneumonia, accounting for 11.7% of serious cases but only 4% of mild/moderate cases (Li et al., 2020a) Obesity has also been linked to the majority of COVID-19 comorbidities, including hypertension, diabetes, and cardiovascular diseases (Balakumar et al., 2016; Kassir, 2020). Obesity prevalence varies widely over the world; for example, obesity is more prevalent in the United States and Europe than in Asian nations (Dietz and Santos-Burgoa, 2020). As a result, the severity of COVID-19 and death rates may differ. When comparing survivors versus non-survivors, research revealed that 88.2% of non-survivors had a BMI above 25 kg/m^2, which was a much larger proportion than survivors (Liu M et al., 2020). Additionally, ACE2 expression was found to be elevated in the adipocytes of obese people, suggesting that it might be a potential target for SARS-CoV-2 infection (Kruglikov and Scherer, 2020). Chronic obstructive pulmonary disease (COPD), among several other comorbidities, and has been linked to poor disease progression. In patients with underlying COPD who were confirmed with COVID-19, a meta-analysis of numerous studies in China indicated a four-fold increase in death (Zhao et al., 2020). As per another meta-analysis study of 1786 COVID-19 patients, hypertension (15.8%), cardiovascular and cerebrovascular disorders (11.7%), and diabetes (9.4%) were reported as the most prevalent comorbidities (Paudel, 2020; Zhou F et al., 2020). While co-existing HIV and hepatitis B infection (1.5%), malignancy (1.5%), respiratory diseases (1.4%), renal diseases (0.8%), and immunodeficiencies (0.01%) were reported as the least prevalent comorbidities (Paudel, 2020).

The elderly COVID-19 patients with chronic comorbidities like diabetes, hypertension, cardiovascular, and lung diseases are at a greater risk of acquiring the serious forms of the disease that usually gets fatal (Sanyaolu et al., 2020). For people above the age of 65 in the United States, cardiac disease is the leading cause of mortality, followed by chronic low respiratory disease in third place and diabetes mellitus in sixth place (Heron, 2019).

The adipokine enzyme DPP4 has also been considered as a crucial factor that can be correlated with worse outcomes post SARS-CoV-2 infliction (Bassendine et al., 2020). MERS-CoV employs this enzyme for entry into the target cells, while SARS-CoV-2 also binds to this enzyme through the S1 domain of the spike proteins (Li et al., 2020b). COVID-19 patients experience dysregulation in DPP4 functioning. DPP4 enzyme is present in salivary glands, liver, kidney, colon epithelial cells, alveolar cells type2, and capillaries as well as in immune cells such as T cells, B cells, and NK cells (Bassendine et al., 2020). The elevated expression of the DPP4 enzyme has been found in the elderly and also in many patients with diabetes or other metabolic disorders. Furthermore, it has been observed that adipose tissue is a source of DPP4 in mice models of diet-induced obesity (Bassendine et al., 2020). The broad expression of DPP4 might explain the vast range of COVID-19 symptoms. The related research, taken together, suggests a possible explanation for the link between pre-existing diseases, comorbidities, and poor clinical outcomes (Bassendine et al., 2020). DPP-4 inhibitors, widely used to treat type 2 diabetes mellitus, may be beneficial to COVID-19 patients with type 2 diabetes, as they can benefit from anti-inflammatory, anti-proliferative, and anti-fibrotic effects, in addition to glycemic regulation (Smelcerovic et al., 2020). Furthermore, DPP4 inhibitors are effective in the prevention and treatment of pulmonary fibrosis, heart disease, and kidney damage, and since these conditions are a long-term consequence of COVID-19, it is plausible to anticipate that DPP-4 inhibitors will be effective in reducing COVID-19's long-term repercussions (Smelcerovic et al., 2020).

THERAPEUTICS

COVID-19 has been linked to an increase in mortality in aged people, which has a great deal of correlation to immunosenescence, comorbidities, and the accompanying

proinflammatory condition that develops as people age (Franceschi et al., 2000; Shaw et al., 2013; Weyand and Goronzy, 2016; Fuentes et al., 2017; Fulop et al., 2018). Senolytics, commonly known as "anti-aging medications," eliminate senescent immune cells that build up over time and overproduce cytokines (Sargiacomo et al., 2020). Senolytics diminish the cytokine storm induced by excessive production of proinflammatory cytokines, may be beneficial to older persons. Drugs with senolytic properties, such as rapamycin and azithromycin, were widely employed as potential therapies for COVID-19, and it may help older persons in particular (Ma and Fang, 2013; Rea et al., 2018; Omarjee et al., 2020; Sargiacomo et al., 2020). Other medications that limit cytokine signaling or reduce inflammation, such as JAK-STAT pathway inhibitors and corticosteroids, may also help to reduce inflammation, particularly in aged COVID-19 patients (Rea et al., 2018; Gozzetti et al., 2020; Jamilloux et al., 2020; Sargiacomo et al., 2020).

Senolytics

Several clinical trials are being conducted to investigate the impact of senolytic drugs in the aging process (Kirkland and Tchkonia, 2020; Bartleson et al., 2021). Senolytics like fisetin, dasatinib, and quercitin (D + Q), remove senescent cells and diminish the SASP's pro-inflammatory and pro-thrombotic actions (Bartleson et al., 2021). Senolytics have been shown to relieve inflammatory symptoms in a few clinical trials. For instance, during idiopathic pulmonary fibrosis which is an age-related lung disease (Justice et al., 2019), D + Q lowered SASP-associated pro-inflammatory cytokines, and a current finding found that fisetin and D + Q lowered SASP and enhanced survivability in a mouse model of β-coronavirus (Camell et al., 2021).

IL-6 Inhibitors

COVID-19 patients exhibit a greater level of serum IL-6, which has been linked to a more severe illness and death. As a result, IL-6 dysregulation might cause problems in recovery. As part of the inflammaging phenotype, older persons have been found to have higher serum IL-6 levels, predisposing them to a more severe COVID-19 manifestation (Rea et al., 2018). The Chinese Health Commission and the Italian Society for Infectious and Tropical Diseases both had suggested the use of anti-IL-6 monoclonal antibodies (mAbs) such as tocilizumab in COVID-19 treatment. Anti-IL-6 mAbs have also been shown to enhance clinical outcomes in COVID-19 patients (Jamilloux et al., 2020). Siltuximab, like tocilizumab, is an anti-IL-6 mAb with a greater affinity for IL-6. Antibiotics like azithromycin, in addition to mAbs, can also be used to block IL-6. In addition to suppressing IL-1 and IL-6 cytokines, azithromycin increases the destruction of senescent cells (Sargiacomo et al., 2020).

Chloroquine/Hydroxychloroquine

The antimalarial, chloroquine (CQ) and its less toxic analog, Hydroxychloroquine (HCQ), have been demonstrated to affect the glycosylation of ACE2 receptor, hence disrupting the viral entry into the host cell (Liu et al., 2020). Furthermore, HCQ has been demonstrated to have anti-inflammatory properties, implying that it may aid in the reduction of pro-inflammatory cytokines release. However, clinical investigations employed with CQ/HCQ treatment have shown unsatisfactory outcomes that imply more danger than benefit. It has been suggested that HCQ and azithromycin, a macrolide antibiotic, may function together to reduce viral load *in vivo* and *in vitro* cell cultures (Andreani et al., 2020). These medications, however, are not without danger. The drugs such as HCQ, CQ, and azithromycin may cause cardiac arrhythmias by prolonging the QT interval (Kamp et al., 2020). Several COVID-19 patients have died as a result of severe cardiac events, presumably because of the overdosing of the above-specified drugs (Kamp et al., 2020). Surprisingly, in randomized controlled studies, HCQ was proven to be ineffective as a COVID-19 prophylactic or therapy (Boulware et al., 2020; Linsell and Bell, 2020). More clinical trials are required to explore the role of HCQ/CQ in COVID-19 patients.

Corticosteroids

Corticosteroids are anti-inflammatory agents. Glucocorticoids were frequently used to treat systemic inflammation in SARS patients during the 2003 outbreak, suggesting that they may be used to treat COVID-19 individuals with identical symptoms (Zhang W. et al., 2020). However, using corticosteroids in the treatment of COVID-19 involves risks such as reduced viral clearance and an increased risk of subsequent bacterial or fungal infections (Russell et al., 2020). Due to inconsistent data and the possibility of bias, more information is needed to fully understand the function of corticosteroid usage in COVID-19 patients.

JAK-STAT Pathway Inhibitors

Ruxolitinib and baricitinib are Janus kinase (JAK) inhibitors, have been discovered as potential COVID-19 treatment drugs. Despite the fact that JAK inhibitors may lower cytokine storms and alleviate COVID-19-induced ARDS, existing research is restricted by small sample sizes and inadequate experimental design (Bajaj et al., 2021).

Remdesivir

Remdesivir, a nucleoside analog that blocks viral replication in SARS-CoV and MERS-CoV, has been discovered as a potential COVID-19 therapeutic option. Remdesivir may significantly minimize recovery time, according to preliminary data from a double-blind, randomized, and controlled experiment (Beigel et al., 2020). Remdesivir was linked to a reduced risk of serious side effects, such as respiratory failure. Provisional data supports the use of remdesivir to cure COVID-19 patients (Beigel et al., 2020). However, new information from the WHO Solidarity experiment, a randomized controlled trial with a huge enrollment of over 11,000 individuals, contradicts remdesivir's success (Consortium et al., 2020). To explain the contradictory conclusions of the study, more evidence is required.

Dipeptidyl Peptidase-4 -Inhibitor

Dipeptidyl peptidase-4 (DPP-4) inhibitor has anti-inflammatory, anti-fibrotic, and anti-adipogenic characteristics, which could be

effective in inhibiting the progression to hyper-inflammation in extreme cases of COVID-19 (Strollo and Pozzilli, 2020; Iacobellis et al., 2020a; Valencia et al., 2020). Some adverse effects may be associated with using DPP4 inhibitors such as gastrointestinal complications, upper respiratory tract infection, headache, and allergic reactions (Pathak and Bridgeman, 2010). However, it has been observed that the usage of DPP-4 inhibitors was linked to decreased mortality in COVID-19 patients (Rakhmat et al., 2021). The use of DPP4 inhibitors, such as gliptins, in COVID-19 patients with or without type 2 diabetes may provide a convenient strategy to minimize viral entrance and reproduction into the airways, as well as to lessen the long-term cytokine storm and inflammation in the lungs has been studied (Solerte et al., 2020).

CONCLUSION AND FUTURE PERSPECTIVES

The COVID-19 pandemic has exhibited a significant demographic bias, in terms of the number of cases and associated fatalities, where the elderly subjects succumb to the infection more predominantly. Apart from genetics and basic comorbidities, aging invokes a variety of physiological alterations in the immune system including immunosenescence and inflammaging, *etc*. As a consequence, the ability of the aged host immune system to combat invading pathogens deteriorates to a great extent. In fact, besides more predispositions to various infections, the elderly subjects generally fail to manifest desirable immune responses against vaccines and other prophylactic programs as well. The aging process modulates the proinflammatory phenotypes, which not only influence the vulnerability of an individual to various invading pathogens, but also to the course of the disease and clinical consequences. Recently, immunosenescence has been correlated with mortality among the elders with COVID-19. It has been suggested that inadequate T cell response, poor antibody generation against SARS-CoV-2, and inflammaging, etc., that severely destroys the homeostasis, ensues in severe organ failure of the aged COVID-19 patients. In order to customize therapies and vaccine approaches in the direction of personalized medicine, a better knowledge of the intricacies related to the immune system of elderly people, is required. The pathogenesis of COVID-19 is tremendously complicated, involving the inhibition of the host's antiviral and innate immune responses, the promotion of oxidative stress, and hyper-inflammation characterized as a "cytokine storm," resulting in acute lung damage, tissue fibrosis, and pneumonia, *etc*. (Mrityunjaya et al., 2020). Several strategies such as vaccines, nutraceuticals, and pharmaceuticals are currently being used to boost both immunity of the susceptible host on one hand as well as inhibition of the replication and vital cellular machinery of the SARS-CoV-2 on the other. There are some approved vaccines, such as Pfizer, Moderna, AstraZeneca, Johnson and Johnson, and Gam-COVID-vac (Sputnik V) (Teijaro and Farber, 2021), that trigger innate and adaptive immune responses against the deadly SARS-CoV-2 virus. Phase III clinical trials have demonstrated 90–95% efficacy of Pfizer/BioNTech (BNT162b2) and Moderna (mRNA-1273) mRNA vaccines, while the AdV vaccines (ChAdOx1nCoV-19), and Gam-COVID-vac (Sputnik V) showed slightly lower efficacy (average 70 and 91%, respectively) (Teijaro and Farber, 2021). However, their effectiveness in elderly subjects is still an enigma and needs time to establish their usefulness. On the other hand, nutraceuticals if used as an immune-boosting, antiviral, antioxidant, and anti-inflammatory agent can be more apposite in imparting desirable combat strategy against SARS-CoV-2 in general, as well as in elderly patients. It is speculated that nutraceuticals such as Zinc (Zn), vitamin D, vitamin C, cinnamaldehyde, curcumin, selenium, probiotics, quercetin, and lactoferrin, etc may help in fighting against COVID-19 (Mrityunjaya et al., 2020). Combining some of these phytonutrients in the proper mix as a nutritional supplement would also aid to boost the immune system, inhibit viral transmission, reduce disease development to the extreme level, and control excessive inflammation, thereby offering both prophylactic and therapeutic assistance against SARS-CoV-2 infection (Mrityunjaya et al., 2020). Some pharmaceutical candidates have been in various phases of research and development, while a few such as favipiravir, remdesivir, and tocilizumab, etc. have been repurposed and authorized for emergent use against the COVID-19 pandemic (Mrityunjaya et al., 2020).

It is critical to learn about the functioning of environmental factors like nutrition, physical activity, co-morbidities, and pharmaceutical therapies, and to the fact that how these modulate the aging immune system's capacity to respond to invading pathogens on one hand and various vaccination programs on the other. The in-depth studies pertaining to COVID-19 have unraveled various relevant host immune system-related biomarkers as well as drug/antigen targets. More research is needed to figure out how to assist the elderly in generating a functional adaptive immune response while also reducing the detrimental pro-inflammatory state of the disease.

AUTHOR CONTRIBUTIONS

SF and MO conceived the idea of the study. SF, FJ, IA, AK and SA drafted the manuscript. SU, AK, SF and MO completed the manuscript.

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Liposomal Encapsulation of Polysaccharides (LEPS) as an Effective Vaccine Strategy to Protect Aged Hosts Against *S. pneumoniae* Infection

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Despite the availability of licensed vaccines, pneumococcal disease caused by the bacteria *Streptococcus pneumoniae* (pneumococcus), remains a serious infectious disease threat globally. Disease manifestations include pneumonia, bacteremia, and meningitis, resulting in over a million deaths annually. Pneumococcal disease disproportionally impacts older adults aged ≥65 years. Interventions are complicated through a combination of complex disease progression and 100 different bacterial capsular polysaccharide serotypes. This has made it challenging to develop a broad vaccine against *S. pneumoniae*, with current options utilizing capsular polysaccharides as the primary antigenic content. However, current vaccines are substantially less effective in protecting the elderly. We previously developed a Liposomal Encapsulation of Polysaccharides (LEPS) vaccine platform, designed around limitations of current pneumococcal vaccines, that allowed the non-covalent coupling of polysaccharide and protein antigen content and protected young hosts against pneumococcal infection in murine models. In this study, we modified the formulation to make it more economical and tested the novel LEPS vaccine in aged hosts. We found that in young mice (2–3 months), LEPS elicited comparable responses to the pneumococcal conjugate vaccine Prevnar-13. Further, LEPS immunization of old mice (18–22 months) induced comparable antibody levels and improved antibody function compared to Prevnar-13. Importantly, LEPS protected old mice against both invasive and lung localized pneumococcal infections. In summary, LEPS is an alternative and effective vaccine strategy that protects aged hosts against different manifestations of pneumococcal disease.

Keywords: pneumococcal disease, aging, vaccines, immunosenescence, antibodies, liposomes

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1 INTRODUCTION

Streptococcus pneumoniae (pneumococcus) is an opportunistic pathogen that asymptomatically resides in the upper respiratory tract of humans but can cause serious life-threatening infections (Kadioglu et al., 2008; Lower Respiratory Infecti, 2018). These include pneumonia which can progress to invasive pneumococcal disease leading to bacteremia, meningitis, and endocarditis (van der Poll and Opal, 2009; Randle et al., 2011). Pneumococcal infections result in more than a million deaths annually worldwide (Lower Respiratory Infecti, 2018), and in the USA alone, the estimated annual direct medical cost associated with pneumococcal disease is approximately $3.5 billion (Huang et al., 2011; Weycker et al., 2016). Pneumococcal infections are more problematic in elderly individuals (above 65 years of age) in terms of both health burden and treatment cost (Chong and Street, 2008; Huang et al., 2011). Despite the availability of vaccines and antibiotics, *S. pneumoniae* remains the leading cause of community acquired bacterial pneumonia and nursing home associated pneumonia in the elderly (Janssens and Krause, 2004; Wroe et al., 2012; Henig and Kaye, 2017). In the USA, older individuals account for 71% of all pneumococcal cases and 82% of associated deaths (Centers for Disease Control and Prevention, 2017). The yearly cost associated with hospitalization in this population will increase significantly as the number of elderly individuals is projected to double in the coming decades (Boe et al., 2017). To further complicate the current health concerns, elderly individuals are more at risk of acquiring drug-resistant infections (CDC, 2021) which are on a rise in *S. pneumoniae* (CDC, 2013).

There are 100 different *S. pneumoniae* serotypes identified based upon capsular polysaccharide content (Weiser et al., 2018; Ganaie et al., 2020), which in part regulates the severity of pneumococcal disease (Obaro and Adegbola, 2002). Currently licensed vaccine formulations consist of capsular polysaccharides from the most common disease-causing *S. pneumoniae* serotypes prevalent globally. The pneumococcal polysaccharide vaccine (Pneumovax 23/PPSV23) consists of polysaccharides from 23 pneumococcal serotypes and elicits a T cell-independent antibody response, while the pneumococcal conjugate vaccine (Prevnar 13/PCV13) is comprised of 13 serotypes covalently linked to a diphtheria toxoid CRM197 carrier protein which produces a T cell-dependent immune response in the host (Bonten et al., 2015). There are several limitations to the current vaccines, one of which is the phenomenon of serotype replacement wherein the targeting of a certain and limited number of pneumococcal serotypes by these vaccines has led to an increased prevalence of non-vaccine serotypes (Hanage, 2008; Berical et al., 2016). In addition, having only polysaccharides-centric vaccine formulations results in serotype-specific antibodies which mainly target nasopharyngeal colonization or the invasive stage of pneumococcal infection during which *S. pneumoniae* upregulate capsule expression to survive the host immune response. This strategy provides only limited host protection given the fact that pneumococcus undergoes transcriptomic changes to alter expression of several factors, including capsule expression, when transitioning from the stage of colonization to pulmonary or systemic infection (Marks et al., 2012; D'Mello et al., 2020). Moreover, the recent emergence of disease-associated non-encapsulated pneumococcal strains carrying antibiotic resistance genes (Keller et al., 2016) highlights the necessity of novel vaccine formulations, which in addition to serotype-specific polysaccharide content, also need to include other pneumococcal antigen(s), ideally the ones shared by multiple *S. pneumoniae* serotypes to broaden vaccine coverage.

In response, we have previously developed an alternative vaccine platform termed Liposomal Encapsulation of Polysaccharides (LEPS) which is designed to address the limitations of current vaccine options (Jones et al., 2017). With LEPS, pneumococcal capsular polysaccharides are localized internally within the liposomal carrier. To date, we have included final LEPS formulations with up to 24 different serotype polysaccharides (Hill et al., 2018). To mimic conjugated vaccine options (such as PCV13), the LEPS vehicle is engineered with a noncovalent surface attachment mechanism to localize protein components. In the past, we have surface localized both CRM197 and a *S. pneumoniae* protein antigen (PncO) that is upregulated during transition of pneumococci from a benign colonizer to a more invasive pathogen and that is well-conserved across pneumococcal strains (Jones et al., 2017; Hill et al., 2018). The LEPS platform thus allows for a broad degree of serotype coverage, provokes the same immune response provided by conjugate vaccines, and can be tailored to include a protein component that can account for the different stages of pneumococcal disease progression.

The efficacy of currently licensed pneumococcal vaccines, defined as prevention of infection by vaccine serotypes, is limited in the elderly. Although protective against bacteremia, PCV13 and PPSV23 show only 45 and 33% protection, respectively, against pneumonia in older individuals (Bonten et al., 2015). This is largely driven by an overall decline in the immune system with aging, also known as immunosenescence, which results in reduced antibody levels and function following vaccination with PPSV23 (Simell et al., 2011) and PCV13 (Jackson et al., 2013) in elderly individuals. The causes of the reduced vaccine response in aging populations are multi-factorial and may be attributed to intrinsic defects in B cells, including reduced repertoire, defects in key transcription factors, and reduction in AID, the enzyme required for class-switch recombination and somatic hypermutation. This results in blunted antibody responses to T cell-independent antigens such as bacterial polysaccharides found in PPSV23. Further, aging is associated with overall defects in T cell signaling and proliferation (Chen et al., 2009; Pinti et al., 2016) and in T-follicular helper cells that aid antibody production by B cells (Vinuesa et al., 2016; Gustafson et al., 2018), which in turn can blunt responses to T cell-dependent vaccines such as PCV13. In addition, the elderly suffer from inflammaging, i.e., low-grade chronic inflammation (Krone et al., 2014) characterized by increased levels of inflammatory cytokines (such as IL-6 and TNF-α) which is known to blunt the ability of the host to respond to acute stimuli and to inhibit immune responses to vaccination (Ridda et al., 2009; Pereira et al., 2020). With the success

demonstrated by the LEPS vaccine in young mice, as assessed for serotype coverage and directed protection from *S. pneumoniae* challenge, we conducted the current study to test the LEPS vaccine in aged hosts in preclinical murine models of infection. We present data with old mice that support the prospect of the LEPS platform for effective prevention of pneumococcal disease within aged hosts.

2 MATERIALS AND METHODS

2.1 Ethics Statement

All animal studies were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. Procedures were reviewed and approved by the University at Buffalo Institutional Animal Care and Use Committee.

2.2 Mice

All the animal work was done in C57BL/6J young (2–3 months) and C57BL/6JN old (18–22 months) male mice. The mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and the National Institute on Aging colonies and co-housed in a specific-pathogen-free facility at the University at Buffalo for 4 weeks prior to performing the studies.

2.3 Bacterial Strains and Growth Conditions

Streptococcus pneumoniae serotypes 4 (TIGR4 strain) and 19F (P1084 strain) were a kind gift from Andrew Camilli (Tufts University). Bacteria were grown to mid-log phase (corresponding to an OD_{650nm} of 0.7–0.8) in Todd–Hewitt broth (BD Biosciences) supplemented with Oxyrase and 0.5% yeast extract at 37°C/5% carbon dioxide. Aliquots were frozen at −80°C in growth media with 20% glycerol. Prior to use, aliquots were thawed on ice, washed and diluted in phosphate buffered saline to required numbers. Bacterial titers were confirmed by plating on tryptic soy agar plates supplemented with 5% sheep blood agar (Hardy Diagnostics).

2.4 Protein Production and Purification

Recombinant production of the PncO protein antigen was accomplished as reported previously (Jones et al., 2017; Hill et al., 2018; Nayerhoda et al., 2019; Nayerhoda et al., 2021). Briefly, *Escherichia coli* strain BL21(DE3) containing a plasmid with the PncO gene was grown in lysogeny broth (LB) medium with ampicillin (100 μM) while shaking overnight at 37°C. The overnight culture was diluted 1:1000 into LB (with ampicillin) and grown under the same conditions to an OD_{600nm} of 0.4–0.5. The culture was then induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG; 300 μM) and incubated with shaking at 30°C for 6 h. The cell culture was harvested by centrifugation for 15 min at 3,200 rcf at 4°C. The resulting pellet was resuspended gently in Buffer A (50 mM Na_2HPO_4, 500 mM NaCl, and 10% glycerol [pH 7.5]), an apostrophe placed in ice, and the suspended cells lysed by sonication using a small, tapered tip in 3 cycles of 45 s on and 60 s off at 50 Amp, 20 kHz. The post-sonication solution was centrifuged for 15 min at 12,000 rcf at 4°C. The supernatant was maintained at 4°C for protein purification with a prepared HisTrap HP column (GE Healthcare). Purified protein samples were concentrated using an Amicon 4 centrifugal filtration tube centrifuged for 5 min at 3600 rcf (4°C), and the final protein concentration was measured via Bradford analysis (Thermo Fisher).

2.5 Liposomal Encapsulation of Polysaccharides Formulation, Assembly, and Assessment

LEPS formulation and assessment was completed as previously reported (Jones et al., 2017; Hill et al., 2018; Nayerhoda et al., 2019; Nayerhoda et al., 2021). Briefly, all lipids were purchased from Avanti, Thermo Fisher, or Sigma Aldrich, and pneumococcal polysaccharides serotype 19F and 4 were obtained from ATCC. 1:2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (DGS-NTA(Ni)), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG(2000)), and cholesterol were dissolved in chloroform with the molar ratio of 3:3:1:0.1:4. Then, 19F and 4 solutions with concentrations of 2.2 μg per dose were separately added to different lipid mixtures and vortexed for 1 min. Both lipid mixtures were fully evaporated using a rotatory evaporator until a thin film was formed and then rehydrated with PBS at 45°C to dissolve the film prior to extrusion through a 200 nm pore size membrane of a handheld extruder. The resulting liposomes encapsulating the polysaccharides were separated from free floating and unencapsulated polysaccharides by centrifugation for 5 min at 1,200 rcf and 4°C using a centrifugal tube with a 300 kDa (Pall Co) filtration device. After readjusting to the initial volume with PBS, 34 μg per of the PncO protein was incubated with the liposome solution for 30 min at room temperature. The centrifugation step with filtered tubes was repeated twice to separate the unbound protein, resulting in the final formulation of liposome encapsulating PS with bound protein on the liposomal surface. To this final formulation, 125 μg per dose of aluminum phosphate was added.

2.6 Mice Immunization and Sera Collection

Mice were immunized via intramuscular injection into the caudal thigh muscle (Tchalla et al., 2020) with 50 μL of the following vaccine formulations: LEPS containing PncO, alum and either serotype 4 or 19F capsular polysaccharide antigen (LEPS); Prevnar 13 (PCV13) (Wyeth Pharmaceuticals); or the controls PBS (Sham) and empty LEPS vector (Empty LEPS plus alum). PCV13 was administered as a single dose, as approved for human use. Booster doses of LEPS or Empty LEPS were given at 2 weeks following the first inoculation. Sera from each mouse were collected from the tail vein at weeks 2 and 4 following the initial vaccination and saved at −80°C for quantification of antibody titers (Bhalla et al., 2021a). Sera from each mouse across three experiments were collected via cardiac puncture 4 weeks following the initial vaccination, pooled per group and saved at −80°C for quantification of antibody function.

2.7 Measurement of Antibody Titer

Enzyme-linked immunosorbent assay (ELISA) was used to determine the antibody titer from the sera samples, as previously described (Bou Ghanem et al., 2018; Tchalla et al., 2020). Briefly, Nunc maxisorp plates were coated overnight at 4°C with type 4 pneumococcal polysaccharide (ATCC) or type 19F pneumococcal polysaccharide (ATCC), each at 2 μg/well, or heat killed *S. pneumoniae* TIGR4 strain at 2 x 10^5 colony forming units (CFU)/well. For type 4 and type 19F polysaccharide conditions, the sera were preabsorbed with a pneumococcal cell wall polysaccharide mixture (CWP-multi, Cederlane) to neutralize noncapsular Abs and added to the plate. Following incubation with sera samples, pneumococcal-specific Abs were detected using horseradish peroxidase–conjugated goat anti-mouse immunoglobulin M (IgM; Invitrogen), IgG (Millipore Sigma), or IgG1, IgG2b, IgG2c, or IgG3 (Southern Biotech) followed by addition of TMB substrate (Thermo Scientific). Readings were taken at OD_{650} using a BioTek reader with a program set for kinetic ELISAs where readings were taken every minute for a total of 10 min. Antibody units were calculated as percentages of a hyperimmune standard sera included in each ELISA. Hyperimmune standard sera were generated as previously described (Bou Ghanem et al., 2018; Bhalla et al., 2021a). Briefly, C57BL/6 young mice were intranasally inoculated with *S. pneumoniae* TIGR4 or 19F over the period of 4 weeks with once weekly inoculations, as previously described (Bou Ghanem et al., 2018). Mice were then immunized with PCV13 at week 4. Approximately, 4 weeks following PCV13 vaccination, mice were euthanized, sera collected and pooled, and aliquots stored at −80°C. Total antibody levels in the circulation were then determined in sera from sham treated controls (naïve) for each age group and the hyperimmune standard serum using antibody quantification kits from Invitrogen for IgG (88-50400), IgM (88-50470), IgG1 (88-50410), IgG2b (88-50430), IgG2c (88-50670), and IgG3 (88-50440). To calculate the total amounts of anti-pneumococcal antibodies, the antibody levels in the naïve sera was subtracted from the hyperimmune standard sera and total antibody levels in the different sera samples were then extrapolated using the measured percentages of the hyperimmune standard sera.

2.8 Polymorphonuclear Leukocytes Isolation

PMNs were isolated from the bone marrow of naïve young and old C57BL/6 mice through density gradient centrifugation, using Histopaque 1119 and Histopaque 1077, as previously described (Swamydas and Lionakis, 2013; Bhalla et al., 2021a). Following isolation, the PMNs were resuspended in Hanks' Balanced Salt Solution (HBSS)/0.1% gelatin without Ca^{2+} and Mg^{2+} and kept on ice until used in subsequent assays.

2.9 Opsonophagocytic Killing

The opsonic capacity of antibodies in the sera was determined using an opsonophagocytic (OPH) killing assay with primary PMNs, as previously described (Standish and Weiser, 2009; Swamydas and Lionakis, 2013; Bhalla et al., 2020a; Siwapornchai et al., 2020; Bhalla et al., 2021a; Bhalla et al., 2021b). Briefly, 1 × 10^3 bacteria (*S. pneumoniae* TIGR4) grown to mid-log phase were incubated with 3% sera from mice immunized with the LEPS formulation containing serotype 4 capsular polysaccharide antigen, PCV13, or the controls (Sham or Empty LEPS vector). Reactions were rotated for 40 min at 37°C to allow opsonization. Following opsonization, bacteria were mixed with 1 × 10^5 PMNs in 100 μl reactions of HBSS/0.1% gelatin. Reactions were then rotated for 45 min at 37°C. Following incubation, the plates were kept on ice for 2 min to stop the reaction. The ability of sera from each treatment group to induce opsonophagocytic killing of bacteria by PMNs was then determined by plating the reaction mixtures on blood agar plates and comparing colony counts and calculating the percent of bacteria killed with respect to a no PMN control under the exact sera conditions.

2.10 Binding Assay Using H292 Cells

The ability of sera to neutralize the binding of *S. pneumoniae* to pulmonary epithelium was determined through an assay using human pulmonary mucoepidermoid carcinoma-derived NCI-H292 (H292) cells (ATCC), as previously described (Bhalla et al., 2020b). H292 cells were grown and maintained following a previously described protocol (Bhowmick et al., 2013; Bhalla et al., 2020b). Approximately, 2.5 × 10^5 epithelial cells were seeded in tissue culture-treated flat bottom 96-well plates (Corning) and allowed to adhere overnight. The following day, cells were washed three times with PBS and infected with the *S. pneumoniae* TIGR4 strain at a multiplicity of infection (MOI) of 10 in antibiotic-free media. Prior to infection, bacteria were opsonized for 40 min at 37°C with 10% sera from mice immunized with serotype 4 LEPS, PCV13, or the controls (Sham or Empty LEPS). The reaction plates were spun down to facilitate cell-bacterial interaction and incubated for 1 h at 37°C/CO_2. The cells were then washed five times with PBS to remove unbound bacteria, lifted with 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA) (Invitrogen), and mixed vigorously to produce a homogeneous solution. Serial dilutions were then plated on blood agar plates to determine the bacterial CFU. The percent of bacteria bound was determined with respect to a no cell control of the same sera condition where bacteria were added to the wells and incubated for an hour under the same experimental conditions. The number of bacteria bound to H292 cells in the Sham sera condition was set as 100% and relative changes in bacterial binding were then calculated for other sera conditions.

2.11 Animal Infections

Mice were infected with *S. pneumoniae*, as previously described (Bou Ghanem et al., 2018; Bhalla et al., 2020a). Briefly, mice were intratracheally (i.t.) challenged with either the serotype 4 (5 × 10^5 CFU) or 19F (2 × 10^7 CFU) strain of *S. pneumoniae*. Following infection with the *S. pneumoniae* serotype 4 strain, mice were monitored daily over 2 weeks for survival and clinical signs of disease (weight loss, activity level, posture, and breathing, scored healthy [0] to severely sick [21], as previously described (Bou Ghanem et al., 2018; Bhalla et al., 2020a)) while bacteremia was determined for up to 72 h post infection. In case of infection with

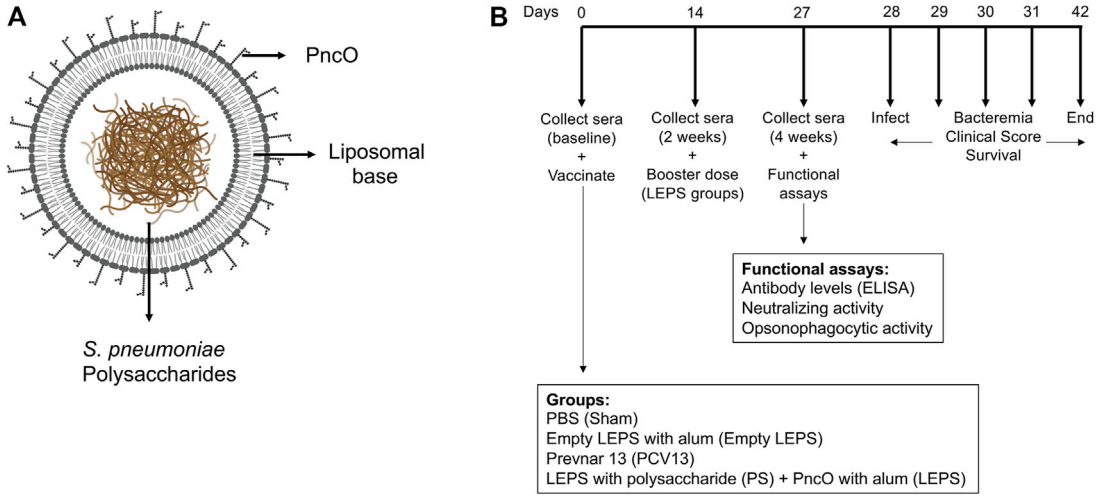

FIGURE 1 | Vaccine design and experimental set-up. **(A)** LEPS design. The LEPS vaccine consists of the liposomal carrier encapsulating bacterial polysaccharide with PncO linked to the surface. **(B)** Vaccination timeline. Mice were mock treated with 50 µl PBS (Sham), empty LEPS with alum (Empty LEPS), or administered either the pneumococcal conjugate vaccine Prevnar 13 (PCV13) or the LEPS vaccine containing bacterial polysaccharide in addition to PncO and alum (LEPS) via intramuscular (*i.m*) injections to the hind legs. Two weeks following the initial vaccination, the LEPS and Empty LEPS groups received a booster dose. Sera were collected at baseline and two and 4 weeks with respect to the initial vaccination to assess antibody levels and function. Four weeks following vaccination mice were challenged intratracheally (*i.t.*) with *S. pneumoniae* and monitored for bacterial burden and clinical signs of disease.

the *S. pneumoniae* serotype 19F strain, mice were scored for clinical signs of disease at twenty-four hours post infection and euthanized to assess bacterial burden in the lung. Lung homogenates and blood were assessed for CFU by plating on blood agar plates.

2.12 Statistical Analysis

All statistical analysis was performed using Prism9 (Graph Pad). Data with bacterial numbers in blood and lungs were log-transformed to normalize distribution. Bar graphs represent the mean values +/− standard deviation (SD) and line graphs represent the mean values +/− 95% confidence interval of the mean (CI). Significant differences were determined with one-way ANOVA followed by Tukey's multiple comparisons test as indicated. Survival was analyzed using the log-rank (Mantel-Cox) test. Differences between fractions were determined by Fisher's exact test. All p values < 0.05 were considered significant.

3 RESULTS

3.1 Liposomal Encapsulation of Polysaccharides and Prevnar-13 Vaccination Induce Comparable Antibody Production in Young Hosts Against *S. pneumoniae*

We had previously shown that a LEPS formulation constructed with surface localized CRM197, to mimic conjugated vaccine options, conferred a similar degree of immunogenicity and protection against *S. pneumoniae* as the pneumococcal conjugate vaccine Prevnar-13 (PCV13) in young mice (Jones et al., 2017). In an effort to simplify and economize, we altered the LEPS vaccine by replacing CRM197 with the pneumococcal protein antigen PncO (**Figure 1A**). To test if the modified LEPS vaccine is immunogenic and still mimics the immunological outcome of PCV13, we first examined the antibody production in young (2 months old) C57BL/6 mice. Mice were immunized by intramuscular injection with the following vaccine formulations 1) LEPS containing capsular polysaccharide conjugated with PncO with alum as adjuvant (LEPS); 2) PCV13; 3) a PBS control (Sham); and 4) an empty LEPS control with alum (Empty LEPS), following the schedule presented in **Figure 1B**. Sera from each mouse was collected over time to measure antibody levels (timeline in **Figure 1B**).

Serum antibody response was measured using ELISAs against purified polysaccharide. We found that LEPS vaccination was able to induce IgM production at levels that were significantly higher than the Empty LEPS and that were comparable to those induced by PCV13 (**Figure 2A**). An important feature of the PCV13 vaccine is the presence of the covalently attached CRM197 protein, which induces T-cell-mediated IgG class switching. To confirm that the LEPS vaccine still elicited IgG class switching (with only PncO as the noncovalently affixed protein conjugate), we next measured total antibody levels as well as the different subtypes of IgG produced. We found that while no IgG was detected in the Empty LEPS treated controls (**Figure 2B**), LEPS vaccination induced IgG production that was comparable to PCV13 (**Figure 2B**). Similar IgG and IgM responses were also detected against heat-killed bacteria (**Supplementary Figure S1**). When we compared IgG subtypes, we found that LEPS and PCV13 triggered similar class switching to predominantly the IgG3 and IgG2b subtypes (**Figures 2C–F**). Overall, these findings confirm that the LEPS vaccine is immunogenic in young hosts and elicits antibody production comparable to PCV13.

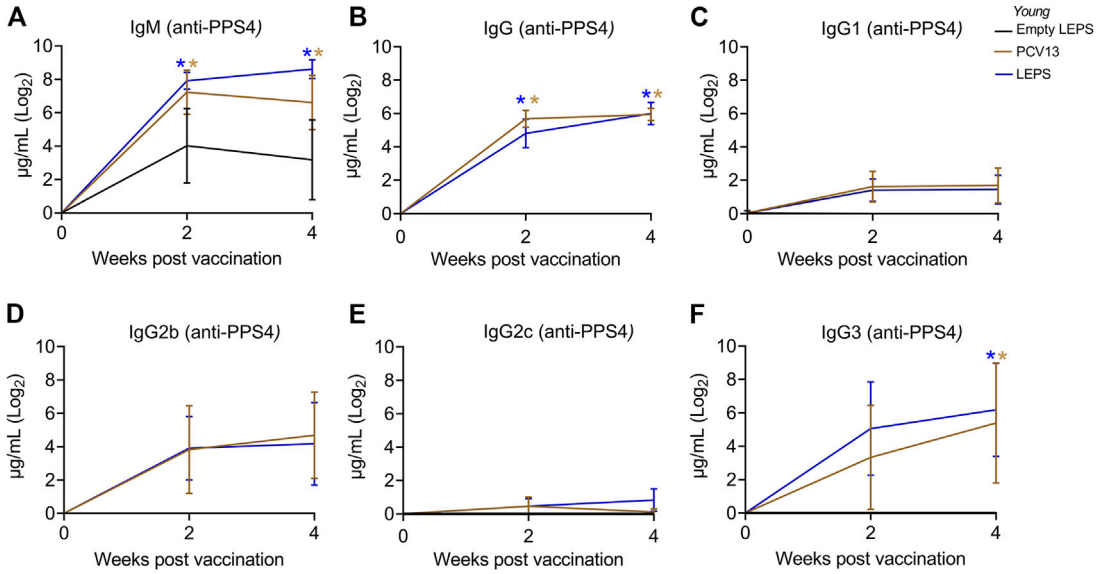

FIGURE 2 | The LEPS vaccine and Prevnar-13 induce comparable levels of antibodies against pneumococcal polysaccharides in young hosts. Young C57BL/6 mice (2-months) were injected *i. m.* with LEPS containing serotype 4 capsular polysaccharide antigen, PCV13, or the controls (Sham or Empty LEPS vector) following the timeline in **Figure 1B**. Total levels of IgM **(A)**, IgG **(B)**, and IgG subtypes including IgG1 **(C)**, IgG2b **(D)**, IgG2c **(E)** and IgG3 **(F)** against purified polysaccharide serotype 4 in the sera were measured over time by ELISA. *p* values were determined by one-way ANOVA followed by Tukey's test. Asterisks (*$p < 0.05$) denote significant differences between the indicated group and the empty LEPS group. Data shown are presented as mean ± CI and are pooled from 3 separate experiments with $n = 10$ mice for Empty LEPS, $n = 10$ mice for LEPS, and $n = 15$ mice for the PCV13 groups.

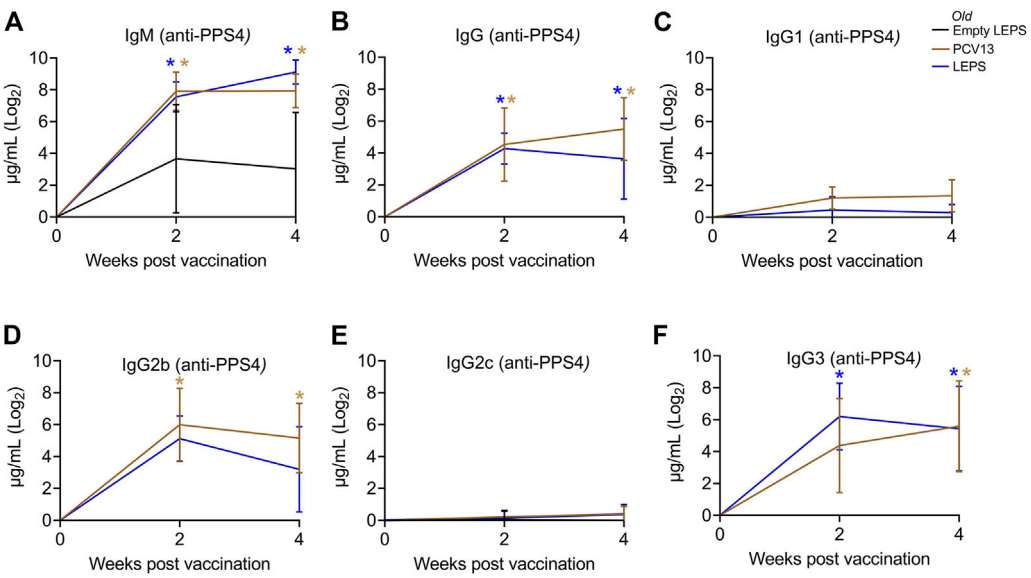

FIGURE 3 | The LEPS vaccine and Prevnar-13 induce comparable levels of antibodies against pneumococcal polysaccharide in old hosts. Old C57BL/6 mice (18–22 months) were injected *i.m.* with LEPS containing serotype 4 capsular polysaccharide antigen, PCV13, or the controls (Sham or Empty LEPS vector) following the timeline in **Figure 1B**. Total levels of IgM **(A)**, IgG **(B)**, and IgG subtypes including IgG1 **(C)**, IgG2b **(D)**, IgG2c **(E)** and IgG3 **(F)** against purified polysaccharide serotype 4 in the sera were measured over time by ELISA. *p* values were determined by one-way ANOVA followed by Tukey's test. Asterisks (*$p < 0.05$) indicate significant differences between the indicated group and the empty LEPS group. Data shown are presented as mean ± CI and are pooled from 3 separate experiments with $n = 5$ mice for Empty LEPS, $n = 10$ mice for LEPS, and $n = 10$ mice for the PCV13 groups.

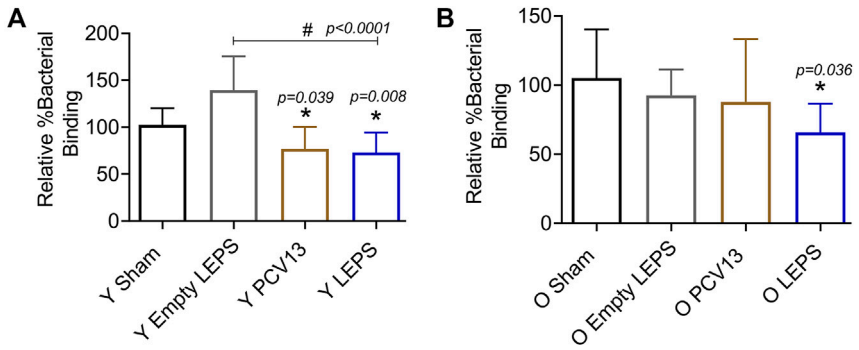

FIGURE 4 | Sera from old mice vaccinated with LEPS, but not Prevnar-13, neutralize the ability of *S. pneumoniae* to bind pulmonary epithelial cells. Sera were collected from mice immunized with LEPS containing serotype 4 capsular polysaccharide antigen, PCV13, or the controls (Sham or Empty LEPS vector) at 4 weeks post vaccination following the timeline indicated in **Figure 1B**. The ability of sera to neutralize bacterial binding to pulmonary epithelial cells was determined. *S. pneumoniae* serotype 4 TIGR4 strain was pre-opsonized for 45 min with 10% sera from young (Y) **(A)** or old (O) **(B)** mice and used to infect H292 cells for 1 h at an MOI of 10. The number of bound bacteria was determined by plating on blood agar plates and the percent bacterial binding calculated with respect to bacteria incubated with sera alone for each opsonization condition. The effect of sera on bacterial binding was then determined relative to the Sham group. Asterisks (*) indicate significant differences with respect to the Sham group and hash signs (#) indicate significant differences between the indicated groups as calculated by one-way ANOVA followed by Tukey's test. Data shown are pooled from four separate experiments where each condition was tested in quadruplicate and presented as mean ± SD.

3.2 The Liposomal Encapsulation of Polysaccharides Vaccine Is Immunogenic in Aged Hosts

Aging is accompanied by immunosenescence which is known to blunt immune responses to vaccines (Frasca et al., 2011; van Werkhoven et al., 2015; Pinti et al., 2016). Given our positive results when using young mice, we next tested if the LEPS vaccine could similarly prompt an immunogenic response in aged hosts. Thus, old (18–22 months) C57BL/6 mice were immunized with the same vaccine formulations used in young mice (**Figure 1B**) and antibody levels against purified polysaccharide were measured. Similar to what we observed in young mice, we found that LEPS vaccination of old mice elicited IgM and IgG production comparable to that of PCV13 (**Figures 3A,B**). Further, LEPS and PCV13 also induced class switching to predominantly IgG2b and IgG3 in old mice (**Figures 3C–F**). Overall, these data indicate that the LEPS vaccination is immunogenic in old mice and the antibody response mounted to LEPS by aged hosts is comparable to that observed in young controls.

3.3 Liposomal Encapsulation of Polysaccharides Vaccination Elicits Enhanced Serum Neutralizing Activity Against *S. pneumoniae* in Aged Hosts

Apart from antibody levels, antibody function is crucial for vaccine efficacy (LaFon and Nahm, 2018). We therefore wanted to assess antibody function following LEPS vaccination. Binding of *S. pneumoniae* to the airway epithelium is an important step that is crucial for host colonization and infection (Cundell et al., 1995; Kadioglu et al., 2008; van der Poll and Opal, 2009; Bhalla et al., 2020b). As such, we tested the ability of antibodies induced by the LEPS vaccine to neutralize bacterial binding to H292 cells (type I and II pneumocytes), a human lung epithelial cell line extensively used to study host-pathogen interactions (Choi et al., 2008; Bhowmick et al., 2013; Yonker et al., 2017; Bhalla et al., 2020b). To do so, we compared the ability of sera collected 4 weeks post-vaccination across the different immunization groups (**Figure 1B**) to block bacterial binding relative to the Sham control group (**Figure 1B**). We found that in young mice, sera from both the PCV13 and LEPS vaccinated groups significantly reduced the binding of a *S. pneumoniae* serotype 4 strain (serotype covered by both vaccines) to H292 cells by 25% (**Figure 4A**). Importantly, sera from the LEPS group caused ≥ 2-fold reduction in bacterial binding compared to the Empty LEPS control (**Figure 4A** and **Supplementary Figure S2A**). The effects observed were not due to the direct bactericidal activity of the sera as all binding is calculated with respect bacteria incubated with sera alone for each opsonization condition (see *Materials and Methods*). These data suggest that antibodies induced by both PCV13 and LEPS in the young mice are capable of neutralizing bacterial adherence to host cells.

The function of antibodies is known to decline with age (Weinberger et al., 2008). In fact, when we measured the ability of sera isolated from old mice to neutralize bacterial binding, we found that unlike what we observed in young hosts, sera from old mice vaccinated with PCV13 did not consistently reduce pneumococcal binding to H292 cells compared to the sham group (**Figure 4B** and **Supplementary Figure S2**). Interestingly, when *S. pneumoniae* was opsonized with sera from old LEPS vaccinated mice, there was a significant 40 and 25% reduction in bacterial binding compared to the Sham and Empty LEPS controls, respectively (**Figure 4B**). This finding suggests that compared to PCV13, LEPS vaccination induces antibodies that are better at neutralizing bacterial binding to the pulmonary epithelium.

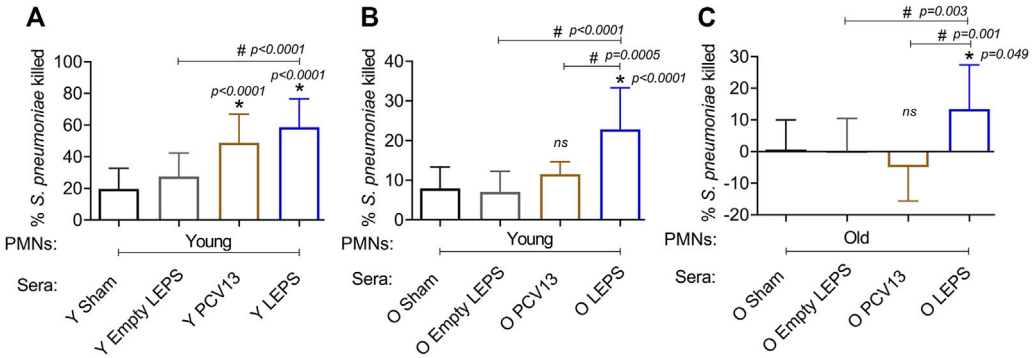

FIGURE 5 | LEPS vaccination of old mice induces sera with better opsonic activity compared to Prevnar-13 vaccination. Sera were collected from young (Y) or old (O) mice immunized with LEPS containing serotype 4 capsular polysaccharide antigen, PCV13, or the controls (Sham or Empty LEPS vector) at 4 weeks post vaccination following the timeline indicated in **Figure 1B**. The ability of sera to induce opsonophagocytic killing of bacteria by PMNs was determined. PMNs were isolated from the bone marrow of naïve young (2 months) **(A,B)** or old (18–22 months) **(C)** C57BL/6 mice and mixed for 45 min at 37°C with *S. pneumoniae* serotype 4 TIGR4 strain pre-opsonized with 3% sera from the indicated groups. Reactions were stopped on ice, and viable CFU were determined after serial dilution and plating. The percentage of bacteria killed was determined with respect to a no PMN control for each condition. Asterisks (*) indicate significant differences with respect to the Sham group (ns: not significant) and hash signs (#) indicate significant differences between the indicated groups as calculated by one-way ANOVA followed by Tukey's test. Data shown are pooled from three separate experiments (n = 3 biological replicates or mice per group) where each condition was tested in triplicate (n = 3 technical replicates) per experiment and presented as mean ± SD.

3.4 Liposomal Encapsulation of Polysaccharides Vaccination Elicits Enhanced Serum Opsonic Activity Against *S. pneumoniae* in Aged Hosts

The opsonic capacity of antibodies is an important correlate of vaccine protectiveness (Song et al., 2013). We therefore measured the ability of sera isolated at 4 weeks post-immunization across the different groups (**Figure 1B**) to induce opsonophagocytic (OPH) killing of a *S. pneumoniae* serotype 4 strain (serotype covered by both vaccines) by primary PMNs from naïve mice, using assays we had previously established (Bhalla et al., 2021a). We found that in young hosts, compared to the control groups, we saw a significant increase in pneumococcal killing by PMNs when the bacteria were opsonized with sera from PCV13 or the LEPS vaccinated groups, with higher opsonophagocytic killing induced by the latter (**Figure 5A**). This suggests that the opsonic activity of sera induced by LEPS immunization matched or exceeded that elicited by PCV13.

We then compared the opsonic activity of sera from old mice. As PMNs function is known to decline with age and can confound the interpretation of the data (Bhalla et al., 2020a; Simmons et al., 2021), we first measured the ability of sera isolated from old mice to elicit opsonophagocytic bacterial killing by PMNs isolated from young mice. We found that while sera from PCV13 immunized old mice failed to significantly improve bacterial killing by PMNs relative to Sham controls (**Figure 5B**), sera from LEPS vaccinated old mice elicited significantly higher opsonophagocytic killing by PMNs than both controls as well as the PCV13 group (**Figure 5B**). To best mimic *in vivo* conditions, we then age matched the sera and PMNs and measured the ability of sera from old hosts to induce bacterial killing by PMNs from old mice. As expected (Bhalla et al., 2020a), sera from the control groups (Sham and Empty LEPS) failed to elicit opsonophagocytic bacterial killing by PMNs from old mice (**Figure 3C**). Surprisingly, when PMNs were challenged with *S. pneumoniae* opsonized with PCV13 sera from old mice, we failed to see bacterial killing and in fact observed bacterial growth in the presence of PMNs (**Figure 5B**). In contrast, we saw a significant 10-fold increase in bacterial killing by PMNs in comparison to the control groups when sera from the LEPS vaccinated old mice were used to opsonize *S. pneumoniae* (**Figure 5B**). Further, PMN-mediated bacterial killing induced by sera from the LEPS group was significantly higher than that seen with sera from the PCV13 group (**Figure 5B**). These findings suggest that compared to PCV13, LEPS vaccination induces antibodies that are better at eliciting opsonophagocytic bacterial killing by immune cells.

3.5 Young Mice Immunized With the Liposomal Encapsulation of Polysaccharides Vaccine Show Resistance to Invasive Pneumococcal Infection

We next wanted to test whether the LEPS vaccination confers host protection against pneumococcal infection. *S. pneumoniae* strains can vary (Kadioglu and Andrew, 2005) and most infections result in primarily pneumonia, but up to 30% of patients with pneumococcal pneumonia also develop bacteremia and have worse prognosis (Lin et al., 2011). Thus, we first tested host protection against invasive infection using the well-characterized serotype 4 clinical isolate *S. pneumoniae* TIGR4 as a model of pneumonia that results in bacteremia (Aaberge et al., 1995; Tettelin et al., 2001).

At week 4 following vaccination (**Figure 1B**), young (2–3 months) C57BL/6 mice were infected with 5×10^5 CFU of *S. pneumoniae* TIGR4 intra-tracheally (*i.t.*) and assessed for clinical scores and bacteremia 24 h post-infection and overall

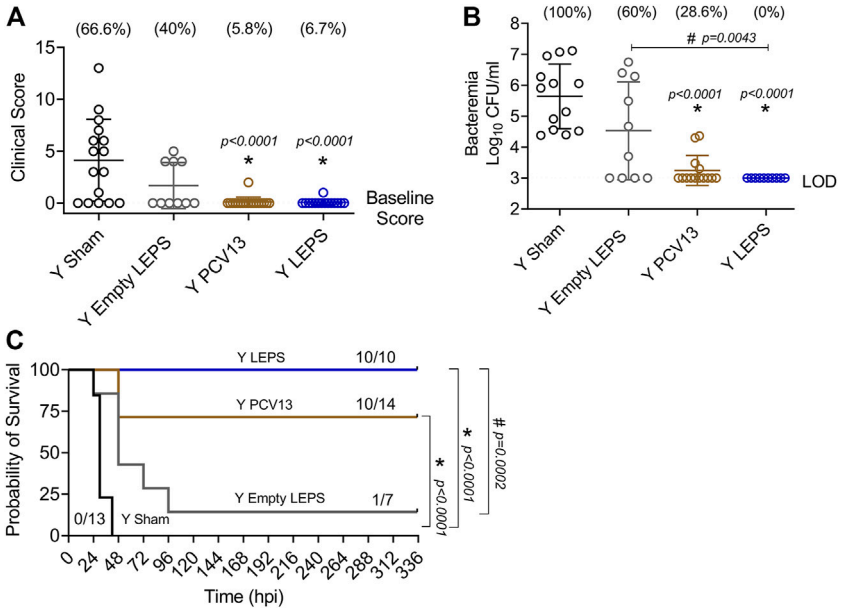

FIGURE 6 | LEPS vaccination confers similar protection to Prevnar13 in young mice against invasive pneumococcal infection. Young (2–3 months) C57BL/6 mice were immunized with LEPS containing serotype 4 capsular polysaccharide antigen, PCV13, or the controls (Sham or Empty LEPS vector). Four weeks later (timeline presented in **Figure 1B**), mice were infected *i.t.* with 5×10^5 CFU of *S. pneumoniae* TIGR4 strain. Clinical scores **(A)** and bacterial numbers in the blood along with the incidence of bacteremia (% noted above each group) **(B)** were determined 24 h post-infection. Survival was also followed over time **(C)**. **(A,B)** Asterisks (*) indicate significant differences with respect to the Sham group, and hash signs (#) indicate significant differences between the indicated groups as calculated by one-way ANOVA followed by Tukey's test. Pooled data are presented as mean ± SD and each dot represents one mouse. **(C)** Asterisks (*) indicate significant differences with respect to the Sham group, and hash signs (#) indicate significant differences between the indicated groups as determined by the log-rank (Mantel-Cox) test. Fractions denote surviving mice. Pooled data from two separate experiments with $n = 13$ mice in the Sham group, $n = 7$ mice in the Empty LEPS group, $n = 14$ mice in the PCV13 group, and $n = 10$ mice in the LEPS group are shown. LOD: limit of detection.

survival over a period of 2 weeks. We found that compared to the Sham group, vaccination of mice with LEPS significantly reduced the disease severity associated with pneumococcal disease (**Figure 6A**). Importantly, this reduction in disease severity was comparable with that observed in mice vaccinated with PCV13 (**Figure 6A**). When we compared bacteremia across different mice groups, both the LEPS and PCV13 vaccine significantly reduced the bacterial burden in the blood (by approximately 1,000-fold) compared to the Sham group (**Figure 6B**). The reduction in blood bacterial burden seen with the LEPS formulation was also significantly lower than the Empty LEPS group (**Figure 6B**). Interestingly, the PCV13 vaccinated group had significantly higher incidence of bacteremia ($p = 0.002$ by Fisher's exact test), where approximately 29% of mice became bacteremic compared to 0% of mice in the LEPS vaccinated group (**Figure 6B**). When we tracked overall survival, we found that all the mice from the Sham group succumbed to pneumococcal infection within 48 h of infection (**Figure 6C**). Mice from the Empty LEPS group showed similar kinetics with more than 50% having died within 48 h of challenge with *S. pneumoniae* with only 14% survival (1/7) by the end of the 2-weeks observation period (**Figure 6C**). Vaccination with PCV13 significantly improved the survival rate of mice with only 25% of mice succumbing to infection (**Figure 6C**). Strikingly, mice immunized with LEPS showed 100% survival (**Figure 6C**), mirroring the effect of this vaccination on clinical score (**Figure 6A**) and bacteremia (**Figure 6B**). Overall, these findings highlight the high efficacy of the LEPS vaccine in preventing invasive pneumococcal infection.

3.6 Liposomal Encapsulation of Polysaccharides Vaccination Protects Old Hosts Against Invasive Pneumococcal Infection

We next compared the ability of the LEPS and PCV13 vaccines in protecting aged hosts against invasive pneumococcal infection. Four weeks following vaccination (**Figure 1B**), old (18–22 months) mice were infected with 5×10^5 CFU of *S. pneumoniae* TIGR4 intra-tracheally (*i.t.*) and assessed for clinical scores and bacteremia 24 h post-infection and survival over time. Mice belonging to both the control groups (Sham and Empty LEPS) showed severe signs of clinical disease (**Figure 7A**). Although vaccination with PCV13 mitigated the overall clinical severity of disease to some extent, half of the mice from this group still experienced clinical symptoms (indicated by high clinical scores) (**Figure 7A**). However, mice immunized with LEPS showed no clinical symptoms at 24 h post-infection (**Figure 7A**). When we compared bacteremia at 24 h post-infection, we found that mice from both control groups (Sham and Empty LEPS) had a high blood bacterial burden with 100% incidence of bacteremia (**Figure 7B**). Compared to the young

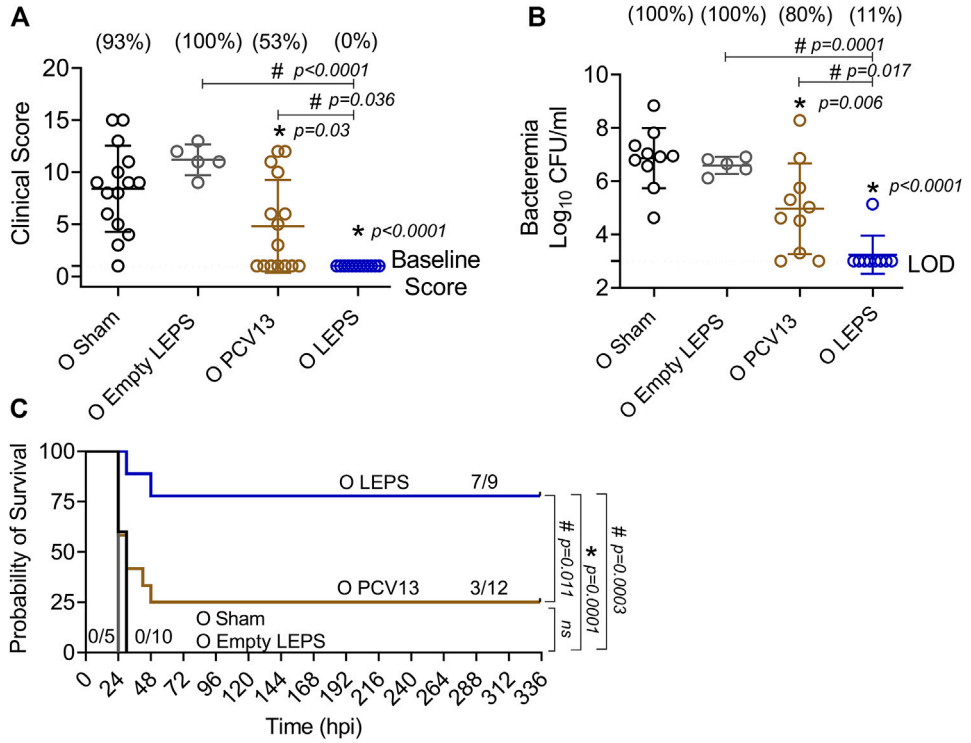

FIGURE 7 | LEPS vaccination protects old mice against invasive pneumococcal infection. Old (18–22 months) C57BL/6 mice were immunized with LEPS containing serotype 4 capsular polysaccharide antigen, PCV13, or the controls (Sham or Empty LEPS vector). Four weeks later (timeline presented in **Figure 1B**), mice were infected intra-tracheally with 5×10^5 CFU of *S. pneumoniae* serotype 4 TIGR4 strain. Clinical scores **(A)** and bacterial numbers in the blood along with the incidence (% noted above each group) of bacteremia **(B)** were determined 24 h post-infection. Survival was also followed over time **(C)**. **(A,B)** Asterisks (*) indicate significant differences with respect to the Sham group, and hash signs (#) indicate significant differences between the indicated groups as calculated by one-way ANOVA followed by Tukey's test. Pooled data are presented as mean ± SD and each dot represents one mouse. **(C)** Asterisks (*) indicate significant differences with respect to the Sham group (ns: not significant), and hash signs (#) indicate significant differences between the indicated groups as determined by the log-rank (Mantel-Cox) test. Fractions denote surviving mice. Pooled data from two separate experiments with $n = 10$ mice in the Sham group, $n = 5$ mice in the Empty LEPS group, $n = 12$ mice in the PCV13 group, and $n = 9$ mice in the LEPS group are shown. LOD: limit of detection.

counterparts (**Figure 6B**), aged-mice experienced 100-fold higher bacterial burden in the circulation, displaying the expected age-associated susceptibility to *S. pneumoniae* infection (Gonçalves et al., 2016; Bhalla et al., 2020a). Although vaccination of old mice with PCV13 significantly reduced the bacterial burden in blood compared to the Sham group, 80% of the animals still became bacteremic (**Figure 7B**). Interestingly, mice immunized with LEPS had more than 1,000-fold reduction in blood bacterial numbers (**Figure 7B**) compared to both control groups. Importantly, protection against bacteremia elicited by LEPS was significantly better than PCV13 as the LEPS group had a 100-fold lower bacterial burden in the blood and a lower incidence of bacteremia ($p = 0.0055$ by Fisher's exact test) where only 11% of mice became bacteremic (**Figure 7B**). When we compared survival, we found that mice in both the control groups succumbed to pneumococcal infection within 24 h of infection (**Figure 7C**). Interestingly, PCV13 failed to fully protect old mice, where we observed only 25% of animals surviving the course of infection, albeit being significantly higher than survival observed in the Sham group (**Figure 7C**). In contrast, we found that the majority of mice immunized with LEPS (75%) survived the infection which was not only higher than both control groups but also significantly higher that the survival of the PCV13 group (**Figure 7C**). These data indicate that while PCV13 fails to protect aged hosts, the LEPS vaccine significantly reversed the age-related susceptibility to invasive pneumococcal infection.

3.7 Liposomal Encapsulation of Polysaccharides Vaccination Protects Old Hosts Against Pneumococcal Pneumonia

The efficacy of pneumococcal vaccines, particularly against pneumonia, decline with aging (Bonten et al., 2015; van Werkhoven et al., 2015). Therefore, we wanted to test the protective capacity of the LEPS vaccine against non-bacteremic pneumonia in aged hosts. To do so, we used a *S. pneumoniae* serotype 19F strain that is less invasive and covered by PCV13 (Marks et al., 2013). C57BL/6 old (18–22 months) mice were injected *i. m.* with the LEPS vaccine containing serotype 19F capsular polysaccharide antigen, PCV13, or the Sham control (**Figure 1B**). At week 4 post immunization (**Figure 1B**), mice were infected (*i.t.*) with 2×10^7 CFU of *S. pneumoniae* 19F and assessed for clinical severity of disease and bacterial burden in the

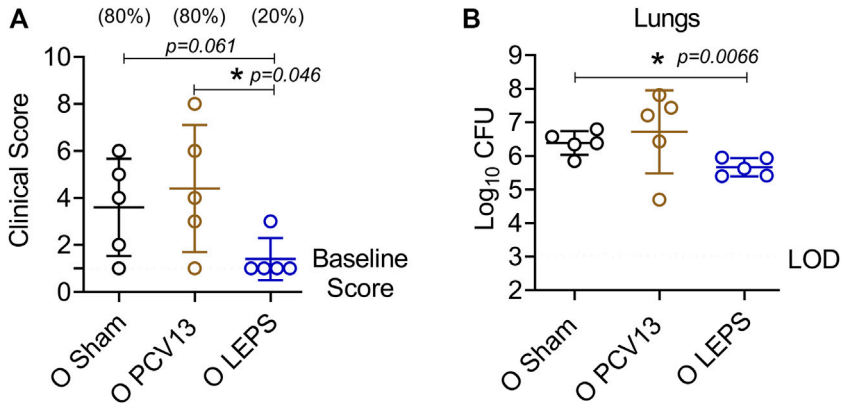

FIGURE 8 | LEPS vaccination protects old mice against pneumococcal pneumonia. Old (18–22 months) C57BL/6 mice were immunized with LEPS containing serotype 19F capsular polysaccharide antigen, PCV13, or the controls (Sham or Empty LEPS vector). Following the timeline presented in **Figure 1B**, 4 weeks after vaccination, mice were infected *i.t.* with 2×10^7 CFU of *S. pneumoniae* serotype 19F. Clinical scores **(A)** and bacterial burden in the lungs **(B)** were determined 24 h post-infection. **(A)** Percentage of mice that displayed clinical signs of pneumonia are in parentheses. **(A,B)** Asterisks (*) indicate significant differences between the indicated groups as determined by one-way ANOVA followed by Tukey's test. Data shown are pooled from $n = 5$ mice per group and presented as mean ± SD. Each dot represents one mouse. LOD: limit of detection.

lung 24 h post-infection. Interestingly, mice vaccinated with PCV13 showed worsening of clinical scores similar to that of the Sham group (**Figure 8A**). In contrast, LEPS immunized mice showed an overall significantly reduced clinical score compared to both the Sham control and PCV13 groups (**Figure 8A**). When we measured the lung bacterial burden, we observed a similar trend, with PCV13 failing to reduce the bacterial numbers compared to the Sham group (**Figure 8B**), while mice that received the LEPS vaccine had approximately 10-fold less bacterial burden in the lungs compared to the Sham controls (**Figure 8B**). Overall, these findings indicate that while vaccination with PCV13 failed to provide any protection in old mice against pneumococcal pneumonia, the LEPS vaccine boosted the ability of aged hosts to control lung infection.

4 DISCUSSION

The current study began with the assessment of the LEPS platform in young mice to first establish protocols and confirm prior success before shifting to aged hosts. Our findings (**Figures 2**, **4**–**6**) support that the LEPS approach is a viable vaccine strategy against *S. pneumoniae* when compared to prior studies from our working group (Jones et al., 2017; Hill et al., 2018). Though in this case, we note two differences to previous efforts. First, we use a different species of mice (C57BL/6 in this study vs. CD-1 mice in prior work). This is relevant as it suggests that the LEPS vaccine platform is effective across different mouse strains with varying genetic backgrounds, supporting future translational efforts across a diverse human population. Second, in the current study, we used the virulent-specific pneumococcus antigen PncO as an effective replacement for CRM197 in comparisons of the LEPS formulation to PCV13. Here, PncO appears to serve a very similar role to CRM197 (i.e., an immunogenic carrier protein) in promoting LEPS vaccine effectiveness both in terms of inducing a functional antibody response (**Figures 2**, **4**, **5**) as well as host protection against invasive pneumococcal disease (**Figure 6**). Similar to prior studies (Jones et al., 2017; Hill et al., 2018), we observed isotype switching to IgG upon vaccination with the modified LEPS that was comparable to Prevnar-13, indicating that PncO matches CRM197 in the capability to induce a T-cell dependent response (Jones et al., 2017). Since PncO serves another crucial purpose in the overall LEPS design (as discussed below), the refined formulation presented here which eliminates the need for CRM197 offers a more economical vaccine strategy against pneumococcal infections.

As pneumococcal disease has a disproportional impact upon the elderly, success of the LEPS platform in young mice, both within the current study and as reported previously (Jones et al., 2017; Hill et al., 2018), thus prompted efforts with aged mice. However, a significant unknown was whether the LEPS platform would perform within aged hosts as the efficacy of vaccines is known to decline with aging (Frasca et al., 2011; Pinti et al., 2016). PPSV is traditionally recommended for the elderly while PCV is recommended for the most vulnerable elderly with underlying conditions (Matanock et al., 2019). Yet, aging leads to defects in T cell-dependent and -independent antibody production (Ridda et al., 2009; Buffa et al., 2011), limiting the efficacy of both vaccines (Ridda et al., 2009; Suzuki et al., 2017; Matanock et al., 2019). This was recapitulated in this study where Prevnar-13 vaccination of old mice induced antibodies with subpar function (**Figures 4**, **5**) resulting in reduced protection against infection (**Figures 6**, **7**) as compared to young controls. PCV13 was administered as a single dose as is clinically recommended, and administering a second dose may be needed to improve antibody quality in aged hosts. In contrast, the LEPS vaccine was equally effective across host age (**Figures 6**, **7**). LEPS immunization of old mice resulted in comparable antibody levels to Prevnar-13 (**Figure 3**) but improved

antibody function (**Figures 4**, **5**). This translated to improved protection against pneumococcal infection (**Figures 7**, **8**) relative to Prevnar-13. Importantly, LEPS immunization protected old mice (clinical score, bacteremia, and overall survival- **Figures 7**, **8**) to a similar degree as it did young mice, suggesting that the LEPS platform is able to overcome the age-driven decline in immune responses. The detailed mechanisms by which LEPS (and its myriad of formulation variables) boosts antibody responses in aged hosts and whether it is directly activating B and/or T cells or acting as an adjuvant to boost antigen uptake and presentation by antigen presenting cells and whether single administration of LEPS would induce similar immunity are all avenues for future studies.

An important finding here is that the LEPS platform not only provided protection against invasive pneumococcal disease (**Figure 7**) but also against pneumococcal pneumonia (**Figure 8**). This is of high clinical relevance as one of the limitations of licensed pneumococcal vaccines is their reduced efficacy against pneumonia in the elderly (Andrews et al., 2012; Assaad et al., 2012). This is largely driven by the fact that current polysaccharide vaccines fail to account for bacterial disease progression. *S. pneumoniae* typically reside as a commensal biofilm within the human nasopharynx and asymptomatic colonization is thought to be a prerequisite of disease (Chao et al., 2014; Weiser et al., 2018). The transition from benign colonizer to lethal pulmonary or systemic pathogen involves changes in transcription profiles and bacterial phenotype (D'Mello et al., 2020), including changes to the surface polysaccharide capsule, which is the target of current vaccines. The LEPS vaccine is built specifically to address this weakness in current vaccine options that only focus on polysaccharide immunogens as the LEPS vehicle also includes PncO (Eskola and Anttila, 1999; Daniels et al., 2016; Hill et al., 2018), an *S. pneumoniae* surface protein antigen (conserved across serotypes) over-represented on those virulent pneumococci that break free of the asymptomatic nasopharynx biofilm, disseminate to other bodily locations (lung, blood), and promote disease. We have yet to fully assess the functionality of the associated PncO protein in aged hosts. As this protein antigen becomes critical during later stages of pneumococcal disease, including those prompted by viral co-infection, we anticipate its relevance to emerge in secondary bacterial pneumoniae studies spurred by viral exposure in aged mice. We intend to pursue such studies in the future and, as warranted, further refine the protein antigen content of LEPS based on *S. pneumoniae* antigens that are specifically upregulated during infection of aged hosts.

In summary, this study establishes the use of the LEPS platform as a viable, effective, and economical vaccine strategy against pneumococcal infection in aged hosts. Many features of LEPS can be readily adjusted, including polysaccharide coverage and content level, the noncovalent attachment mechanism of the associated protein, and the base lipid composition. As such, we expect future optimization of the LEPS formulation to further build upon the results obtained in this work. The protein component of LEPS, in particular, holds extended potential as several protein antigens can be combined into one LEPS system for a multiplier effect in valency and for serotype-independent protection. As the elderly are projected to reach two billion worldwide by 2050 (Boe et al., 2017), the LEPS platform provides a timely intervention against serious pneumococcal infections that can be easily adapted to target other respiratory-tract pathogens that infect this vulnerable population.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the University at Buffalo Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

MB conducted the research, analyzed data, and wrote the paper. RN conducted research and analyzed data. ET, DP, AA, and SS conducted research. BP and EBG designed and supervised the research and wrote and edited the paper. All authors read and approved the final manuscript. MB and RN share first authorship. EBG and BP contributed equally to this work and share senior and corresponding authorship.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fragi.2021.798868/full#supplementary-material

Supplementary Figure S1 | LEPS and Prevnar-13 vaccination induce comparable antibody production against *S. pneumoniae*. C57BL/6 young (2 months) and old (18-22 months) mice were injected i.m. with LEPS containing serotype 4 capsular polysaccharide antigen, PCV13, or the controls (Sham or Empty LEPS vector) following the timeline presented in Fig.1B. Levels of IgM and IgG in the sera against heat killed (HKB) S. pneumoniae TIGR4 were measured by ELISA. Antibody units were determined based on a hyperimmune standard included in each ELISA plate. Data shown are presented as the mean ± CI and are pooled from 3 separate experiments. For the

young groups (A-B), data from n=10 mice for Empty LEPS, n=10 mice for LEPS, and n=15 mice for PCV13 are pooled. For the old groups (C-D), data from n=5 mice for Empty LEPS, n=10 mice for LEPS, and n=10 mice for PCV13 are pooled.

Supplementary Figure S2 | Effect of different sera on the number of *S. pneumoniae* bound to pulmonary epithelial cells. Sera were collected from mice immunized with LEPS containing serotype 4 capsular polysaccharide antigen, PCV13, or the controls (Sham or Empty LEPS vector) at four weeks post vaccination following the timeline indicated in Fig. 1B. The ability of sera to neutralize bacterial binding to pulmonary epithelial cells was determined. S. pneumoniae serotype 4 TIGR4 strain was pre-opsonized for 45 minutes with 10% sera from young (Y) (A) or old (O) (B) mice and used to infect H292 cells for 1 hour at an MOI of 10. The number of bound bacteria was determined by plating on blood agar plates. The graph represents the average number of bound bacteria per experiment for each opsonization condition.

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Reduced *Bordetella pertussis*-specific CD4$^+$ T-Cell Responses at Older Age

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Pertussis, a human-specific respiratory infectious disease caused by the Gram-negative bacterium *Bordetella pertussis* (Bp), remains endemic with epidemic years despite high vaccination coverage. Whereas pertussis vaccines and natural infection with Bp confer immune protection, the duration of protection varies and is not lifelong. Recent evidence indicates a considerable underestimation of the pertussis burden among older adults. Whereas the impact of increasing age on Bp-specific humoral immunity has been demonstrated, little is known on immunosenescence of CD4$^+$ T-cell responses in the context of Bp. Here, we aimed to address whether increasing age impacts responsiveness of the Bp-specific CD4$^+$ T-cells in the memory pool following a clinically symptomatic pertussis infection in whole cell vaccine-primed pediatric and adult cases. Cytokine and proliferative responses and phenotypical profiles of CD4$^+$ T cells specific for Bp antigens at an early and late convalescent timepoint were compared. Responses of various Th cytokines, including IFNγ, were significantly lower in older adults at early and late timepoints post diagnosis. In addition, we found lower frequencies of Bp-specific proliferated CD4$^+$ T cells in older adults, in the absence of differences in replication profile. Phenotyping of Bp-specific CD4$^+$ T cells suggested reduced expression of activation markers rather than increased expression of co-inhibitory markers. Altogether, our findings show that the magnitude and functionality of the Bp-specific memory CD4$^+$ T-cell pool decrease at older age. Declined CD4$^+$ T-cell responsiveness to Bp is suggested to contribute to the burden of pertussis in older adults.

Keywords: ***Bordetella pertussis*, IFNγ ELISpot, CD4$^+$ T-cell cytokines, proliferation, infection, aging**

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INTRODUCTION

Pertussis is an acute and severe disease of the respiratory tract and is caused by the highly infectious and human-specific Gram-negative coccobacillus *Bordetella pertussis* (Bp). Pertussis can cause serious illness in people of all ages (Hewlett and Edwards, 2005) and can even lead to fatality in newborns and young infants without protective (maternal) antibody levels (Paddock et al., 2008). It is a vaccine preventable disease, but despite high vaccine coverage, it remains endemic with outbreaks every three to 5 years (van der Maas et al., 2013; Tan et al., 2015). For a long time, pertussis was considered to be a childhood disease (Gordon and Hood, 1951), but substantial evidence has been put forward that it also affects adolescents, adults and elderly (reviewed in (Rothstein and Edwards, 2005; Kandeil et al., 2019)). The true number of pertussis cases in older age groups are likely underestimated due to under identification (McGirr et al., 2013) by missed diagnoses or no medical visit, as severity of symptoms is

lower compared to infants. A large burden of disease may therefore occur in older (vulnerable) adults and elderly, facilitating ongoing Bp transmission.

Pioneering studies in mice have shown that especially $CD4^+$ T cells producing IFNγ are imperative for controlling Bp bacterial load (Mills et al., 1993; Barbic et al., 1997; Mahon et al., 1997; Leef et al., 2000) and reviewed in (Lambert et al., 2019). Moreover, transferred $CD4^+$ T cells but not $CD8^+$ T cells were shown to clear Bp infection in convalescent mice (Mills et al., 1993). Neither natural infection, nor vaccination provide life-long immunity to protect against pertussis (Wearing and Rohani, 2009), however epidemiological studies indicate more durable responses induced by natural infection and whole cell pertussis vaccination as compared to acellular pertussis vaccination (Klein et al., 2012; Sheridan et al., 2012; Klein et al., 2013). More sustained protection is associated with skewed $CD4^+$ T-cell programming: natural infection and priming with whole cell pertussis vaccination induce programming towards T helper (Th1) and Th17 $CD4^+$ T cells, as opposed to Th2/Th17 skewed $CD4^+$ T-cell memory responses induced by acellular pertussis vaccination (Ryan et al., 1998; Esposito et al., 2001; Mascart et al., 2003; Vermeulen et al., 2010; Warfel et al., 2014).

Whereas waning immunity has been suggested to explain the occurrence of pertussis in all age groups (reviewed in (van Twillert et al., 2015)), especially aging of the immune system or 'immunosenescence' (Gruver et al., 2007) may be a major factor that contributes to pertussis incidence amongst older adults. Immunosenescence of the T-cell population has been widely studied and can result in dysfunctional T-cell responses, which in turn may contribute to increased susceptibility to infection and poor vaccination responses (Akbar et al., 2016). Within the global T-cell population, the total number of T cells declines, memory T cells accumulate, and the naïve T-cell pool reduces (reviewed in (Chinn et al., 2012; Dixit, 2012)), which negatively affects the response to new pathogenic encounters. Moreover, in mice and in humans, immunosenescence of $CD4^+$ and $CD8^+$ T cells has been associated with a rise of co-inhibitory marker expression, such as PD-1 (Lages et al., 2010), CTLA-4 (Leng et al., 2002), TIGIT (Song et al., 2018), and the transcription factor Helios (Pieren et al., 2021). Currently, Bp-specific immune responses in the population are mostly memory-based, in view of high vaccination coverage during many decades, as well as endemic and epidemic circulation of Bp. It is not known, however, whether antigen-specific $CD4^+$ T-cell memory to Bp is subject to aging. To advance our understanding of age-related differences in Bp-specific $CD4^+$ T-cell responsiveness, we compared cytokine profiles, proliferative capacity and phenotype of Bp-specific $CD4^+$ T-cell populations detectable in time after a clinically symptomatic Bp infection in children, adults and older adult participants in a unique cohort of ex-pertussis cases, sharing a history of primary whole cell pertussis vaccination during infancy.

MATERIAL AND METHODS

Ethics Statement

Participants were included from two clinical studies. The first clinical study comprised a cross-sectional observational study in Dutch symptomatic pertussis cases (Specifieke Kinkhoest Immuniteit; SKI; 2008–2012) (Han et al., 2013; van Twillert et al., 2014). This study was approved by the accredited Review Board STEG, followed by management of the METC UMC Utrecht (CCMO nr: NL16334.040.07). The second clinical study was a longitudinal observational study in Dutch symptomatic pertussis cases (Immfact; 2014–2020). This study was approved by the accredited Medical Research Ethics Committee "METC Noord-Holland" (Alkmaar, the Netherlands) followed by management of the METC MEC-U (Utrecht, the Netherlands) (CCMO nr: NL4679.094.13). All participants provided written informed consent for the collection of samples, the usage of a completed questionnaire regarding clinical symptoms and vaccination history, and the subsequent immunological analyses. Informed written consent for minor participants was provided by both parents or guardians of participants. These studies were conducted in compliance with the principles of the Declaration of Helsinki.

Study Population

Participants from the SKI study consisted of 58 clinically symptomatic (ex) pertussis patients who donated blood at a single known, either early or late, time point after their laboratory confirmed diagnosis. For analysis, subjects were classified into a young group (median age of 12 years; range 11–15 years, n = 21), referred to as youngsters (Y), and an adult group referred to as (A) (median age of 38 years; range 25–56 years, n = 37). Youngsters and adults were subcategorized according to the time elapsed between their date of clinical pertussis diagnosis and date of blood sampling (τ, in months), indicated as sampled in *early phase* ($\tau1 < 4$ months, or in *late phase* $\tau2 \geq 8$ months, no upper limit for inclusion). Late phase cases were excluded on serological criteria, if having an IgG plasma level specific for Pertussis Toxin (PT) $\geq$ 62.5 IU/ml, indicative for an additional non-diagnosed exposure (de Melker et al., 2000; de Greeff et al., 2010). Participants of the Immfact study consisted of 16 clinically symptomatic pertussis patients who donated blood at two longitudinal time points after their laboratory confirmed diagnosis. Here, youngsters (Y) with a whole cell priming background (median age of 16 years, range 12–23 years, n = 9) and older adults (O) (median age of 71 years, range 60–78, n = 7) were sampled at $\tau1 \leq 3$ months post diagnosis (*early phase*) as well as at $8 \leq \tau2 \leq 12$ months post diagnosis (*late phase*). For details of cohorts, see **Supplementary Figure S1**. All participants from both clinical cohorts and of all age groups were primed during their first year of life and according to their birth cohort with multiple doses of the Dutch whole cell vaccine (in use until the year 2005 when the Dutch National Immunization Programme switched to an acellular primary pertussis vaccine for infants). As by their birth cohort and the Dutch national immunization programme, participants did not receive any pertussis booster dose(s), except for three younger participants from the Immfact cohort. At the age of 4-years these participants received an acellular pertussis booster dose at school entrance, which was programmatically introduced in the Netherlands in 2001. The Dutch National Immunization Programme does not include further adolescent or adult acellular pertussis booster doses. Maternal pertussis immunization was only introduced in December 2019.

Pertussis Protein Antigens and Peptide Pools

P.69 pertactin (PRN) was recombinantly expressed and purified from *E. coli* as described previously (Hijnen et al., 2005). PT and Filamentous Hemagglutinin (FHA) were both obtained from Kaketsuken, Japan. Presence of *E. coli* LPS or *B. pertussis* LOS was ruled out (<0.015 EU/ml) based on Limulus Amebocyte Lysate (LAL) testing. Pools of synthetic immunogenic Bp peptides were purchased; Bp132, a peptide pool consisting of 132 immunogenic peptides derived from PT, FHA, PRN and Fimbriae 2/3 (Bancroft et al., 2016) (Pepscan); PT peptide pool, consisting of 18-mers spanning the PT S1 subunit with 12 amino acids overlap (in-house synthesis, P. Hoogerhout); FHA peptide pool and PRN peptide pool, consisting of only the FHA or PRN-specific peptides from the Bp132 peptide pool, respectively (Pepscan).

Blood Sampling and PBMC Isolation

Venous blood samples were collected in the SKI study using CPT Mononuclear cell preparation tubes (BD) and in the Immfact study using vacutainer blood collection tubes (BD). Peripheral blood mononuclear cells (PBMCs) were isolated using standard procedures and frozen in 10% DMSO. Samples were kept at −80°C overnight and then stored at −135°C.

In vitro T-Cell Re-Stimulation

PBMCs were quickly thawed at 37°C and *in vitro* cultured as described elsewhere (Schure et al., 2012) with minor modifications. Briefly, SKI PBMC samples were cultured for 5 days at 37°C, 5% CO_2, in 96-well U-bottom plates at 3.0×10^5 viable cells in 150 μL/well in replicate wells per condition in AIM-V medium (Gibco, Invitrogen, United States) containing 5% human AB serum (Harlan, United Kingdom) (AIM-V+) only (negative control), or in the presence of 5 μg/ml of PT (heat-inactivated), FHA or PRN, or of 5 μg/ml of pokeweed mitogen (Sigma Chemicals, United States) (positive control). Alternatively, Immfact PBMC samples were labelled with CellTrace Violet (Thermofisher) at a final concentration of 0.5 μM and cultured for 6 days at 37°C, 5% CO_2, in loosely capped 5 ml tubes (Falcon, BD) at 2.0×10^6 in 1 ml/tube in RPMI 1640 medium (Gibco, Invitrogen, United States) containing 5% human AB serum (Harlan, United Kingdom; RPMI+) in medium only (negative control) or in the presence of PT S1 peptide pool, Bp132, PRN peptide pool, FHA peptide pool (all at 0.1 μM per individual peptide) or anti-CD3 and CD28 antibodies (at 0.5 μg/ml and 1 μg/ml, respectively, positive control). For both SKI and Immfact samples aliquots of culture supernatants were collected on day 5 and stored at −80°C for subsequent quantification of cytokine levels. After culture, SKI and Immfact PBMCs were harvested on day 5 and day 6, respectively, for further analyses. Depending on the amount of PBMCs available per sample, one or more *B. pertussis* antigenic stimulations were tested apart from the negative control.

IFNγ ELISpot Assay

5-stimulated PBMC (SKI) samples were spun down and after collection of supernatants, cells were reconstituted in culture medium and transferred to anti-human IFNγ (Mabtech, Sweden) coated ELISpot plates (Millipore, United States) at a starting concentration of 1.4×10^5 cells in 150 μL/well and three times two-fold serially diluted. Plates were incubated for approximately 20 h at 37°C, 5% CO_2, then after four washing steps and one cell-lysing step, incubated with 1 μg/ml anti-human IFNγ (Mabtech), followed by peroxidase labeled extravidin (Sigma) and BCIP/NBT (KPL, United States). After development, the plates were air-dried at room temperature prior to analysis of numbers of IFNγ spot forming cells (SFC), assessed by an automatic computer-assisted ImmunoScan-Pro reader (CTL Europe, Germany). Results are expressed as IFNγ SFC/100.000 PBMCs with a lower detection limit of 0.1 IFNγ SFC/100.000 PBMCs.

Flow Cytometry

CellTrace-labelled day 0 and day 6-stimulated Immfact PBMCs were washed and labelled at 4°C for cell surface and intracellular markers with the following antibodies: anti-CD3-Alexa700 (clone SK7), anti-CD4-BV711 (clone OKT-04), anti-CD8-BV785 (clone RPA-T8), anti-CD45RO-PerCP-Cy5.5 (clone UCHL1), anti-FoxP3-Alexa647 (clone 259D), anti-PD-1-BV605 (clone EH12.2H7), anti-CD127-BV650 (clone A019D5), anti-CTLA-4-PE (clone L3D10), anti-TIGIT-PE/efluor-610 (clone MBSA43), anti-Helios-PE-Cy7 (clone 22F6), anti-CD27-BUV395 (clone L128), anti-CD25-BUV737 (clone 2A3) and Live-dead ZOMBIE NEAR IR-APC-Cy7. Acquisition was performed on a BD LSR Fortessa X-20 (BD Biosciences, Franklin Lakes, NJ, United States). Subsets of $CD4^+$ T cells were identified based on combined marker expression on gated $CD3^+CD4^+$ cells. Frequencies and phenotypes of proliferating antigen-specific $CD4^+$ T cells were determined by analyzing sequential halving of the CellTrace Violet fluorescence intensity combined with performing surface phenotypic and intracellular marker labelling. Percentages of proliferated $CD4^+$ T cells found after medium stimulation conditions were subtracted from percentages of proliferated $CD4^+$ T cells after Bp-antigen stimulation, when comparing proliferative $CD4^+$ T-cell responses between groups. For both replication index calculations (by proliferation modeling) as well as gating analyses, FlowJo software was used (Tree Star, Ashland, OR, United States).

Cytokine Multiplex Bead-Based Immunoassay

Concentrations of the cytokines IL-2, IFNγ, TNF-α, IL-5, IL-13, IL-17A and IL-10 in day 5 culture supernatants were determined in pg/mL according to manufacturer's instructions using a commercial multiplex bead-based immunoassay kit (Bio-Rad, United States) (SKI samples) or using the LEGENDplex Human Th cytokines kit (BioLegend) (Immfact samples), and expressed in [pg/ml]. Measurements and data analysis were performed with a Bio-Plex 200, using Bio-Plex Manager software or flow cytometry (FACS Canto II), respectively.

Dimensionality reduced analyses

Dimensionality-reduced analyses (viSNE) of flow cytometry data were performed in Cytobank (www.Cytobank.org). Prior to the viSNE plots, $CD4^+$ proliferating events were exported and pooled per culture condition, age group and timepoint. The number of

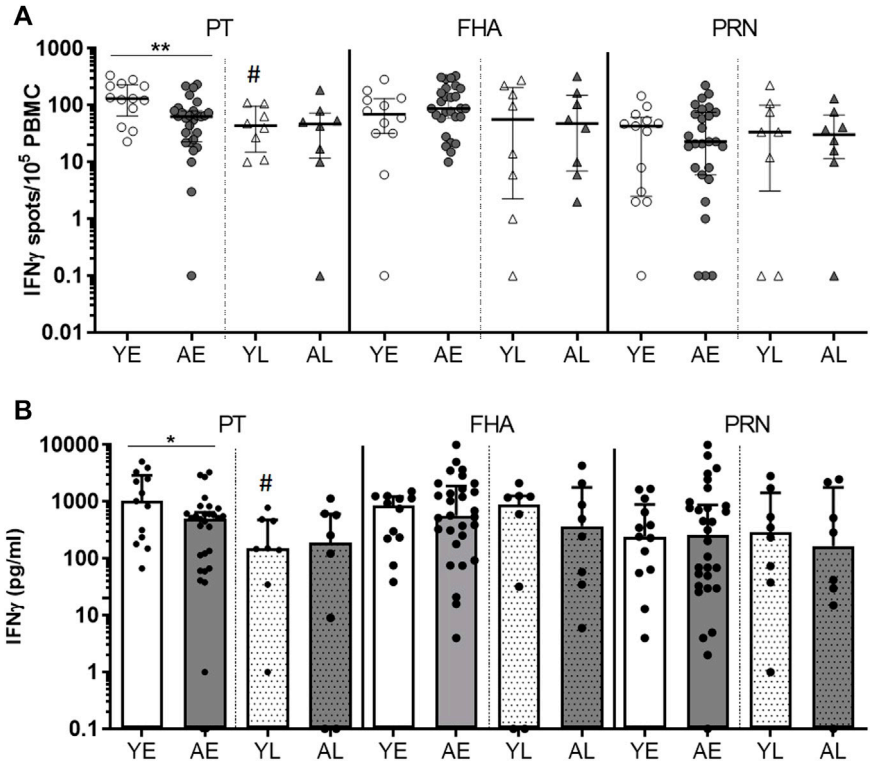

FIGURE 1 | Adults show lower frequencies of PT-specific IFNγ-producing cells and IFNγ levels in the early phase after clinical pertussis (SKI). PBMCs of youngsters ("Y") and adults ("A"), from an early timepoint ('E'; within 4 months after diagnosis), and late timepoint ("L", 8–80 months) after clinical infection were stimulated with Bp proteins PT **(left panels)**, FHA **(middle panels)** and PRN **(right panels)** for 5 days. **(A)** Numbers of IFNγ-spot forming cells per 10^5 PBMCs measured by ELISpot. **(B)** IFNγ levels in culture supernatants by Luminex and expressed as [pg/ml]. Symbols show individual cases, while lines and bars indicate medians and interquartile ranges. Statistical significance was calculated with Mann Whitney *U*-test. *$p < 0.05$, **$p < 0.01$; # = significantly lower compared to the early phase.

cells included in the analysis was 2.3×10^5 and was equal for youngsters and older adults.

Statistical Analysis

For data analysis and visualization of data, GraphPad Prism (GraphPad Software version 8.4.1) was used. Statistical significance of differences was analyzed with the nonparametric Mann-Whitney *t*-test, when comparing stimulated and unstimulated conditions of samples, and the Wilcoxon matched pairs signed-rank test, when comparing longitudinal samples from individuals. For all analyses, *p* values <0.05 were considered statistically significant.

RESULTS

Adults Show Lower Frequencies of PT-Specific IFNγ-Producing Cells and IFNγ Levels in the Early Phase After Clinical Pertussis

Production of IFNγ is an important parameter for an effective immune response to a Bp infection (Mills et al., 1993; Ryan et al., 1997; Hafler and Pohl-Koppe, 1998). We first set out to explore IFNγ production in total PBMC cultures stimulated with Bp protein antigens to compare these responses between youngsters and adults. To determine the impact of age on functionality of Bp-specific responses, we studied IFNγ responses in early and late phase post-infection PBMC samples from youngsters and adult pertussis patients of the SKI cohort with a similar whole cell pertussis vaccine priming background. Production of IFNγ of 5-day cultured PBMCs stimulated with protein antigens Pertussis Toxin (PT), filamentous hemagglutinin (FHA) and pertactin (PRN) were determined by ELISpot. Geomean number of IFNγ SFC ranged between 11–118 per 1×10^5 PBMCs, depending on the stimulation, age and timepoint. The frequency of IFNγ-producing cells in response to PT was significantly lower in adults compared to youngsters in the early phase (**Figure 1A**). Waning of the PT response from the early to late phase was observed in youngsters but not in adults. In the early phase response to FHA, adults showed comparable median frequencies of IFNγ-producing cells (**Figure 1A**), which did not decline over time. In response to PRN, no age-related differences in numbers or patterns over time were found for IFNγ-producing cells (**Figure 1A**). Similar to findings on the number of IFNγ-producing cells, the level of IFNγ measured in

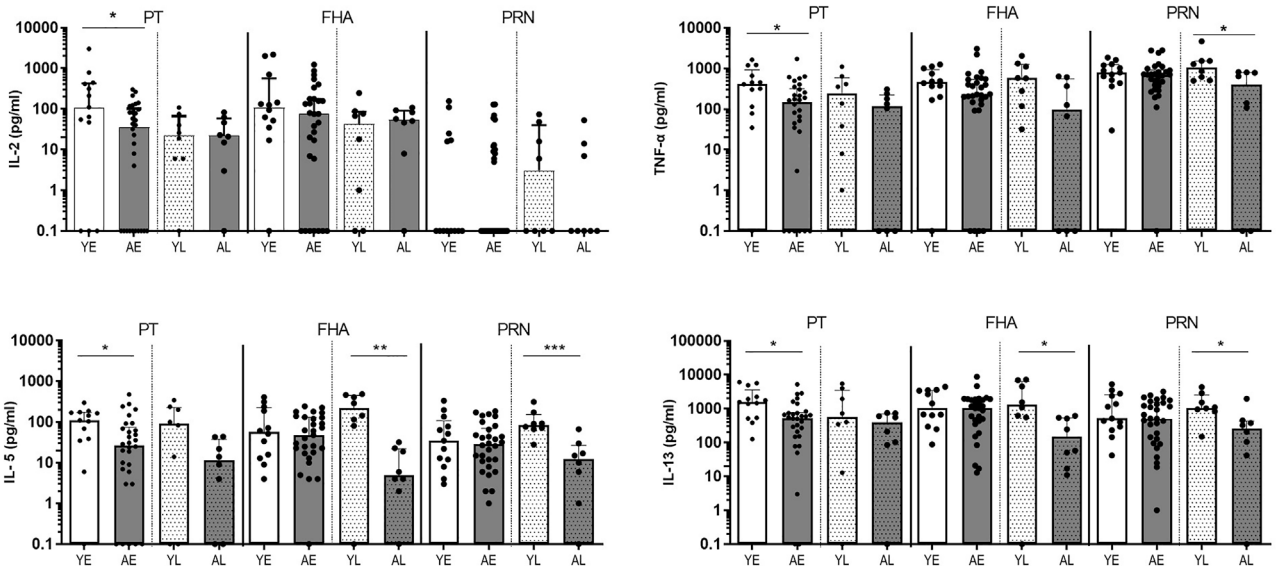

FIGURE 2 | Adults show lower levels of secreted Th-type cytokines after pertussis infection (SKI). Bar graphs show the concentration of IL-2, TNF-α, IL-5, and IL-13 in supernatants of PBMCs *in vitro* stimulated with Bp proteins PT **(left panels)**, FHA **(middle panels)** and PRN **(right panels)** in youngsters ("Y", white bars) and adults ("A", dark bars) in early ("E", open bars) and late ("L", dotted bars) phase after clinical diagnosis. Dots show individual cases while bars indicate medians and interquartile range. Statistical significance was calculated with Mann Whitney *U*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

culture supernatants was significantly lower in PT-stimulated samples of adults compared to youngsters in the early phase of the response (**Figure 1B**). In summary, these data suggest age-related differences in IFNγ responses to PT in the early phase after clinical pertussis infection.

Adults Show Lower Levels of Secreted Th-type Cytokines After Pertussis Infection

Next, we extended our analyses of cytokines produced in total PBMC cultures stimulated with protein antigens in youngsters and adults from the SKI cohort by measuring Th1 (IL-2, TNF-α), Th2 (IL-5, IL-13) (**Figure 2**), Th17 (IL-17) and regulatory (IL-10) associated cytokines (**Supplementary Figure S2**). Overall, adults presented with lower levels of cytokines than youngsters in both early and late phase after clinical pertussis infection. We found significantly lower levels of Th1-associated cytokines IL-2 and TNF-α, and Th2-associated cytokines IL-5 and IL-13 in PT-stimulated culture supernatants in adults compared to youngsters in the early phase (**Figure 2**). In FHA-stimulated samples, significantly lower levels of Th2 associated IL-5 and IL-13 cytokines were found in the late phase in adults compared to youngsters (**Figure 2**). In addition, PRN-stimulated culture supernatants had significantly lower levels of IL-5, IL-13 and TNF-α in the late phase. Levels of IL-17A and IL-10 were comparable between the two age groups (**Supplementary Figure S2**). Together, these data indicate that in various phases after clinical pertussis infection, Bp specific cytokine responses, typically mediated by $CD4^+$ T cells, are reduced at older age.

Longitudinal follow up confirms lower levels of Th-type cytokines after Bp infection in older adults The lower Bp-antigen specific cytokine responses found in the older age group suggested that either the proportion of Bp-antigen specific functional memory $CD4^+$ T cells responding in this group was smaller, or its proliferative capacity was impaired compared to youngsters. Immunosenescence of $CD4^+$ T-cell proliferation has been described at increased age (Jiang et al., 2007). To shed more light on the mechanism, we designed a flow-based assay, allowing the analysis of Bp antigen-specific proliferation of $CD4^+$ T cells, as well as exploring several cellular markers linked to T-cell immunosenescence. Here, we selected paired samples obtained at fixed time points in the early and late phase post clinical pertussis from whole cell vaccine primed youngsters and older adults in the Immfact study (**Supplementary Figure S1**). In contrast to stimulations with whole protein antigen, we now used peptide pools of separate and combined Bp antigens of which the epitopes have previously been described as immunogenic and optimal for binding to MHC class II (Bancroft et al., 2016) and therefore allowed us to assess the response of antigen-specific $CD4^+$ T cells. First, we compared cytokine production in 5 days culture supernatants of PBMCs from youngsters and older adults post clinical pertussis infection longitudinally (**Figure 3**, **Supplementary Figure S3**). In older adults, IFNγ and IL-13 levels were significantly lower in response to peptide pools Bp132, PT S1 and FHA in the late phase. In addition, lower levels of TNF-α were detected in response to Bp132 (in the late phase) and FHA (in the early phase). Stimulation with PRN peptide pools revealed lower levels of IL-13 in older adults in the early phase. Thus, despite various differences in study design and experimental approach between the SKI and Immfact samples, we observed a similar trend towards lower Th-type cytokine production in response to Bp antigens with increasing age.

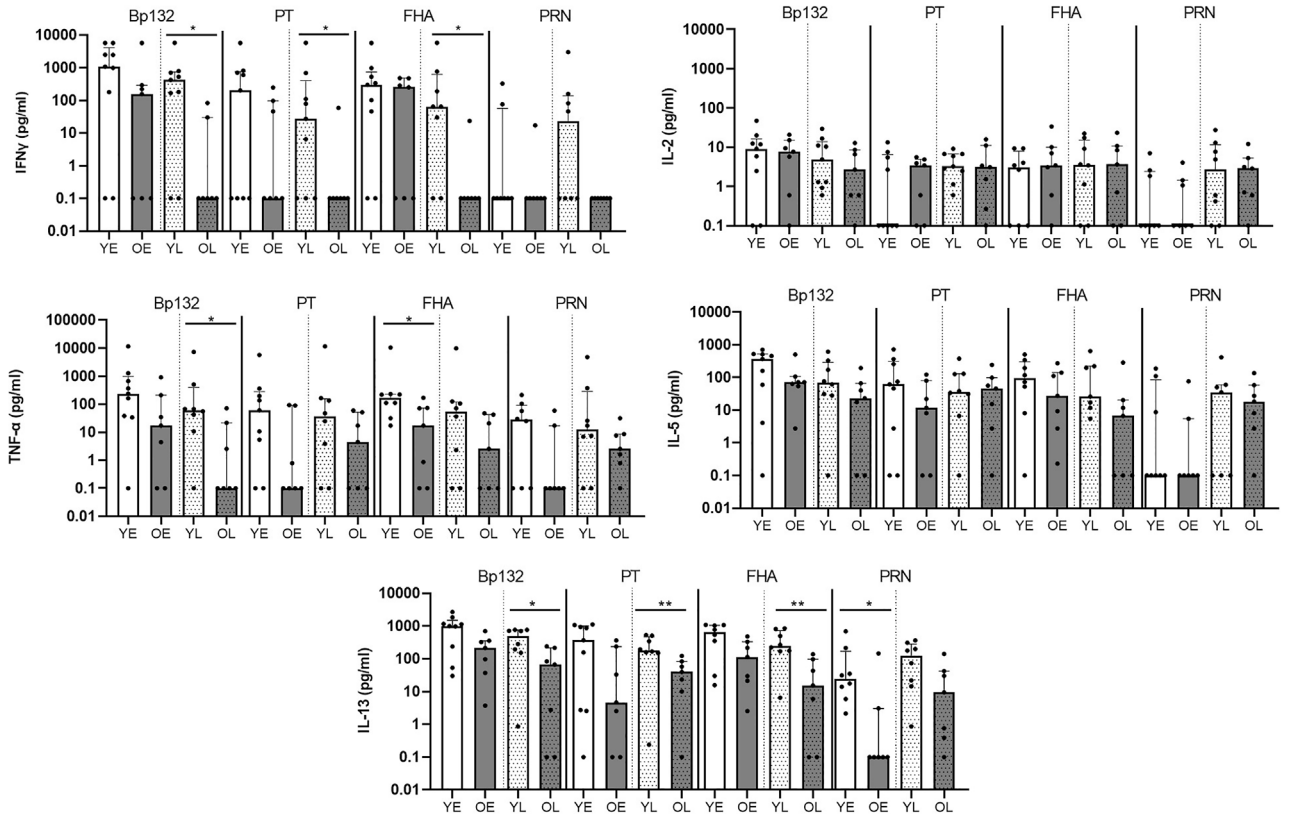

FIGURE 3 | Longitudinal follow up of older adults confirms lower cytokine production after Bp infection (Immfact). Bar graphs show the concentrations of IL-2, TNF-α, IL-5, and IL-13 in supernatants of PBMCs *in vitro* stimulated with Bp peptide pools Bp132, PT S1 (shown as PT), FHA and PRN, as indicated, in youngsters ("Y", white bars) and older adults ("O", dark bars) in early ("E", open bars) and late ("L", dotted bars) phase after clinical diagnosis. Dots show individual cases while bars indicate medians and interquartile range. Statistical significance was calculated with Mann Whitney *U*-test. $^{*}p < 0.05$.

Similar Frequencies of Naïve and Memory Subsets and Regulatory Cells Within the CD4$^+$ T-Cell Compartment of Youngsters and Older Adults Following Clinical Bp Infection

We then explored CD4$^+$ T-cell populations of the Immfact PBMC samples for the various flow-cytometric read-outs of our assay. To first assess whether youngsters and older adults of the Immfact cohort display differences in the frequencies of global naïve and memory CD4$^+$ T-cell subsets in their peripheral blood, we analysed the CD4$^+$ T-cell composition of PBMC samples prior to stimulation based on expression of CD27 and CD45RO (**Figure 4A**, gating strategy in **Supplementary Figure S4**). We found comparable percentages of central memory (T_{cm}, CD45RO$^+$CD27$^+$), effector memory (T_{em}, CD45RO$^+$CD27$^-$) and terminally differentiated (T_{emra}, CD45RO$^-$CD27$^-$) subsets between the age groups at both early and late phase, only the percentage of naïve CD4$^+$ T cells was significantly reduced in older adults compared to youngsters in the late phase (**Figure 4B**). After 6-day stimulation with Bp peptide pools Bp132, PT S1, FHA and PRN, we found the global composition of the CD4$^+$ T-cell compartment to be comparable between youngsters and older adults and between early (**Figure 4C**) and late phase samples (**Figure 4D**).

Additionally, regulatory T cells (Tregs) have been suggested to accumulate with increasing age (Gregg et al., 2005; Lages et al., 2008; Elyahu et al., 2019). Therefore, we analysed the frequency of Tregs, defined as either CD127$^-$CD25$^+$ or FoxP3$^+$CD25$^+$ CD4$^+$ T cells prior to stimulation (Seddiki et al., 2006; Yu et al., 2012; Rodríguez-Perea et al., 2016). Tregs were in the range of 5–8% of the CD4$^+$ T cells and frequencies were comparable between youngsters and older adults (**Supplementary Figure S5**. Altogether, these analyses show that youngsters and older adults from the Immfact cohort do not differ in the proportions of memory T-cell subsets (based on both phenotypes) before and after Bp-peptide stimulation, nor in *ex vivo* Tregs.

Reduced Proliferative Responses of Bp-Specific CD4$^+$ T Cells at Older Age

Next, we investigated whether age had an impact on proliferative responses of antigen-specific CD4$^+$ T cells in youngsters and older adults. To determine frequencies of proliferating CD4$^+$ T cells, PBMCs were CellTrace labelled, stimulated with Bp antigen peptide pools for 6 days, and assessed for CellTrace Violet diminution (**Figure 5A**). Our data showed a general trend of lower frequencies of CD4$^+$ T cells proliferating to Bp antigen in

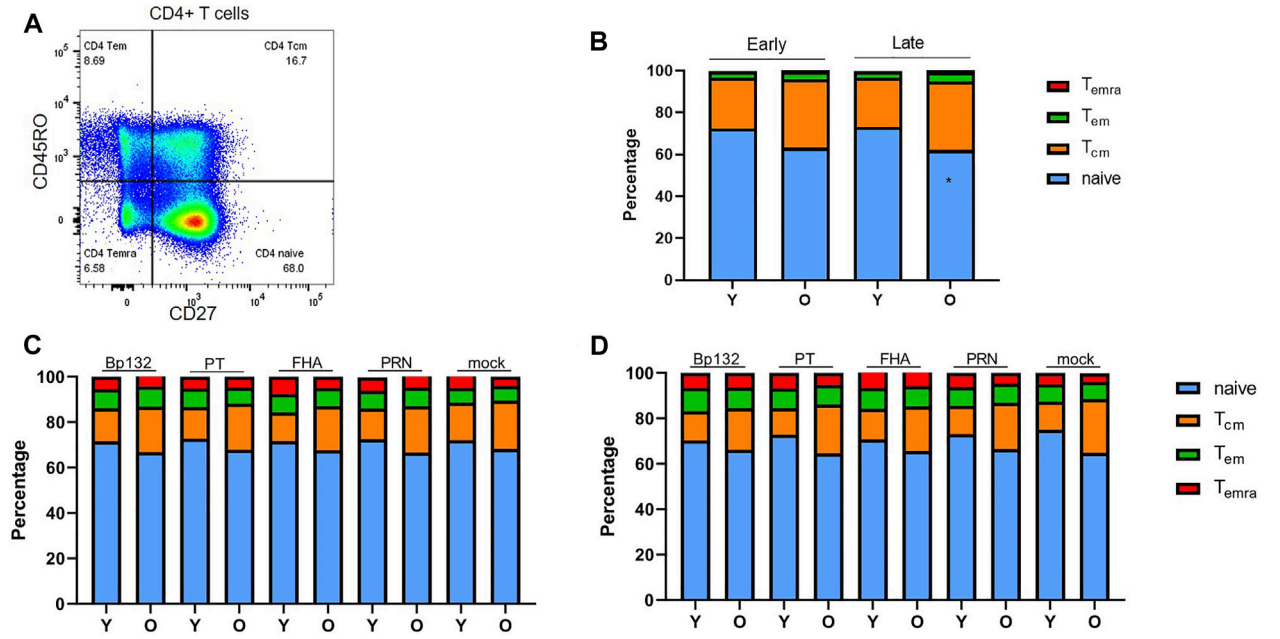

FIGURE 4 | Frequencies of naïve and memory cells within the CD4$^+$ T-cell population in longitudinal PBMC samples of youngsters (Y) and older adults (O) pre- and post *in vitro* culture. **(A)** Gating of naive and memory subsets based on CD45RO and CD27 expression. **(B)** Proportion of naïve/Tem/Tcm/Temra subsets within the CD4$^+$ T-cell population at early and late phase prior to stimulation; **(C)** after 6-day antigenic stimulation in the early phase; and **(D)** after 6-day antigenic stimulation in the late phase. Stacked bars indicate median relative percentage of subsets per age group and timepoint. Statistical significance of proportional differences between CD4$^+$ T-cell subsets of youngsters and older adults was calculated with Mann Whitney *U*-test. $^*p < 0.05$.

the older adults, which was significant for the Bp132 and PT S1 peptide pool early after clinical pertussis infection (**Figure 5B**). In the late phase, no age-related differences were found. We did observe waning of proliferative responses in youngsters in time, as frequencies of proliferated CD4$^+$ T cells to Bp132 and PT S1 peptide pools were significantly lower in the late phase post infection compared to the early phase (**Figure 5B**). Patterns of Bp-specific CD4$^+$ T-cell proliferation in older adults in response to FHA or PRN were comparable to those observed in youngsters. The proliferative response was further investigated by calculating the replication index (fold-expansion of proliferating cells). The replication index was comparable between youngsters and older adults (**Figure 5C**), suggesting that responding CD4$^+$ T cells undergo equal rounds of cell divisions in adults compared to young individuals. Thus, these data show that the magnitude of the proliferative response of the CD4$^+$ T-cell compartment to Bp antigens Bp132 and PT S1 declined with age but rounds of divisions in response to all Bp antigens per cell were similar. This suggests maintenance of a smaller memory pool of Bp-specific CD4$^+$ T cells for recall responses as a hallmark of immunosenescence.

Minor Phenotypical Differences of Proliferated CD4$^+$ T cells Between Youngsters and Older Adults

Next, we assessed whether lower cytokine production and a lower proliferative response were accompanied by an altered phenotypical profile of responding CD4$^+$ T cells. We hypothesized that antigen-specific, proliferating CD4$^+$ T cells of older adults express higher levels of co-inhibitory receptors CTLA-4, PD-1, and TIGIT and lower levels of the activation marker CD25 (the IL-2-alpha receptor). In contrast, we found that expression of CTLA-4 by proliferated CD4$^+$ T cells in the early phase in youngsters was significantly higher compared to older adults in response to Bp132, whereas the percentage of CTLA-4$^+$ proliferating CD4$^+$ T cells varied but was comparable between the two age groups (**Supplementary Figures S6A,B**). Expression of the activation marker CD25 on proliferated CD4$^+$ T cells was comparable between youngsters and older adults both in the early and late phase, as well as were frequencies of CD25$^+$ proliferated CD4$^+$ T cells, both in the early and late phase (**Supplementary Figures S6C,D**). Likewise, analysis of expression and frequencies of PD-1$^+$ (**Supplementary Figure S7A–B**) and TIGIT$^+$ (**Supplementary Figure S7C–D**) cells within proliferating CD4$^+$ T cells did not reveal age-related differences.

Lastly, we applied dimensionality reduction analyses (viSNE) to proliferated CD4$^+$ T cells of pooled datafiles of youngsters and older adults in the early phase to reveal any differences based on combined marker expression between proliferated CD4$^+$ T cells of youngsters and older adults (**Figures 6A,B**). Cluster analysis showed a potentially interesting cluster, cluster d, that was significantly lower in older adults compared to youngsters (**Figure 6C**). Based on subsequent heatmap visualization of marker expression in the clusters (**Figure 6D**), this cluster was positive for markers including

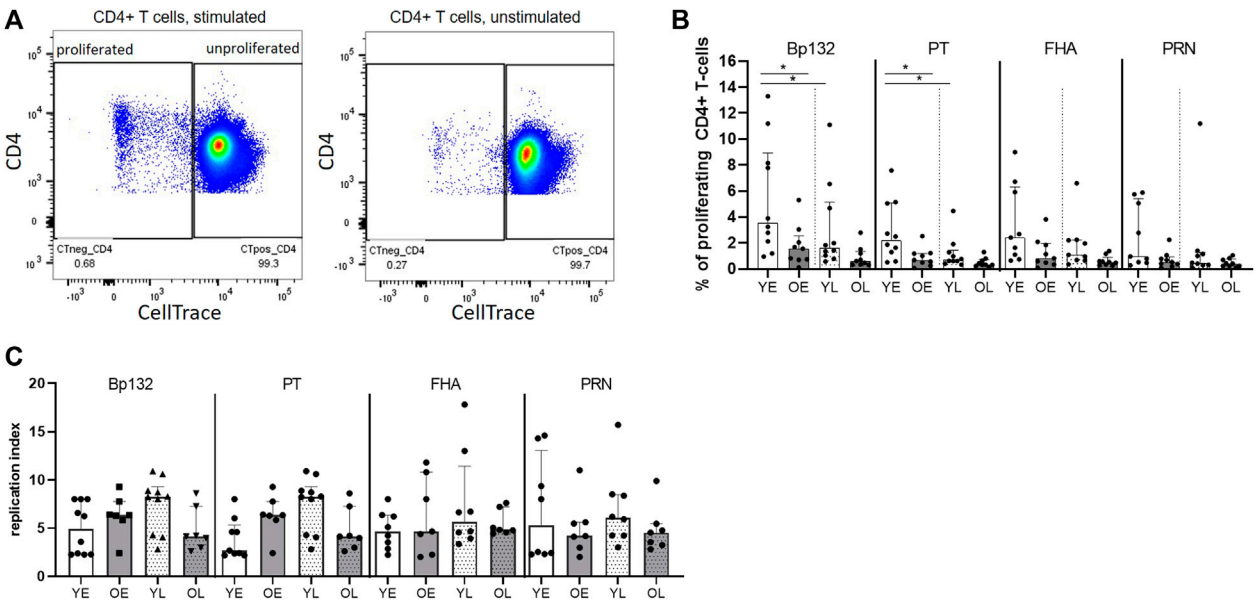

FIGURE 5 | Reduced proliferative response of Bp-specific CD4$^+$ T cells at older age. PBMCs of youngsters (Y) and older adults (O) of early (E) and late (L) timepoints were labelled with a proliferation dye and subsequently assessed on sequential halving of fluorescent intensity after 6-days stimulation Bp peptide pools Bp132, PT S1 (shown as PT), FHA, and PRN. **(A)** Gating strategy of proliferating and non-proliferating CD4$^+$ T cells as shown for Bp132 peptide pool-stimulated (left plot) and medium (unstimulated) (right plot) conditions. **(B)** Frequencies of proliferating CD4$^+$ T cells post stimulation. **(C)** Replication index. Dots show background subtracted frequencies of individual cases, while bars represent medians and interquartile range youngsters (Y), white bars; older adults (O), dark bars; early (E) phase samples, open bars; late (L) phase samples, dotted bars, as indicated. Statistical significance was calculated with Mann Whitney *U*-test and Wilcoxon matched pairs signed-rank test. * = $p < 0.05$.

CD25, CTLA-4 and TIGIT and showed somewhat higher Helios expression compared to other clusters. Additionally, we applied the gating approach derived from cluster analysis of proliferated CD4$^+$ T cells after pneumococcal antigen stimulation using a similar phenotypical marker panel (He et al., unpublished) and found significant higher proportion of CD4$^+$ T cells that were CD127$^-$FoxP3$^-$Helios$^+$ (**Figure 6E**). Helios is considered a marker associated with (CD4$^+$) T-cell activation (Akimova et al., 2011; Bengsch et al., 2018). In summary, unbiased viSNE cluster analysis of Bp-specific CD4$^+$ T cells that have proliferated did not reveal phenotypical differences between youngsters and older adults based on the expression of co-inhibitory receptors CTLA-4, PD-1, and TIGIT or of activation marker CD25. The significantly higher frequency of CD127$^-$FoxP3$^-$Helios$^+$ proliferated CD4$^+$ T cells in youngsters may however suggest a reduced activation status at older adult age. Altogether, these data indicate that older adult Bp cases show features of CD4$^+$ T-cell Bp-specific immunosenescence following clinical infection, highlighted by significantly lower cytokine and proliferative responses than younger adolescent counterparts. Moreover, absence of age-related differences in fold-expansion and co-inhibitory marker expression of Bp-specific proliferated CD4$^+$ T cells, with a significant reduction of an activated CD4$^+$ T-cell subset at older age, suggest that the immunosenescence can largely be attributed to a reduced size and thereby responsiveness of the maintained Bp-specific memory CD4$^+$ T-cell pool.

DISCUSSION

Bp-specific CD4$^+$ T cells are key players in durable protection against Bp. In this study, we have shown for the first time that memory CD4$^+$ T-cell responsiveness to Bp-specific antigens is impaired at older age. Our major findings are that both cytokine and proliferative responses of specific CD4$^+$ T cells in the early contraction phase and the late maintenance phase after an *in vivo* recall by a clinical Bp infection were reduced in adult cases compared to younger cases who had a similar background of primary whole cell pertussis vaccination. These observations could either be explained by a reduced capacity to proliferate per CD4$^+$ T cell, resulting in less cells to produce cytokines, or by maintenance of a reduced memory pool of Bp specific CD4$^+$ T cells that could expand and produce cytokines in response to Bp, at older age. Evidence indicating a similar fold-expansion of proliferated CD4$^+$ T cells of the age sub cohorts favoured the reduced memory pool hypothesis at older age, with the responding CD4$^+$ T cells showing some hallmarks of reduced activation, but no co-inhibitory or regulatory features of immunosenescence.

IFNγ is essential in the clearance of pertussis infection (Barbic et al., 1997), potentiating bacterial opsonophagocytosis and killing by phagocytes (Lambert et al., 2019). Here, lower levels of IFNγ spots and corresponding concentrations in the supernatant to PT stimulation were found in adults compared to youngsters, possibly implicating reduced capacity to combat

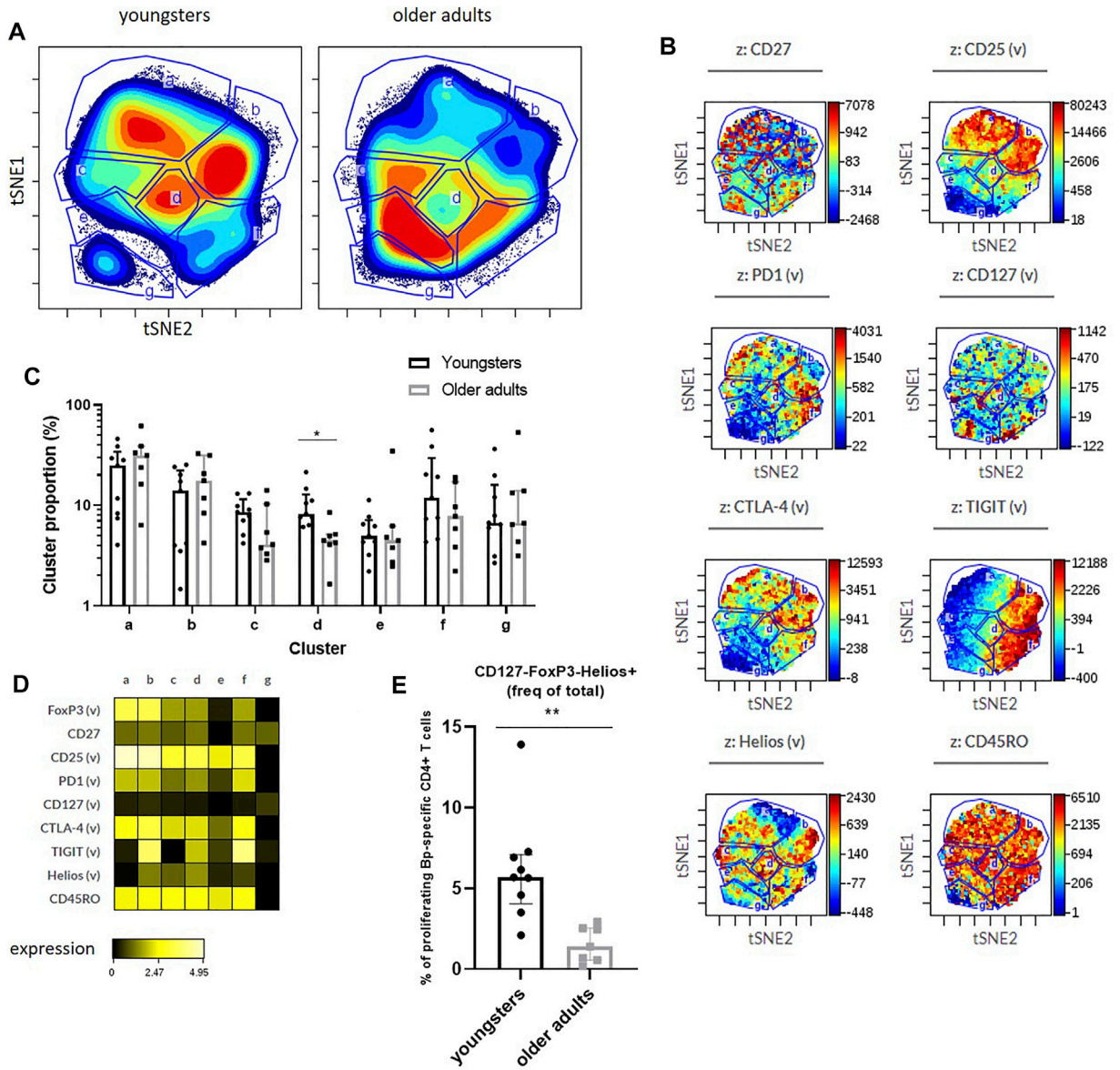

FIGURE 6 | Minor age-related phenotypical differences of proliferated CD4$^+$ T cells responding to Bp132 stimulation in youngsters and older adults in the early phase. **(A)** Cell density maps show phenotyping analysis by dimensionality reduced single cell data by viSNE show clustering of populations within the proliferating cells of youngsters and older adults of pooled flow cytometry datafiles pre-gated on proliferating CD4$^+$ T cells and identified clusters indicated with letters a-g. **(B)** Dot-plot *z*-axis shows the location and intensities of each marker within the identified clusters. **(C)** Frequencies of identified viSNE clusters, based on their identifying marker expression as indicated, were compared on individual levels via FlowJo analysis. Bars represent median frequency of proliferated CD4$^+$ T cells with interquartile range. Cluster proportions in youngsters and older adults as indicated identified by vISNE analyses. **(D)** Heatmap showing Arcsinh-transformed median expression of markers within each cluster of the viSNE plots of youngsters and older adults. **(E)** Frequencies of CD127$^-$FoxP3$^-$Helios$^+$ proliferated CD4$^+$ T cells in youngsters and older adults. Statistical significance was calculated with Mann Whitney *U*-test. * = $p < 0.05$, ** = $p < 0.01$.

the Bp infection. Comparable IFNγ responses were found between age groups after FHA and PRN stimulation. This may be explained by differences in immunogenicity or *in vivo* expression of these antigens, or the fact that they are less unique for Bp than PT (Ashworth et al., 1982; Novotny et al., 1991). Extended analyses of cytokines in culture supernatants indicated that in the capacity of infected youngsters and older persons to produce a wide range of other Th cytokines, age again impacting the magnitude. Both in the early and late phase after clinical Bp infection, lower levels of produced cytokines, including IL-2, TNF-α, IL-5 and IL-13, were found with increasing age. The reduced cytokine levels with older age were found regardless of the type of stimulation or technical approach, and this was already apparent in the relatively younger adult participants from the SKI study and was confirmed in older adults of the Immfact study. Our approach and available clinical

material in this study did not allow for intracellular cytokine analysis to identify cytokine producing cells. Although $CD8^+$ T cells were shown by Dirix *et al.* to contribute to IFNγ responses to FHA stimulation, $CD4^+$ T cells were regarded the main source (Dirix et al., 2012). In our study reduced IFNγ responses were also found against the Bp132 peptide pool, optimized for MHC class II restricted T-cell responses, and besides IFNγ other Th-type cytokine responses were found to be impacted by age. Therefore we regard $CD4^+$ T cells as the main cell type responsible for the cytokine profiles observed. Further research is needed to elucidate mechanisms of immunosenescence of cytokine production or polyfunctionality at the single Bp-specific $CD4^+$ T-cell level. Altogether, our data corroborate with a smaller maintenance and size of the Bp-specific $CD4^+$ memory T-cell response, leading to reduced functionality at older age.

Reduced ($CD4^+$) T-cell proliferative responsiveness is a hallmark of immunosenescence (Jiang et al., 2007). In the flow-based part of our study, we found lower frequencies of proliferated $CD4^+$ T cells in response to *in vitro* Bp132 and PT S1 peptide pool stimulation in the early phase after clinical Bp infection at older age, but cells did not seem to differ in fold-expansion while proliferating, compared to their younger counterparts. Age-related limitations in levels of secreted IL-2, a cytokine that drives the proliferative response of activated T cells (Boyman and Sprent, 2012), could be a factor determining a smaller population of Bp-specific $CD4^+$ T cells to clonally expand after activation. Our data were inconclusive with regards to this cytokine, since reduced IL-2 levels were measured in day-5 culture supernatants of PT-stimulated adult samples from the SKI-study, in contrast to Bp132 or PTS1-stimulated older adult samples from the Immfact study. CD25 expression on $CD4^+$ T cells was heterogenous across youngsters and older adults, yet not significantly different. Hence, the data do not indicate reduced availability of the IL-2 receptor alpha chain. As a mechanism for reduced $CD4^+$ T-cell responsiveness, accumulation of Tregs, main cells expressing high levels of CD25 and consuming IL-2 in human peripheral blood, at older age has been put forward (Gregg et al., 2005; Lages et al., 2008; van der Geest et al., 2014), although some studies find unaltered (Valmori et al., 2005; Hwang et al., 2009) frequencies of Tregs with higher age. In our study we did not observe an increased frequency of Tregs after clinical infection in older adults compared to youngsters, not favouring of a major role for Tregs in IL-2 consumption or reduced $CD4^+$ memory T-cell proliferation in older adults in our study.

Using hypothesis-driven and unbiased viSNE cluster analysis of the flow-cytometric dataset we explored the hypothesis that enhanced expression of co-inhibitory receptors CTLA-4, PD-1, and/or TIGIT on responding $CD4^+$ T cells at older age was associated with reduced Bp-specific $CD4^+$ T-cell responsiveness, as reported to explain impaired T-cell proliferation within the T-cell population at older age in mice and humans (Leng et al., 2002; Lages et al., 2010; Bengsch et al., 2018; Song et al., 2018). In contrast to our hypothesis, we found that phenotypical markers for T-cell activation and co-inhibition did not significantly differ between youngsters and older adults, instead proliferated Bp-specific $CD4^+$ T cells of youngsters had a higher MFI of CTLA-4 expression in response to Bp132, possibly in response to stronger activation. Notably, the proliferated population of youngsters also contained of a significantly higher frequency of $CD127^-FoxP3^-Helios^+$ cells, considered to represent ($CD4^+$) T-cell activation (Gregg et al., 2005; Lages et al., 2008). Thus, our data seem to indicate that immunosenescence observed in the proliferative responsiveness of Bp-specific $CD4^+$ T cells at older age is not associated with increased hallmarks of negative regulation but rather with decreased phenotypical markers that are associated with activation.

Our data suggest that immunosenescence in the Bp-specific $CD4^+$ T-cell response may occur already in relatively young adults. Likewise, in a cohort of 18–49 year old individuals, influenza-specific T-cell responses were shown to become less diverse and less cross-reactive with age (Subbramanian et al., 2010), a trend which extended into shorter-lived booster T-cell responses in a cohort of older adults (≥70 years old) vaccinees (Mahnke et al., 2011). We earlier documented reduced in Bp-specific proliferative responsiveness in ex-pertussis cases, using a 3H thymidine assay and whole PT or single PT and PRN $CD4^+$ T-cell epitopes as antigenic stimulation, starting at the age of 30 years (Han et al., 2013; van Twillert et al., 2015). Together our data now suggest that decline of responsiveness may reflect a gradual loss in the diversity of epitopes recognized.

Adults in these studies may have encountered *B. pertussis* antigens more frequently through natural exposure, considering the existing circulation. Repeated encounters with *B. pertussis* could possibly lead to end-stage differentiation of memory $CD4^+$ T cells with a CCR7-$CD27^-$phenotype, associated with shorter telomeres and less proliferative capacity than early-stage $CCR7^+CD27^+$ memory $CD4^+$ T cells (Fritsch et al., 2005). Also, inherent to the study design, in adults Bp-specific $CD4^+$ memory T cells primed through childhood vaccination experienced a substantially longer maintenance period preceding the clinical pertussis event, studied in this work, compared to their counterparts in the younger group. Together, these factors may confound effects of true immunosenescence, a limitation of these kind of clinical pathogen-specific and age-related clinical cohort studies.

Another limitation of our study is that the early phase clinical samples in the SKI and Immfact studies were taken only at a time point between 0.5 and 3 months post-diagnosis, respectively, likely missing peak levels reached during the expansion phase of specific $CD4^+$ memory T-cell subsets that could occur within days after infection. Instead, levels of responding cells represent the contraction phase as they are still elevated and further declining to long-term maintenance levels. In addition, sampling within the SKI study was cross-sectional and was variable with regards to median time elapsed since diagnosis between younger and older age cohorts, as opposed to Immfact sampling which was longitudinal and homogeneous in time frames between age cohorts. It is however unlikely that enhanced late phase cytokine production in younger SKI samples could be attributed to

differential sampling times, as the median late point sampling time of youngsters in the SKI study was longer after diagnosis compared to the adults. Additionally, the type of pertussis vaccine for primary vaccination, whole cell or acellular vaccine, is known to have a long-lasting impact on important hallmarks of subsequent Bp-specific $CD4^+$ T-cell immunity in response to recall (reviewed in (Ausiello et al., 2019), recently confirmed by (da Silva Antunes et al., 2021)). Primary vaccination for all patients in our study was with whole cell vaccine, however several Immfact youngsters received an additional aP booster dose at pre-school age. These subjects did not cluster for their cytokine or proliferative responses, but we cannot fully exclude the influence of this aP booster vaccination. Lastly, the observational natural infection studies SKI and Immfact provide unique cohorts of subjects to study hallmarks of Bp-specific $CD4^+$ T-cell immunity after recovery from a clinical infection. However, the number of ex-patients eligible for the selected age strata of this work was small in both clinical cohorts. Small group size may have limited the power to find age effects in our data. Further in depth research at the single Bp-specific $CD4^+$ cell level on additional samples are needed to confirm and extent these findings.

In addition to epidemiological studies, serosurveillance studies have highlighted (symptomatic) infections in the elderly as well (Hodder et al., 2000; de Greeff et al., 2010). High-dimensional evaluation of antibody levels following a Bp infection at older age compared to younger age indicates that antibody specificity, isotype and subclass of antibodies are all impacted by age and primary vaccination background (van Twillert et al., 2017). No correlate of protection has been established for pertussis, yet. The fact that pertussis occurs in all age groups, with underreporting in elderly, highlights the importance of further understanding the Bp-specific immune responses and changes therein with progressing age. As a possible intervention to enforce pertussis specific immunity, it was recently shown that older adults do mount both antibody and $CD4^+$ T-cell booster responses to an acellular pertussis booster dose (Lambert et al., 2020; Versteegen et al., 2021). Our study here, however, for the first time supports the hypothesis that Bp-specific memory $CD4^+$ T-cell immunity is subject to aging, possibly contributing to a more rapid loss of protective immune mechanisms with increasing age. For a more effective control of pertussis, also in the growing elderly population, induction and maintenance of protective $CD4^+$ T-cell responses to Bp should be better understood.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the accredited Review Board STEG, followed by management of the METC UMC Utrecht. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

IT, WH and CE designed the study. EL, IT, LB and MP performed experiments. EL and IT analysed the data. EL, IT, WH, DP and CE interpreted the data. EL, DP and CE edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fragi.2021.737870/full#supplementary-material

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