IMMUNOLOGY OF AGING

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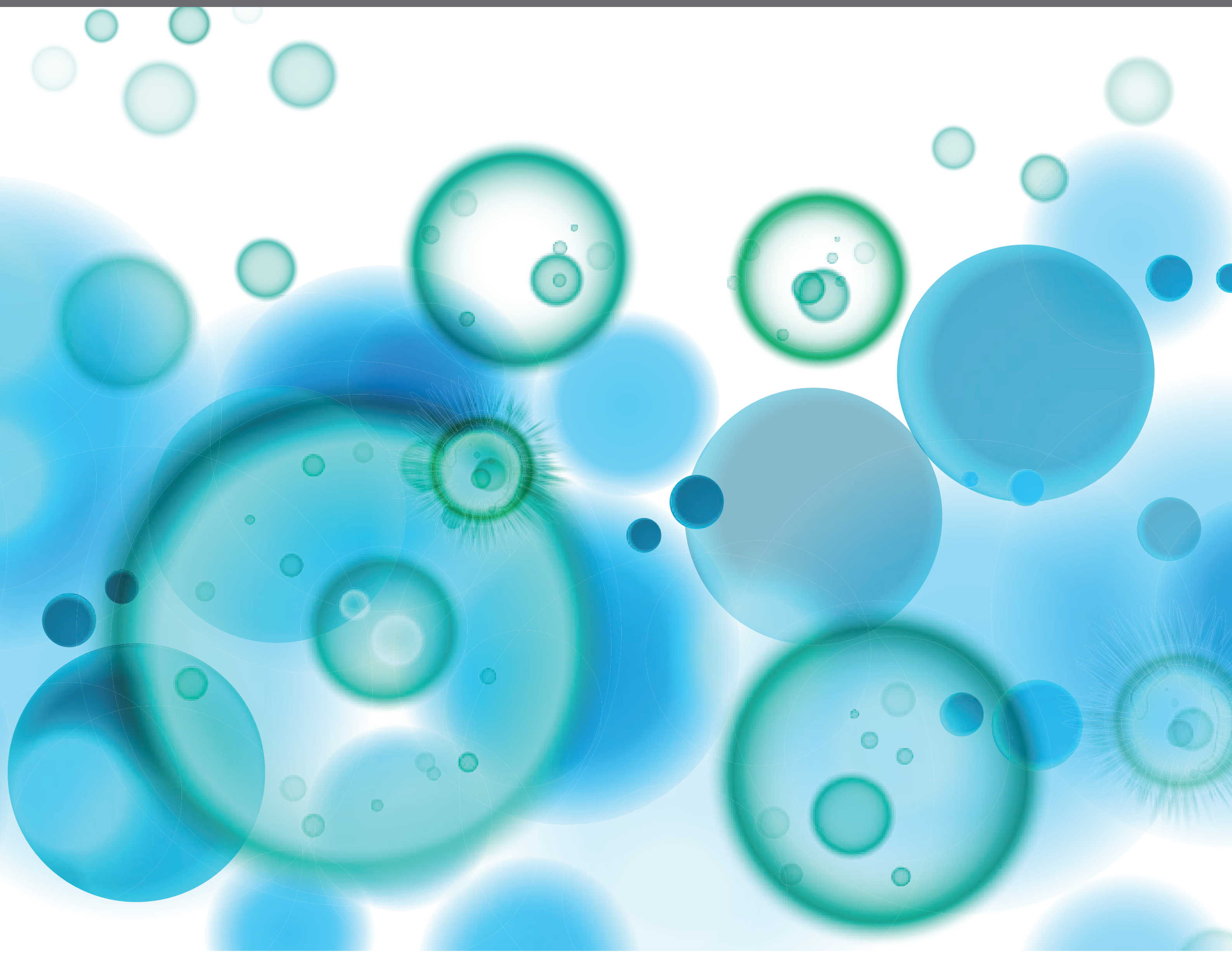

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IMMUNOLOGY OF AGING

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EDITORIAL
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Editorial: Immunology of Aging

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Keywords: immunosenescence, immunogerontology, aging, adaptive immunity, innate immunity

Editorial on the Research Topic

Immunology of Aging

Aging represents a paradox of immunodeficiency and inflammation (inflamm-aging) and autoimmunity (1). Over the lifespan there are changes in the architecture and functioning of the immune system often termed "Immunosenescence." Recently, there have been major developments in understanding the cellular and molecular bases, and genetic and epigenetic changes, in the innate and the adaptive immune system during aging, and the interactions between these separate arms of vertebrate immunity. The 13 papers in this collection "Immunology of Aging" represent a wide range of investigations by prominent experts in the field, focusing primarily on human aging and disease.

Limited longitudinal studies have begun to reveal biomarkers of immune aging, which may be considered to constitute an "immune risk profile" (IRP) predicting mortality and frailty in the very elderly, as first established in the pioneering Swedish OCTO study (2). Hallmark parameters of the IRP may also be associated with poorer responses to vaccination. The usually asymptomatic infection with the widespread persistent beta-herpesvirus HHV5 (Cytomegalovirus, CMV) has an enormous impact on these immune biomarkers, but according to the circumstances and depending on what is measured, this can translate into a detrimental or a beneficial effect (3). The prevalence of CMV infection in populations in industrialized countries increases with age, and within individuals the degree of immune commitment to anti-CMV responses also increases with age. This may cause pathology by maintaining higher systemic levels of inflammatory mediators ("inflammaging") and decreasing the "immunological space" available for immune cells with other specificities, or it may exert beneficial "adjuvant-like" effects. Modalities to prevent or reverse immunosenescence may therefore need to include targeting infectious agents such as CMV in a robustly personalized manner.Because of the increasing recognition that CMV has a marked impact on immune parameters commonly associated with age, it is crucial to dissect out whether age or CMV is responsible for altering biomarkers predictive of health status (e.g., frailty) or other important parameters such as response to vaccination (especially seasonal influenza). Hence several of the papers in this collection focus on the effects of CMV on immune parameters.

In their original article, Jackson et al. explore whether T cell responsiveness to a range of CMV proteins is different in younger and older healthy people and whether relaxation of anti-CMV immunosurveillance in later life could contribute to disease. They found that CMV-specific CD4+

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T cells secreting the anti-inflammatory cytokine IL 10 were predominantly directed to latency-associated CMV proteins and that these responses were not greater in the elderly than the young. However, the frequency of IFN-γ-secreting CD4+ T cells correlated with latent viral genome copy number in monocytes. They conclude that viremia is rare in the elderly due to the maintenance of T cell responsiveness but that CMV can be an important comorbidity factor in people who are not perfectly healthy and that this could be reflected in the IL 10:IFN-γ ratio of CD4+ anti-CMV T cell responses (Jackson et al.). The potential importance and possible prognostic utility of the IL 10:IFN-γ ratio is also emphasized in the paper by Merani et al. on responsiveness to influenza. This review article summarizes the impact of aging and CMV infection on immune cell function, the response to influenza infection and vaccination, and how the current understanding of aging and CMV can be used to design a more effective influenza vaccine for older adults which will also need to focus on generating appropriate T cell responses, as illustrated in the paper by Jackson et al. Mouse models may also offer insight into how to improve seasonal influenza vaccine responsiveness, as illustrated by Bartley et al. in their consideration of the impact of the gut microbiome and caloric restriction on sublethal influenza infection.

Further complications in analyzing the impact of CMV may arise because most human data are derived from studies using peripheral blood. However, as illustrated by Pangrazzi et al. the bone marrow harbors large amounts of late-stage differentiated CD8 T cells possibly because the production of IL 15 is greater in CMV-infected individuals; this does parallel what is seen in the peripheral blood. Finally, the original article by Hassouneh et al. describes detailed phenotyping results for all peripheral T cells, including CD4+, CD8+, CD4CD8-double negative and NKT cells as well, showing subtle differences between the expression of some surface molecules. For example, expression of the NK-associated receptor CD161 is similar in CMV-seropositive and seronegative young subjects but is different in the elderly, illustrating that CMV effects may be different at different ages. In fact, late-stage differentiated T cells, especially CD8+ T cells (often described in the literature as "senescent") commonly express surface molecules expressed by (non-T) NK cells. Such receptors include CD85j, which is discussed by Gustafson et al. as an important checkpoint regulator controlling the expansion of CMV-specific CD8+ T cells during aging.

The large accumulations of CMV-specific T cells, also in the bone marrow, may contribute to the state of inflammaging, but it is likely that other immune (and non-immune) cells are also major contributors. Cells of the innate immune system far outnumber those of adaptive immunity and may also be heavily influenced by the presence of CMV, contributing to inflammaging, as discussed by Franceschi et al. in the context of "trained innate immunity". An evolutionarily more recent persistent virus, HIV, may have some very similar effects, as also discussed in this paper which suggests that the sum total of a person's prior exposures and immunological memory therefor, dubbed the "immunobiography" mostly determines their later-life immune status (Franceschi et al.). The impact of HIV itself is the subject of the original article by Kirk et al. on serum inflammatory mediators in HIV-infected-vs.-non-infected subjects, concluding that chronic infection with HIV, despite its pharmaceutical control, exacerbated the age-associated higher levels of mediators like IL 6 and CRP.

The generally harmful effects of chronic inflammation are not limited to those mediators produced as a result of chronic infection. The article by Frasca and Blomberg starkly illustrates the immune and inflammatory impact of obesity on many health parameters, including decreased and dysregulated B cell function and antibody production as well as inflammaging. Not only does immune dysregulation contribute to inflammaging, but chronicity of inflammatory exposure, as opposed to the necessity of acute inflammation for immune response generation, also negatively influences immune function. Mechanisms whereby this detrimental effect could be mediated are discussed in the review article by Jose et al. focusing on telomere maintenance and telomerase expression. Heightened chronic inflammatory status is also likely to affect innate immune cells as well as the T and B cell effects discussed by Frasca and Blomberg. Much interest in the context of cancer and immunotherapy has recently been raised by studies on so-called myeloid-derived suppressor cells (MDSCs) which may also be relevant in other situations. This is discussed in the context of Alzheimer's Disease by Le Page et al. suggesting that in this instance, unlike in cancer, they may play a positive role. Another type of myeloid cell, essential for the functioning of adaptive immunity in its role as "professional" antigen-presenting cell, may be affected by inflammaging and may itself contribute to this state. Thus, as reviewed by Agrawal et al. dendritic cells (DCs) from older subjects secrete more pro-inflammatory and less anti-inflammatory cytokines and are altered in many other ways, contributing to dysregulated immunity. A further problem related to DC function and antigen presentation relates to the architecture and functionality of the lymph node in elderly subjects, where adaptive immune responses are triggered. As reviewed by Thompson et al. attempts to modulate immunity in the elderly and restore appropriate immune function would need to address this important "checkpoint" too.

The papers collected in this series illustrate only some of the many facets of immune aging, a rapidly developing field now being increasingly recognized as central not only to immune function *per se* in the elderly, but to their general condition of health or frailty.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Latent Cytomegalovirus (CMV) Infection Does Not Detrimentally Alter T Cell Responses in the Healthy Old, But Increased Latent CMV Carriage Is Related to Expanded CMV-Specific T Cells

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Human cytomegalovirus (HCMV) primary infection and periodic reactivation of latent virus is generally well controlled by T-cell responses in healthy people. In older donors, overt HCMV disease is not generally seen despite the association of HCMV infection with increased risk of mortality. However, increases in HCMV DNA in urine of older people suggest that, although the immune response retains functionality, immunomodulation of the immune response due to lifelong viral carriage may alter its efficacy. Viral transcription is limited during latency to a handful of viral genes and there is both an IFNγ and cellular IL-10 CD4+ T-cell response to HCMV latency-associated proteins. Production of cIL-10 by HCMV-specific CD4+ T-cells is a candidate for aging-related immunomodulation. To address whether long-term carriage of HCMV changes the balance of cIL-10 and IFNγ-secreting T-cell populations, we recruited a large donor cohort aged 23–78 years and correlated T-cell responses to 11 HCMV proteins with age, HCMV IgG levels, latent HCMV load in CD14+ monocytes, and T-cell numbers in the blood. IFNγ responses by CD4+ and CD8+ T-cells to all HCMV proteins were detected, with no age-related increase in this cohort. IL-10-secreting CD4+ T cell responses were predominant to latency-associated proteins but did not increase with age. Quantification of HCMV genomes in CD14+ monocytes, a known site of latent HCMV carriage, did not reveal any increase in viral genome copies in older donors. Importantly, there was a significant positive correlation between the latent viral genome copy number and the breadth and magnitude of the IFNγ T-cell response to HCMV proteins. This study suggests in healthy aged donors that HCMV-specific changes in the T cell compartment were not affected by age and were effective, as viremia was a very rare event. Evidence from studies of unwell aged has shown HCMV to be an important comorbidity factor, surveillance of latent HCMV load and low-level viremia in blood and body fluids, alongside typical immunological measures and assessment of the antiviral capacity of the HCMV-specific immune cell function would be informative in determining if antiviral treatment of HCMV replication in the old maybe beneficial.

Keywords: human cytomegalovirus, immunology of aging, viral latency, human cytomegalovirus-specific T-cells, IFNγ production, cIL-10+CD4+ T cells, latent viral load

INTRODUCTION

A consequence of aging in the human population is a decline in immune function, often described as immune senescence, which includes a loss of adaptive immune cells and an increase in inflammatory cytokines resulting in dysregulation of the immune response (1). There is now evidence from a number of studies that, after the age of 65 years, the age-associated loss of immune function results in individuals becoming more susceptible to infectious diseases as well as increased morbidity and mortality from autoimmune disorders (2, 3). Infection with human cytomegalovirus (HCMV) is characterized by its lifelong persistence in the infected individual due, in part, to its ability to establish a latent infection in bone marrow stem cells and myeloid cells (4). Despite a robust immune response to the primary infection, the large number of immune evasion molecules encoded by HCMV allows it to establish its latent life cycle (5). Primary HCMV infection and reactivation from latency is generally well controlled in healthy individuals; however, when the immune system is compromised, or under developed, it can become a significant problem (6, 7). A potential impact of lifelong persistence of HCMV is its effect on the host immune response with aging. A number of longitudinal and population cohort studies have suggested that HCMV seropositivity was linked to age-related (i) increase in susceptibility to infections, (ii) poor response to vaccinations, and (iii) increased risk of all-cause mortality compared to age-matched HCMV seronegative individuals—which has been termed the immune risk phenotype (IRP) (8–13). Analysis of a number of large population cohorts recruited for cancer, dementia, and nutritional studies in the UK and USA have also shown a significant association between HCMV seropositivity and mortality from cardiovascular related disease (14–18). However, other studies have shown no such age-related correlation between HCMV seropositivity and declines in immune responses to either novel infections (19, 20) or responses to vaccination (21). Similarly, a study measuring frailty in older people saw a positive association with inflammatory cytokines but not HCMV infection (22) perhaps consistent with studies that have shown that rises in inflammatory cytokines in the serum of older donors is not primarily driven by HCMV (23).

It has been observed that infection with HCMV changes the composition of the $CD4^+$ and $CD8^+$ memory T cell repertoires; this includes an expansion of the T cell population, which have lost expression of the co-stimulatory molecules CD27 and CD28 but also show re-expression of CD45RA and co-expression of the carbohydrate HNK-1 (CD57) [reviewed in Ref. (24)]. Such T cells are considered to be a highly differentiated phenotype (25), and potentially dysfunctional as they often lose the ability to secrete cytokines and have limited proliferative capacity (11, 26). It has been suggested that expanded populations of highly differentiated T cells in HCMV seropositive older donors may be detrimental to the infected individual (27–29). However, such increases in these highly differentiated T cells is also observed in young HCMV positive individuals (30) and it is, also, now clear that these highly differentiated T cells are still functional and, with the correct co-stimulation, can proliferate (31, 32). Similarly, HCMV-specific T cells have been shown to produce multiple antiviral cytokines and have efficient cytotoxic capacity despite a highly differentiated phenotype (33–35). Furthermore, older HCMV seropositive individuals do not appear to suffer from overt HCMV disease from reactivating virus or HCMV re-infection which suggests that the immune response of older people retains the ability to control virus replication (36). Despite older HCMV seropositive donors having functional HCMV-specific immune responses, there does appear to be age-related increases in levels of viral DNA detectable in urine (36) and blood (37). This suggests that the immune response in older people may be altered, possibly due to lifelong carriage of the virus, and that immunomodulation of the HCMV-specific immune response, as either a direct consequence of the viral infection or bystander effects, results in reduced clearance of reactivating virus in older people (5).

Latent carriage of HCMV in $CD34^+$ progenitor cells and their myeloid derivatives is characterized by repression of viral immediate Early (IE) gene transcription with a restricted gene expression profile, which cannot support production of infectious virus. A number of viral genes have been identified as being transcribed during HCMV latent infection, including UL138 (38), LUNA (latent undefined nuclear antigen; UL81-82as) (39, 40), US28 (41), UL111A (vIL-10) (42), and UL144 (43). Analysis of the secreted cellular proteins (cell secretome) of experimentally latently infected $CD34^+$ and $CD14^+$ cells have identified the induced expression of chemokines, which can recruit T cells as well as the cellular cytokines IL-10 and TGF-β, both of which can modulate the activity of T cells which have migrated to the environment surrounding the latent infection (44). HCMV-specific $CD4^+$ T cells have been identified that either secrete cIL-10 or have a regulatory cell phenotype (45–49) and, in the mouse, it has been shown that $CD4^+$ T regulatory cells (T_{regs}) and IL-10 secretion can reduce viral clearance and increase persistence in murine cytomegalovirus (MCMV) (49, 50). Additionally, there is evidence that the frequency of HCMV-specific inducible T_{regs} is increased in older individuals (47), alongside an overall increase in frequency of T regulatory cells in old age (51, 52). Previously, we have identified $CD4^+$ T cells specific for peptides to two of the latency-associated proteins, UL138 and LUNA, which secrete cIL-10 and also possess Th1 antiviral effector functions (53). We have also shown that the UL138-specific $CD4^+$ T cells recognize experimentally latently infected $CD14^+$ monocytes, secrete cIL-10, and suppress T cell function.

With these observations in mind, we hypothesized that the long-term carriage of HCMV could create an immunomodulatory environment to help prevent clearance of the virus by skewing the $CD4^+$ T cell compartment toward a suppressive or regulatory cIL-10-producing phenotype. We also wanted to assess whether the same environment had an impact on the frequency of HCMV-specific $CD8^+$ T cells within a large old aged donor cohort, who have carried HCMV for longer compared to younger seropositive donors. Additionally, within the study, we wanted to measure the levels of latent viral genome carriage and determine if infectious virus was detectable and relate this to changes in the T cell response. To address these questions, we conducted a study on a large healthy donor cohort, which encompassed a broad age range (23–78 years) of both HCMV seropositive and negative donors. We performed absolute cell counts, measured

HCMV-specific antibody levels, assayed viral genome copy number in total peripheral blood and in CD14+ cells, as well as measuring the CD8+ specific production of IFNγ and CD4+ specific production of IFNγ and IL-10 in response to stimulation by overlapping peptide pools to 11 HCMV proteins (5 latency associated and 6 lytic only expressed proteins). The study group exhibited typical age-related decline in both absolute CD4+ and CD8+ naïve T cell numbers and HCMV seropositive donors had increased absolute numbers of T cells with a differentiated phenotype compared to seronegative donors. We did not see an inversion of the CD4:CD8 ratio within this donor cohort, a characteristic associated with the IRP, although CD4:CD8 ratio was decreased in HCMV seropositive donors compared to seronegative. In contrast to studies in other donor cohorts, we did not see an age-related expansion of the HCMV IgG response or an influence of donor age on either the breadth or magnitude of the T cell responses (24, 54). We detected both CD4+ and CD8+ specific IFNγ responses to all 11 HCMV proteins analyzed and also detected more limited CD4+ specific IL-10 responses to the same proteins, and we also confirmed our previous observations that CD4+ specific IL-10 responses are more common toward latency-associated proteins. We were able to detect latent HCMV genomes in isolated peripheral blood CD14+ monocytes in 45% of donors but, in contrast to previous reports (54), we did not observe an increase in HCMV copy number in donors aged over 70 years old. Importantly, we did see a significant association between the levels of HCMV detected in CD14+ monocytes and both the breadth and magnitude of the CD8+ T cell responses to HCMV proteins, irrespective of donor age. Overall it is our opinion that larger latent HCMV reservoirs will lead to increased HCMV reactivation and dissemination events, which in normal healthy individuals will stimulate secondary HCMV-specific T cell responses, thus driving increases in T cell frequency and differentiation status.

MATERIALS AND METHODS

Ethics and Donor Cohort Information

The study donor cohort was recruited by the National Institute of Health Research Cambridge BioResource, using their Biobank of volunteers, who predominantly are local to Cambridge or live in the East Anglian Region of the UK. Ethical approval was obtained from University of Cambridge Human Biology Research Ethics Committee. Informed written consent was obtained from all donors in accordance with the Declaration of Helsinki (HBREC.2014.07). Known HCMV seropositive and seronegative donors were recruited in three age groups; young (18–40 years), middle (41–64 years), and old (65+ years) were included in this study. Volunteers being treated with oral or intravenous immunomodulatory drugs (including steroids, tacrolimus, cyclosporins, azathioprines, mycophenolate, methotrexate, rituximab, and cyclophosphamide) within the last 3 months, undergoing injected rheumatoid arthritis treatment including anti-TNFα agents and anyone actively, or within the last 24 months, being treated with cancer chemotherapy were excluded from the study. 119 HCMV seropositive and seronegative donors were included in this study, the age range of the recruited donor cohort was 23–76 years, 70 donors were female and 49 donors were male. Further characteristics of the studied donor cohort are detailed in **Table 1**. In total, a 50 ml peripheral blood sample was collected from each donor, comprising 1.2 ml clotted blood, 1.2 ml EDTA treated blood, and 47.6 ml lithium heparin treated blood samples.

Peripheral Blood Mononuclear Cell (PBMC) Isolation

Peripheral blood mononuclear cells were isolated from the heparinized blood samples using Lymphoprep (Axis-shield, Oslo, Norway) density gradient centrifugation.

TABLE 1 | ARIA cohort donor characteristics.

		All ages		Young (<40 years)	Middle (41–64 years)	Old (>65 years)
		Human cytomegalovirus (HCMV) +ve	HCMV −ve	HCMV +ve	HCMV +ve	HCMV +ve
Donors (*n*)	All	105	14	33	31	41
	M	44	5	14	14	16
	F	61	9	19	17	25
Age (years) (mean ± SD)	All	54.4 ± 15.6	51.4 ± 14.4	34.6 ± 5.1	54.5 ± 6.0	70.2 ± 3.1
	M	54.3 ± 16.1	46.2 ± 12.7	34.7 ± 5.5	53.9 ± 6.5	71.7 ± 2.8
	F	54.5 ± 15.3	54.3 ± 14.4	34.6 ± 4.8	54.9 ± 5.5	69.3 ± 2.8
HCMV IgG (ISR) (mean ± SD)	All	3.78 ± 1.28	0.28 ± 0.14	3.66 ± 1.32	3.81 ± 0.99	3.85 ± 1.42
	M	3.67 ± 0.95	0.25 ± 0.10	3.15 ± 0.74	4.06 ± 0.74	3.78 ± 1.06
	F	3.86 ± 1.47	0.29 ± 0.15	4.03 ± 1.51	3.60 ± 1.12	3.90 ± 1.61
HCMV DNAemia (copies/ml blood) (mean ± SD)	All	2.6 ± 26.7[a]	Undetected	Undetected	Undetected	6.7 ± 42.4[a]
	M	6.3 ± 41.0[a]	Undetected	Undetected	Undetected	17.2 ± 66.6[a]
	F	Undetected	Undetected	Undetected	Undetected	Undetected
CD4:8 ratio (mean ± SD)	All	2.25 ± 1.61	3.60 ± 1.80	2.04 ± 0.85	1.96 ± 0.85	2.63 ± 2.29
	M	2.10 ± 1.00	4.00 ± 2.10	1.80 ± 0.70	2.00 ± 1.00	2.30 ± 1.20
	F	2.40 ± 1.90	3.40 ± 1.50	2.20 ± 0.90	1.90 ± 0.70	2.90 ± 2.80

Summary of the number of donors and age ranges, serum HCMV IgG levels [immune status ratio (ISR)], blood HCMV DNA copies and the CD4:CD8 ratio (generated from absolute count data).

[a]HCMV DNAemia detected in n = 1 old male donor.

Absolute Count Protocol

50 µl of the EDTA treated whole blood sample was transferred to Becton Dickinson Trucount tubes (BD Biosciences, Oxford, UK) and stained with a pre-mixed antibody cocktail containing CD45-VioBlue, CD3-VioGreen (Miltenyi Biotec, Bisley, UK.), CD4-Brilliant Violet 605, CD8-PerCP-Cy5.5, CD28-PE, CD27-APC-Cy7, CD45RA-FITC, CD25-APC, and CD127-PE-Cy7 (BioLegend, San Diego, CA, USA). Following staining, the red blood cells were lysed and the cells fixed using FACS Lysing solution (BD Biosciences). The samples were stored at −80°C until acquisition (55). Samples were acquired on a LSR Fortessa (BD Biosciences) along with Fluorescence Minus One (FMO) controls using FACS Diva software (BD Biosciences). Samples were then analyzed using FlowJo software (Treestar, OR, USA), first the trucount bead population was identified and then the trucount bead negative population (i.e., cells) were analyzed by gating for single cells, then $CD45^{hi}$ lymphocytes, $CD3^+$ T cells, $CD4^+$ and $CD8^+$ expressing cells. The $CD4^+$ and $CD8^+$ T cell populations were further subdivided into four memory populations defined by expression of CD27 and CD45RA, and four differentiation populations defined by expression of CD27 and CD28 were identified, and in $CD4^+$ T cells, a T_{reg} population defined as $CD25^{hi}$ and $CD127^{lo}$ were identified, gate and quadrant positions were identified using the FMO controls. A representative gating strategy and the formula used to calculate the absolute cell counts is illustrated in Figure S1 in Supplementary Material, the event number for all populations, and trucount beads were exported to an excel sheet where the number of cells per microliter of blood for each T cell subset was calculated according to manufacturer instructions.

HCMV IgG Antibody Levels Protocol

Human cytomegalovirus serostatus was confirmed using serum from the clotted blood sample and HCMV IgG levels determined using an IgG enzyme-linked immunosorbent (EIA) assay, HCMV Captia (Trinity Biotech, Didcot, UK) following manufacturer's instructions, on serum derived from clotted blood samples. The EIA assay is semi-quantitative, containing negative, positive and calibrator controls which allow the computation of an immune status ratio (ISR) value for the amount of HCMV IgG present in the sample. In addition to the manufacturer controls and quality control protocols, a known positive serum sample was also run to check inter-assay variability was acceptable.

HCMV ORF Peptide Mixes

8 HCMV ORF encoded proteins [UL55 (gB), UL82 (pp71), UL122 (IE2), UL123 (IE1), US3, UL138, US28, and UL111A(vIL-10)] were selected and peptide libraries comprising consecutive 15mer peptides overlapping by 10 amino acid were synthesized by ProImmune PEPScreen (Oxford, UK) from sequences detailed in the Sylwester et al. study (56). A further 3 HCMV ORF encoded proteins [UL83 (pp65), UL144 (which incorporated known strain variants) and LUNA (UL81-82as)] 15mer peptide libraries were synthesized by JPT Peptide Technologies GmbH (Berlin, Germany). The individual lyophilized peptides from each ORF library were reconstituted and used as previously described (57).

Depletion of $CD4^+$ and $CD8^+$ T Cells from PBMCs

Peripheral blood mononuclear cells were depleted of either $CD4^+$ or $CD8^+$ T cells by MACS using anti-$CD4^+$ or anti-$CD8^+$ direct beads (Miltenyi Biotec), according to manufacturer's instructions, and separated on either LS columns (Miltenyi Biotec) or by using an AutoMACS Pro (Miltenyi Biotec). Efficiency of depletion was determined by staining cells with a CD3-FITC, CD4-PE, and CD8-PerCPCy5.5 antibody mix (all BioLegend) and analyzed by flow cytometry. Depletions performed in this manner resulted in mean 3.8% residual $CD8^+$ T cells and 8.6% residual $CD4^+$ T cells (from $n = 61$ donors).

Dual FluoroSpot Assays

2×10^5 PBMC depleted of either $CD8^+$ or $CD4^+$ T cells suspended in X-VIVO 15 (Lonza, Slough, UK) supplemented with 5% Human AB serum (Sigma Aldrich) were incubated in pre-coated FluoroSpot plates [Human IFNγ and IL-10 FluoroSpot (Mabtech AB, Nacka Strand, Sweden)] in triplicate with ORF mix peptides (final peptide concentration 2 µg/ml/peptide) and an unstimulated and positive control mix [containing anti-CD3 (Mabtech AB), Staphylococcus Enterotoxin B, Phytohemagglutinin, Pokeweed Mitogen, and Lipopolysaccharide (all Sigma-Aldrich)] at 37°C in a humidified CO_2 atmosphere for 48 h. The cells and medium were decanted from the plate and the assay developed following the manufacturer's instructions. Developed plates were read using an AID iSpot reader (Oxford Biosystems, Oxford, UK) and counted using AID EliSpot v7 software (Autoimmun Diagnostika GmbH, Strasberg, Germany) using distinct counting protocols for IFNγ and IL-10 secretion. Donor results were discounted from further analysis if there was greater than 1,000 spot forming units (sfu) background secretion of IFNγ or IL-10 in the unstimulated wells, additionally the sfu response in the positive control wells had to be at least 100 sfu (IFNγ) or 50 sfu (IL-10) greater than the background sfu. All data were then corrected for background cytokine production and the positive response cutoff for IFNγ and the IL-10 responses was determined by comparing the distribution of the responses from HCMV seropositive and seronegative donors to all HCMV proteins and the positive control. This analysis determined that the positive response for IFNγ and IL-10 was greater than 100 sfu/million, this threshold is indicated in **Figures 3A**, **4A** and **5A** (dashed line).

Measurement of HCMV DNAemia in Whole Blood

A 1 ml EDTA treated whole blood sample was stored at −20°C for each donor. DNA was isolated from the whole blood sample using the QIAamp DNA Blood Midi Kit (Qiagen, Manchester, UK) following the manufacturer's instructions. Extracted DNA samples were stored at −20°C until required. The detection of HCMV by real-time quantitative PCR method using the StepOne Real-Time PCR system (Applied Biosystems, ThermoFisher Scientific) was performed using a method adapted from Ref. (58). Real-time amplification of HCMV DNA used glycoprotein B-specific primers [5′-GAGGACAACGAAATCCTGTTGGGCA-3′ [gB1] and

5′-GTCGACGGTGGAGATACTGCTGAGG-3′ [gB2] (59)], and detection with a TaqMan probe [5′ 6-FAM- CAATCATGCGT TTGAAGAGGTAGTCCA-BHQ1 3′ [gBP3] (58)] mixed with ABI Universal Mastermix (Applied Biosystems, ThermoFisher Scientific), the final assay volume was 25 µl, which includes a 5 µl donor or control sample. PCR cycling conditions were 2 min at 50°C, 10 min at 95°C, and 45 cycles of 15 s at 95°C and 60 s at 60°C, all donor samples were screened in duplicate with a high (50,000 copies/ml) and low (500 copies/ml) positive control samples [whole EDTA treated blood spiked with HCMV genomes from the World Health Organization (WHO) international standard (60) (National Institute for Biological Standards and Control, Potters Bar, UK)], run in triplicate. Samples with detectable HCMV DNA were repeated in triplicate in a real-time amplification including a standard curve in triplicate of 1–10^4 HCMV genomes (WHO International Standard) in addition to the high and low positive controls. The HCMV DNA load was calculated using the StepOne Software (Applied Biosystems, ThermoFisher Scientific) and reported as HCMV copies per milliliter blood.

Latent Viral Load Digital PCR

$CD14^+$ monocytes were extracted using $CD14^+$ magnetic beads and MS columns (Miltenyi Biotec) from PBMC isolated from 20 ml of heparinized peripheral blood in a HCMV clean facility. The monocytes were enumerated, dry pelleted, and stored at −80°C prior to DNA extraction. DNA was extracted from the cell pellet in a 1:1 mixture of PCR solutions A (100 mM KCl, 10 mM Tris–HCl pH 8.3, and 2.5 mM $MgCl_2$) and B (10 mM Tris–HCl pH 8.3, 2.5 mM $MgCl_2$, 1% Tween 20, 1% Non-idet P-40, and 0.4 mg/ml Proteinase K) at a final concentration equivalent to 10,000 cells/µl, for 60 min at 60°C followed by a 10 min 95°C incubation (61), extracted DNA samples were stored at −80°C until required. Measurement of HCMV DNA in extracted $CD14^+$ cells was assessed using a droplet digital PCR method (54). Using the QX200 droplet digital PCR system (Bio-Rad, Watford, UK), a reaction mixture containing 2 µl of donor $CD14^+$ DNA (equivalent to 20,000 cells) or positive control sample was mixed with PCR grade water, 2× digital droplet PCR (ddPCR) supermix for probes (Bio-Rad), FAM labeled HCMV primer and probe (from Human CMV HHV5 kit for qPCR using a glycoprotein B target, PrimerDesign, Southampton, UK) and HEX labeled RPP30 copy number assay for ddPCR (Bio-Rad). Droplets were generated with droplet generation oil (Bio-Rad) in the QX200 droplet generator (Bio-Rad), then the sample was loaded into a 96-well PCR plate (Eppendorf, Stevenage, UK), sealed with a PX1 PCR Plate sealer (Bio-Rad) and PCR amplification was performed using a C1000 Touch Thermocycler (Bio-Rad), for 10 min at 95°C followed by 40 cycles of 30 s at 94°C and 60 s at 60°C. Following PCR amplification, the PCR plate was loaded onto the QX200 Droplet Reader (Bio-Rad) where the presence or absence of PCR product in each droplet was read and analyzed by QuantaSoft software (Bio-Rad), which gives the result of the number of virus copies per microliter of PCR reaction. All donor $CD14^+$ DNA samples were run in either quadruplicate or triplicate. The RPP30 copy number primer probe enabled the determination of the cell number included in the reaction and the HCMV viral load number was adjusted according to this and expressed as HCMV copies per million $CD14^+$ cells.

Statistics

Statistical analysis was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA, USA). Correlation was assessed by Pearson or Spearman correlation according to the distribution of the data. Multiple data sets groups were compared using a one-way ANOVA Kruskal–Wallis test with *post hoc* Dunn's multiple comparisons or selected Mann–Whitney U comparisons using an adjusted p value ($p \leq 0.05/n$ comparisons) to correct for multiple testing false discovery.

RESULTS

Characterization of the ARIA Study Donor Cohort

To determine whether long-term carriage of HCMV alters the HCMV-specific T cell response, with respect to cytokine secretion or state of T cell differentiation, and whether any identified changes impact on latent HCMV viral carriage and/or levels of HCMV IgG, we designed an age cross-sectional study. Donors were placed into three age groups: young (age ≤40 years), middle aged (age 41–64 years), and old (age ≥65 years) and also grouped on the basis of their HCMV serostatus. Potential donors were excluded from the study if they were currently taking, or had taken in the previous 3 months, any immunomodulatory or monoclonal antibody treatments or if they were currently cancer sufferers or had any form of cancer in the previous 24 months. In total, 119 individuals from the three age groups were included in this analysis: age range, virological and immunological parameters (HCMV IgG levels, HCMV DNA copies per milliliter whole blood and the CD4:CD8 ratio) for the donor cohort are detailed in **Table 1**. Correlation of the levels of HCMV IgG (ISR) (summarized for the three age groups in **Table 1**) within HCMV seropositive (HCMV +ve) donors with age did not show a significant accumulation with age {Pearson $r = 0.1012$ [95% confidence interval (CI): −0.0923, 0.2873], $p = 0.3043$}. Neither was there a significant decrease in the CD4:CD8 ratio within the HCMV +ve donor group with age [Spearman $r_s = 0.08563$ (95% CI: −0.1135, 0.2781), $p = 0.3851$].

The composition of the $CD8^+$ and $CD4^+$ T cell compartments, in whole blood isolated directly *ex vivo,* were enumerated and compared between donor age and HCMV serostatus. **Figure 1** summarizes the impact of increasing age on T cell numbers in the entire donor cohort. This analysis shows that both $CD8^+$ and $CD4^+$ T cell numbers significantly decrease with age (**Figure 1B**, Spearman $r_s = -0.255$, $p = 0.005$ and **Figure 1D**, Spearman $r_s = -0.207$, $p = 0.024$, respectively), which was likely due to the significant loss of naïve $CD8^+$ and $CD4^+$ T cells (**Figures 1C,E**) with no corresponding increase in numbers of memory T cell populations (Figure S2 in Supplementary Material). Enumeration of $CD4^+$ T regulatory cells present in the peripheral blood of all donors, based on the expression of CD127 and CD25 (62), showed that there was no effect of age on the size of this cell population (**Figure 1F**). When comparing the impact of HCMV infection, in donors of all ages, on the numbers of differentiated T cell subsets [representative donor phenotype staining is shown (**Figure 2A**)],

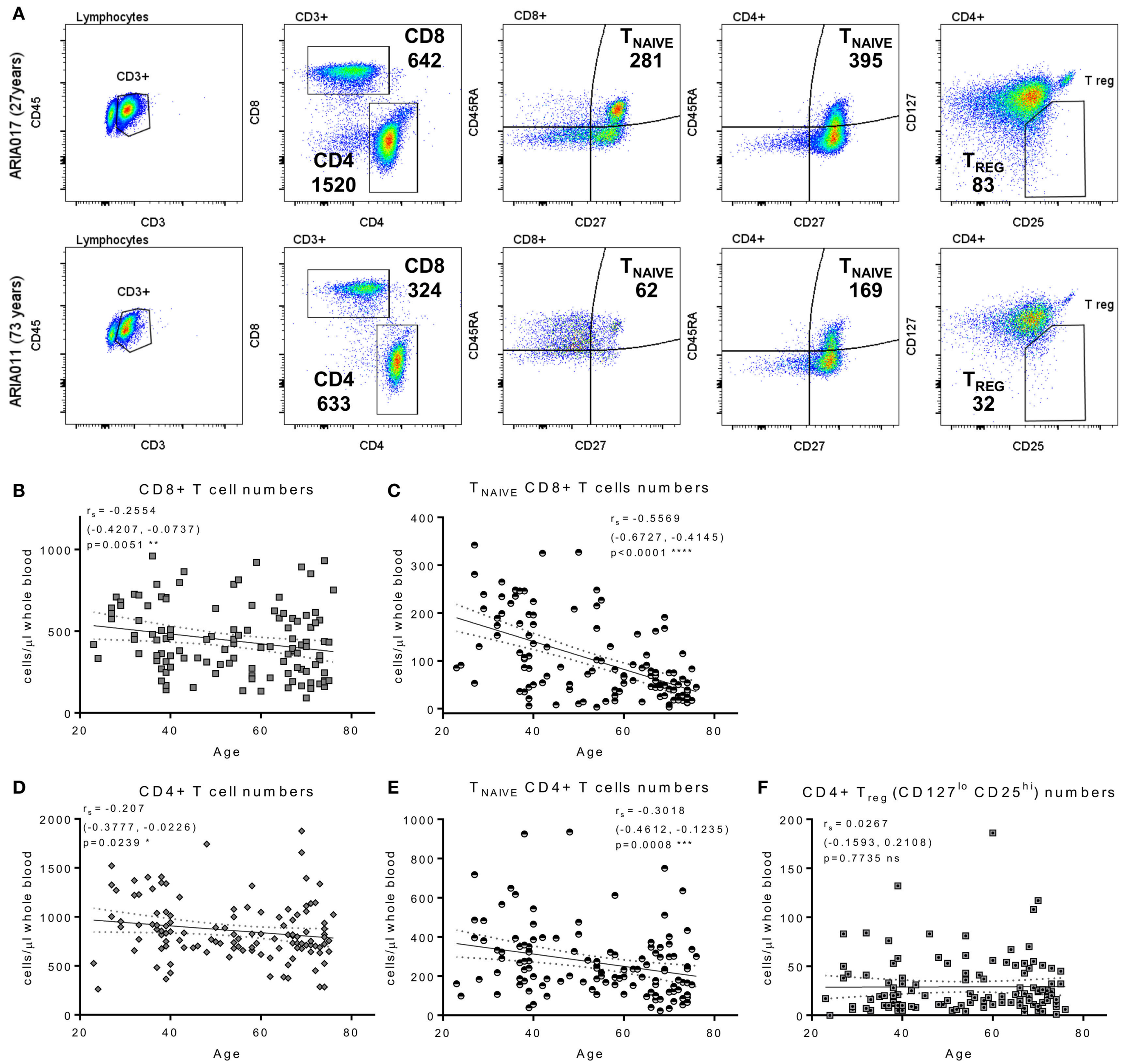

FIGURE 1 | Impact of aging on T cell numbers. EDTA treated whole blood was stained with a panel of phenotyping antibodies in order to enumerate CD4+ and CD8+ T cells and their subsets. Representative dot plots from a young and old donor showing CD4+ and CD8+ T cell gates, naïve T cells subset were defined by CD27+ and CD45RA+ (T_{NAIVE}) expression, and CD4+ T regulatory cells (T_{reg}—CD25^{hi}, CD127^{lo}); the number of cells per microliter of whole blood present for each gated population of interest are also indicated **(A)**. Graphs illustrating the numbers of total CD8+ T cells **(B)**, T_{NAIVE} CD8+ T cells **(C)**, total CD4+ T cells **(D)**, T_{NAIVE} CD4+ T cells **(E)**, and CD4+ T_{reg} cells **(F)** of the entire ARIA cohort (n = 119) correlated to donor age. The relationship of T cell subset numbers with donor age was analyzed using Spearman rank correlation with the results indicated on each graph [r_s (95% confidence interval) and p value]. There was a significant decrease in total and T_{NAIVE} CD4+ and CD8+ T cells with age, CD4+ T_{reg} numbers showed no significant difference.

we observed a significant expansion of the effector memory (T_{EM}—CD27⁻CD45RA⁻) population in both CD8+ (**Figure 2B**) and CD4+ T cells (**Figure 2D**). Within CD8+ T cells only, we also saw a significant increase in the highly differentiated T_{EMRA} (CD27⁻CD45RA+) and CD27⁻CD28⁻ (LATE) populations (**Figures 2B,C**), which was not observed in CD4+ T cells (**Figures 2D,E**). A key component of the IRP, which is associated with HCMV infection, is the inversion of the CD4:CD8 ratio (<1), we only saw this phenomenon in 10% of the seropositive donor group. However, we observed that overall the CD4:CD8 ratio was significantly decreased in HCMV seropositive donors compared to seronegatives (**Figures 2F,G**).

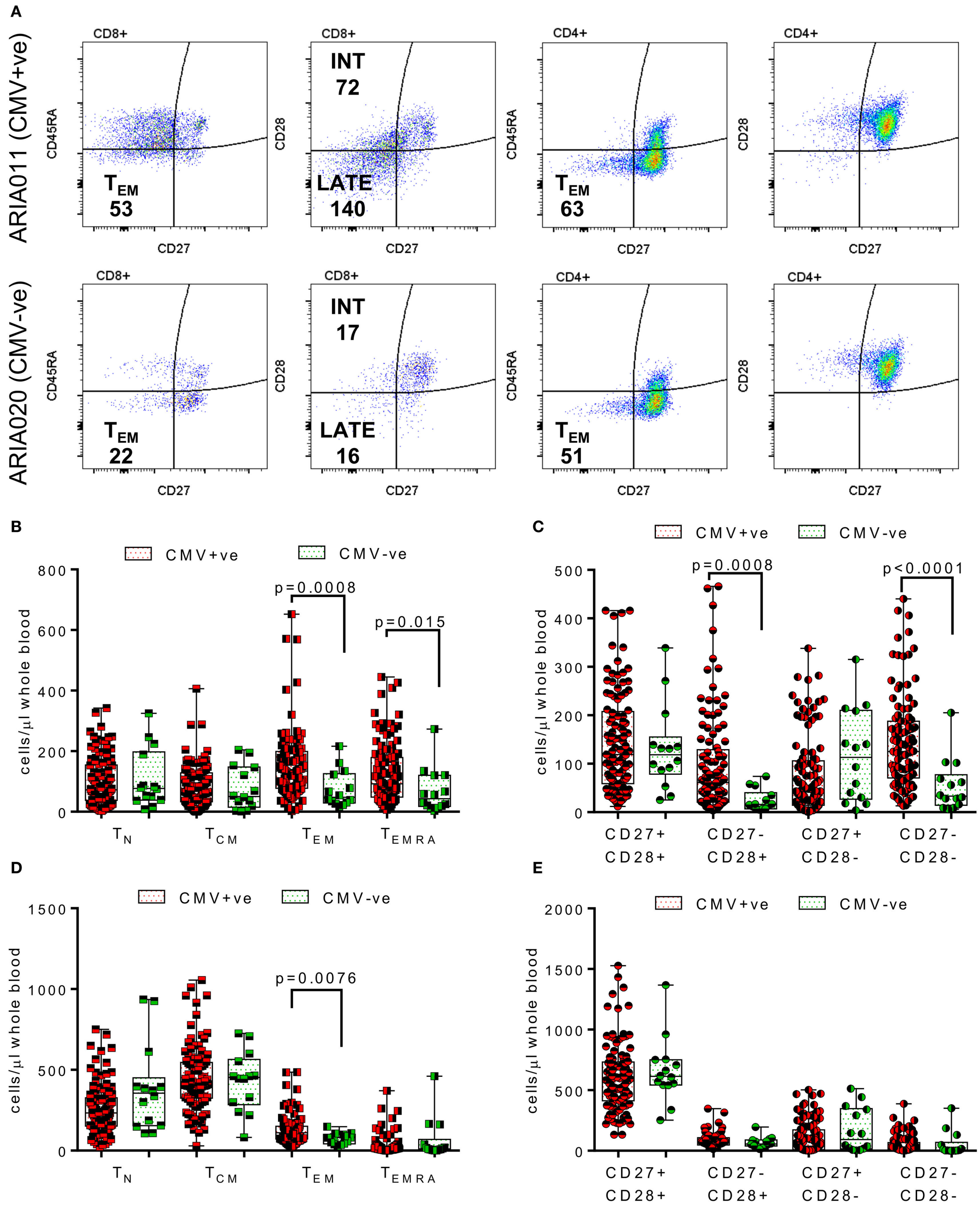

FIGURE 2 | Continued

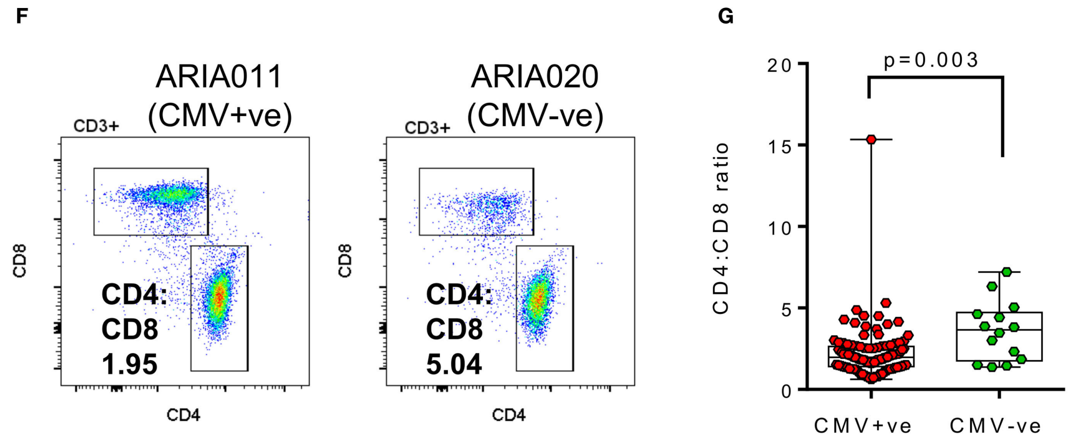

FIGURE 2 | Impact of human cytomegalovirus (HCMV) carriage on T cell numbers. EDTA treated whole blood was stained with a panel of phenotyping antibodies in order to enumerate CD4$^+$ and CD8$^+$ T cells and their subsets. Representative dot plots from a HCMV seropositive (HCMV +ve) and HCMV seronegative (HCMV-ve) age-matched donors are illustrated showing the memory (as defined by CD27 and CD45RA expression) and differentiation level (as defined by CD27 and CD28) phenotype of both CD4$^+$ and CD8$^+$ T cells; the number of cells per microliter of whole blood for effector memory (T_{EM}—CD27$^-$CD45RA$^-$) CD4$^+$ and CD8$^+$ T cells and Intermediate (INT—CD27$^-$CD28$^+$) and Late (LATE—CD27$^-$CD28$^-$) differentiated CD8$^+$ T cells are shown **(A)**. Box and whisker plots comparing cell numbers of the memory **(B,D)** and differentiation phenotypes **(C,E)** of CD8$^+$ T cells and CD4$^+$ T cells, respectively, between HCMV +ve (red) and HCMV-ve (green) donors are shown. The differences between the two groups were analyzed by a Kruskal–Wallis one-way ANOVA test with *post hoc* Mann–Whitney *U*-test performed with significant results set as $p \leq 0.015$ shown on each graph. A representative CD4 vs CD8 dot plot from the same donors with their respective CD4:CD8 ratio indicated are shown **(F)**, the comparison of CD4:CD8 ratios for all seropositive vs seronegative donors are also shown **(G)** with the significant decrease in the CD4:CD8 ratio in HCMV positive donors indicated (Mann–Whitney test).

Magnitude and Breadth of T Cell Responses to HCMV Proteins Remain Stable with Donor Age

To establish whether HCMV latent and lytic protein specific T cells are maintained and are functional during long-term carriage of the virus, we analyzed T cell responses to five viral genes known to be expressed during HCMV latent infection: UL138 (38), LUNA (39, 40), US28 (41), UL111A (vIL-10) (42), and UL144 (43), two of which (UL138 and LUNA), we have previously shown elicit both an IFNγ and IL-10 CD4$^+$ T cell response (53). We also wanted to measure the range of T cell responses in a large donor cohort to a number of viral proteins expressed during lytic infection; we have previously identified both CD4$^+$ and CD8$^+$ T cells producing IFNγ from many donors to six HCMV lytic proteins pp65, IE1, IE2, gB, pp71, and US3 (57, 63). Using FluoroSpot methodology, we were able to measure CD8$^+$ T cell IFNγ responses and both IFNγ and IL-10 CD4$^+$ T cell responses to overlapping peptide pools of these 11 HCMV proteins. Both HCMV seropositive and seronegative donors of all ages were included in these antigen-specific screens and, after discounting samples following quality control [high spontaneous cytokine spot forming unit (sfu) counts in unstimulated wells or failure of positive control stimulation], 98 donors were included in the CD8$^+$ T cell analysis, 99 donors in the CD4$^+$ T cell IFNγ analysis, and 73 donors in the CD4$^+$ T cell IL-10 analysis.

Figure 3 summarizes the results from the screen of 98 donors for CD8$^+$ IFNγ T cell responses. A majority of the HCMV seropositive donors analyzed had an above threshold (100 sfu/million) CD8$^+$ IFNγ T cell response to the six lytic proteins analyzed as well as responses to the latency-associated proteins UL144 and US28 proteins (**Figure 3A**). We noted positive CD8$^+$ T cell responses to LUNA (31.8% of donors) and UL138 (29.6% of donors), which while present in our previous study, using an enzymatic ELISPOT method, were below the positive response threshold (53) because this was a much less sensitive detection system. The frequency of individual donors who produced CD8$^+$ T cell responses to 1 or more HCMV proteins is presented as pie charts for the lytic expressed proteins (**Figure 3B**), latency-associated proteins (**Figure 3E**) and for all HCMV proteins (**Figure 3H**). These analyses shows that a majority of the donors produced a response to 5 or 6 lytic proteins (51.6%—blue and deep pink segments **Figure 3B**), that 29.7% of the donor cohort responded to 4 or 5 of the latency-associated proteins (green and blue segments **Figure 3E**) and, overall, 47.2% of the cohort responded to 8 or more HCMV proteins (orange, dark green, teal, and purple segments, **Figure 3H**). The broad range of responses to lytic, latent and all HCMV proteins observed were also maintained with age (**Figures 3C,F,I**, respectively). An analysis of whether increasing age alters the magnitude of the CD8$^+$ T cell IFNγ response to HCMV revealed no impact on the 11 individual proteins (data not shown) or the summed responses to lytic (**Figure 3D**), latent (**Figure 3G**), or all (**Figure 3J**) HCMV proteins examined.

We also examined the CD4$^+$ T cell responses of the donor cohort to the same 11 HCMV proteins in 99 donors. As observed for the CD8$^+$ T cell responses, the majority of the HCMV seropositive donor cohort produced an above threshold IFNγ response to all the lytic expressed proteins but also latency-associated UL144 and US28 (**Figure 4B**). The responses to

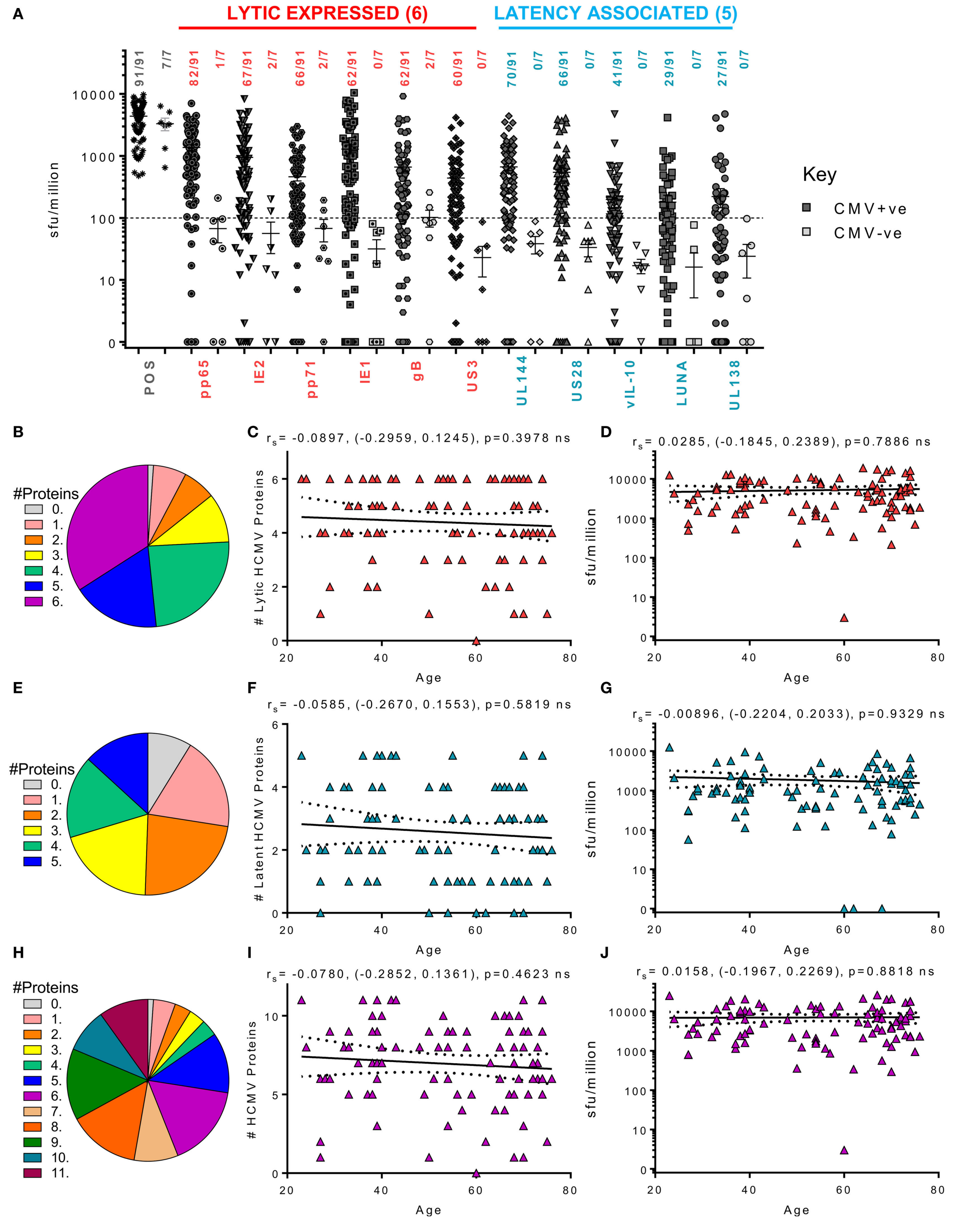

FIGURE 3 | Continued

FIGURE 3 | Continued
Magnitude and breadth of CD8+ T cell IFNγ response to human cytomegalovirus (HCMV) proteins. The IFNγ secreting CD8+ T cell response to 6 HCMV proteins only expressed during lytic infection: pp65, IE2, pp71, IE1, gB, US3, and 5 HCMV latency-associated proteins: UL144, US28, vIL-10, LUNA, and UL138 were measured in a cohort of 91 HCMV seropositive and 7 seronegative donors. The production of IFNγ was measured using an IFNγ FluoroSpot detection method; with the results converted to spot forming units/million cells (sfu/million) with background counts subtracted. The response to the lytic expressed proteins (red), latency associated (blue), and the positive control by all 98 donors are summarized **(A)** with HCMV seropositive donors (dark) and HCMV seronegative donors (light) both illustrated. The positive response threshold cutoff of 100 sfu/million is shown (dashed line) and the proportion of donors with a positive response to each HCMV protein is indicated. The proportion of the 91 seropositive donors producing a positive response to 1 or more of the 6 Lytic expressed proteins **(B)**, 5 latency-associated proteins **(E)** or all 11 HCMV proteins **(H)** are summarized as pie charts with the key to segments for each graph shown. Graphs illustrating the breadth of HCMV seropositive donors response to HCMV proteins correlated with age are illustrated for lytic expressed **(C)**, latency associated **(F)**, and all 11 proteins **(I)**; also shown is the summed IFNγ response to lytic **(D)**, latent **(G)**, and all proteins **(J)** correlated with age. Spearman rank correlation [Spearman r_s (95% confidence intervals) and *p* values] results are indicated on each graph.

the lytic expressed proteins by CD4+ T cells have already been reported in a subset of this donor cohort (63); however, the observation that both UL144 and US28 proteins induce T cell responses in the majority of HCMV seropositive donors has not previously been reported. Only 29.6% of the donor cohort examined produced an above threshold CD4+ IFNγ response to UL138, LUNA, and vIL-10 latency-associated proteins; this is a similar frequency to that seen in the CD8+ T cell compartment and not dissimilar to the percentage of responding donors for UL138 and LUNA CD4+ T cell responses previously reported in a small-scale study (53). The ability of individual donors to mount CD4+ IFNγ responses to multiple HCMV proteins is summarized as pie charts (**Figures 4B,E,H**). In contrast to the CD8+ T cell IFNγ response routinely seen to 5 or 6 lytic proteins, fewer donors were capable of mounting responses to 5 or 6 of the lytic expressed HCMV proteins (43.9%—blue and deep pink segments, **Figure 4B**). This trend was maintained in response to the latent proteins (22% responding to 4 or 5 proteins—green and blue segments, **Figure 4E**) and, overall, only 33% of the donor cohort responded to 8 or more of the examined HCMV proteins (**Figure 4H**—orange, dark green, teal, and purple segments). Despite this lower proportion of HCMV seropositive donors responding to many HCMV proteins, the overall breadth of the CD4+ IFNγ T cell response remained stable with increasing donor age which shows that there was no significant increase or decrease in the number of proteins an individual responded to within the lytic (**Figure 4C**) or latent group of proteins (**Figure 4F**) or to all 11 proteins examined (**Figure 4I**). Also, we did not observe an effect of donor age on the magnitude of the response to the individual HCMV proteins (data not shown) or to the summed responses to the 6 lytic proteins (**Figure 4D**), 5 latent proteins (**Figure 4G**), or to the summed response of all 11 proteins (**Figure 4J**).

We next examined the ability of CD4+ T cells to produce cIL-10 following stimulation with our 11 candidate HCMV proteins. Cellular IL-10 levels were measured in 73 HCMV donors from the cohort (these donors having passed the quality control thresholds outlined in the methods). Although we have already shown that lytically expressed proteins pp71 and US3 can induce cIL-10 production by CD4+ T cells in a small subset of this donor cohort (63), in this larger donor cohort, pp71 (38.8%), US3 (32.8%), and pp65 (23.8%) are the most common lytic proteins to trigger an above threshold cIL-10 CD4+ T cell response. The latency-associated proteins, US28 (34.3%), LUNA (31.3%), and UL138 (26.8%), also frequently induced a CD4+ specific cIL-10 response in this donor cohort. In contrast to the ability of donors to produce IFNγ T cell responses to multiple HCMV proteins, a positive cIL-10 response to any 1 of the 11 HCMV proteins examined was absent in 19 of 67 seropositive donors (gray segment—**Figure 5H**) and no donors produced responses to more than 9 of the 11 HCMV proteins. When examining the response to the 6 lytic proteins, about half of the 67 donors (49.3%) did not produce a cIL-10 response (gray segment—**Figure 5B**). Despite this more limited breadth of the response, 70% of the donors examined produced an above threshold cIL-10 response to 1 or more HCMV protein. The ability of an individual donor to produce a cIL-10 response to HCMV proteins was not affected by age (**Figures 5C,F,I**) and neither was the magnitude of the responses to each of the 11 HCMV proteins (data not shown). The relationship of the total cIL-10 responses, for each donor, to the 6 lytic proteins (**Figure 5D**), 5 latent proteins (**Figure 5G**), and all 11 proteins (**Figure 5J**) was also stable with donor age. Overall, the data presented show that the breadth and magnitude of the IFNγ and cIL-10 HCMV-specific T cell responses, within this donor cohort, do not show any impact of either increasing donor age or putative long-term carriage of the virus on these HCMV-specific T cell responses.

CD4+ T Cells Specific for LUNA, UL138, pp71, US3, and US28 Proteins Are More Frequently Biased toward Expression of cIL-10 than IFN γ and This Was Not Affected by Donor Age

Using the FluoroSpot technology, we were able to ask whether CD4+ T cell responses to our candidate, HCMV proteins was dominated by either IFNγ or IL-10 secretion or whether it was comprised of cells that secrete both cytokines. **Figure 6** shows the relative cytokine composition of the CD4+ T cell response to each of the 11 HCMV proteins examined for donors who generated an above threshold response (>100 sfu/million) for either cytokine. Overall, we found that IFNγ and cIL-10 are generally produced by distinct populations of CD4+ T cells, as dual secretors were very rare (red bars—**Figure 6**). The CD4+ T cell responses to UL144 (**Figure 6D**), gB (**Figure 6J**), pp65 (**Figure 6H**), IE1 (**Figure 6K**), and IE2 (**Figure 6I**) proteins were dominated by IFNγ secretion. In contrast, the donor cohort responses to the proteins UL138 (**Figure 6C**), LUNA (**Figure 6B**), US28 (**Figure 6A**), vIL-10 (**Figure 6E**), pp71 (**Figure 6F**), and US3 (**Figure 6G**) showed more cIL-10 secretors (white spotted bars). Although there was no significant change

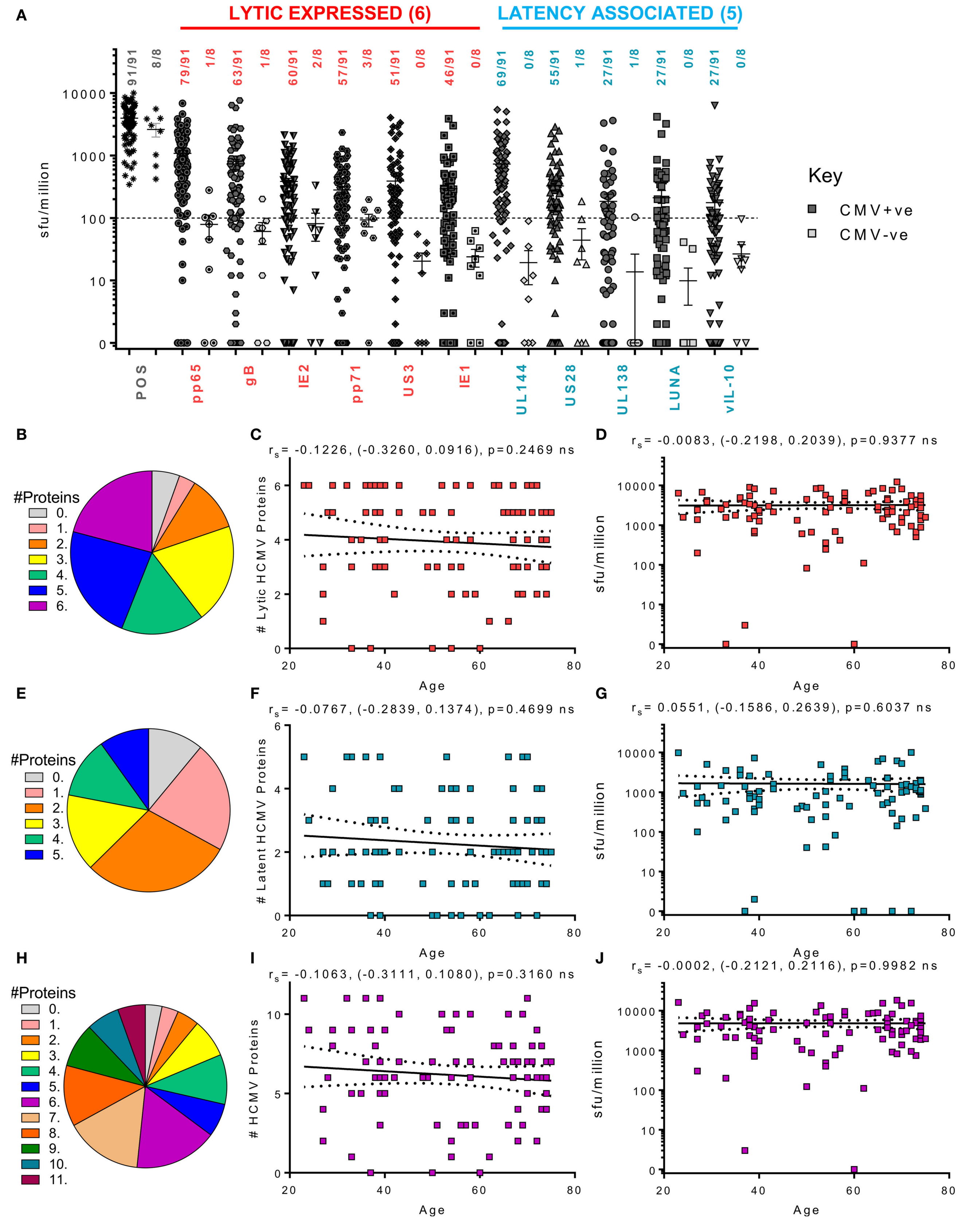

FIGURE 4 | Continued

FIGURE 4 | Continued
Magnitude and breadth of $CD4^+$ T cell IFNγ response to human cytomegalovirus (HCMV) proteins. The IFNγ-secreting $CD4^+$ T cell response to 6 HCMV proteins only expressed during lytic infection: pp65, IE2, pp71, IE1, gB, and US3 (red) and 5 HCMV latency-associated proteins: UL144, US28, vIL-10, LUNA, and UL138 (blue) were measured in a cohort of 91 HCMV seropositive and 8 seronegative donors. The production of IFNγ was measured using an IFNγ FluoroSpot method; with the results converted to spot forming units/million cells (sfu/million) with background counts then subtracted. The response to the HCMV proteins and the positive control by all 99 donors are summarized **(A)** with HCMV seropositive donors (dark) and HCMV seronegative donors (light) both illustrated. The positive response threshold cutoff of 100 sfu/million (dashed line) and the proportion of donors with an above threshold response to each HCMV protein is indicated. The proportion of the 91 seropositive donors producing a positive IFNγ response to 1 or more of the 6 Lytic expressed proteins **(B)**, 5 latency-associated proteins **(E)** or all 11 HCMV proteins **(H)** are summarized as pie charts with the key to segment color for each graph shown. Graphs illustrating the breadth of HCMV seropositive donors IFNγ response to HCMV proteins correlated with age are illustrated for lytic expressed **(C)**, latency associated **(F)**, and all 11 proteins **(I)**; also shown is the summed IFNγ response to lytic **(D)**, latent **(G)**, and all proteins **(J)** correlated with age. Spearman rank correlation [Spearman r_s (95% confidence intervals) and p values] results are indicated on each graph.

in the magnitude of the $CD4^+$ T cell IL-10 response to HCMV proteins with age (summarized **Figure 5**), we were interested to see if there was a change in the proportion of IFNγ and IL-10 secretion by $CD4^+$ T cells within individuals during long-term viral carriage. The data presented in **Figure 6** are arranged with donor age along the *x*-axis and does not show any obvious changes in the composition of the positive $CD4^+$ T cell response. Analysis of the proportion of donors in which the majority of the $CD4^+$ T cell responses was secretion of cIL-10 (i.e., greater than 50% of the total $CD4^+$ T cell response of the individual to each HCMV protein) revealed that for LUNA 48.5% of responding donors had a dominant cIL-10 response (Figure S3A in Supplementary Material). UL138, pp71, US3, and US28 also elicited a greater than 50% IL-10 response in more than a third of the donor cohort (42.8, 38.4, 34, and 33.3%, respectively; Figure S3A in Supplementary Material). When looking at the breadth of the cIL-10 dominant responses with donor age, there was no significant increase in the breadth of HCMV proteins and individual produced a majority cIL-10 response toward for all proteins (Figure S3B in Supplementary Material), lytic proteins (Figure S3C in Supplementary Material), or latent-associated proteins (Figure S3D in Supplementary Material).

The Magnitude of Latent HCMV DNA Load in $CD14^+$ Monocytes Is Not Affected by Donor Age in the ARIA Cohort

In addition to assessing the effect of increasing age on the T cell response to HCMV lytic and latent expressed proteins, the other principle aim of this study was to determine if there was an age-related effect on latent viral load. Consequently, we screened whole blood of all donors in the study for the presence of HCMV DNA using a quantitative real-time PCR assay. No viral DNA was detectable in the 14 HCMV seronegative donors and of the 105 HCMV seropositive donors, viral genome was only detected in 1 of these (274 copies/ml whole blood). The donor with detectable HCMV in whole blood also had an inverted CD4:CD8 ratio and above average numbers of differentiated memory $CD8^+$ T cells, data summarized in Figure S4 in Supplementary Material. During latent HCMV infection, virus is known to reside in $CD34^+$ hematopoietic stem cells and derivative $CD14^+$ monocytes (64). Using a sensitive ddPCR approach (54), we quantified the number of copies of HCMV present in isolated $CD14^+$ monocytes from all donors. In total, we assessed 108 HCMV seropositives and negatives for HCMV DNA present in $CD14^+$ cells; of these, no copies of viral genome were detected in the 14 HCMV seronegative donors. We did, however, detect HCMV genomes in 43 of 94 (45.7%) of $CD14^+$ monocytes from HCMV seropositive donors (51 of 94 were below the level of detection of this assay, 1 genome in 60,000 cells); the latent viral load (copies HCMV/million $CD14^+$ cells) for the 94 seropositive donors, relative to donor age, is summarized in **Figure 7**. Within this ARIA donor cohort, we did not observe a significant relationship between age and the magnitude of the latent viral load.

High Latent Viral Loads in $CD14^+$ Monocytes Were Associated with both Increased Breadth and Frequency of IFNγ-Secreting HCMV-Specific T Cells

Human cytomegalovirus is latently carried in $CD34^+$ hematopoietic progenitor cells and subsequently in the periphery by monocyte derivatives from these cells (65). Virus reactivation from these myeloid lineage cells would activate HCMV-specific T cells and could drive increased frequencies, as well as potentially seeding more cells in the latent reservoir. Theoretically, increased frequency of latently infected cells could result in increased virus reactivation events, potentially resulting in induction of more T cell stimulation and, possibly, an increase in HCMV-specific antibody levels during lifelong persistence. Consequently, we assessed whether there was an association between HCMV-specific IgG levels and latent viral load, but these measures were unrelated (data not shown). We then assessed whether there was an association between the latent viral load and the $CD8^+$ and $CD4^+$ T cell responses to the individual HCMV proteins as well as to the magnitude and breadth of the total responses of each donor. We did not observe an association between latent load and the cIL-10 $CD4^+$ response and there was only a significant association between the magnitude and breadth of the $CD4^+$ IFNγ response to the subset of 6 lytic proteins and increased latent viral load (data not shown). There was a significant association with the summed total of the $CD8^+$ T cell response to lytic (**Figure 8B**), latent (**Figure 8D**), and all proteins (**Figure 8F**). Also, high viral copy latent load correlated significantly to the breadth of the $CD8^+$ T cell responses to lytic (**Figure 8A**) and all HCMV proteins (**Figure 8E**), but not to the latent proteins only (**Figure 8C**).

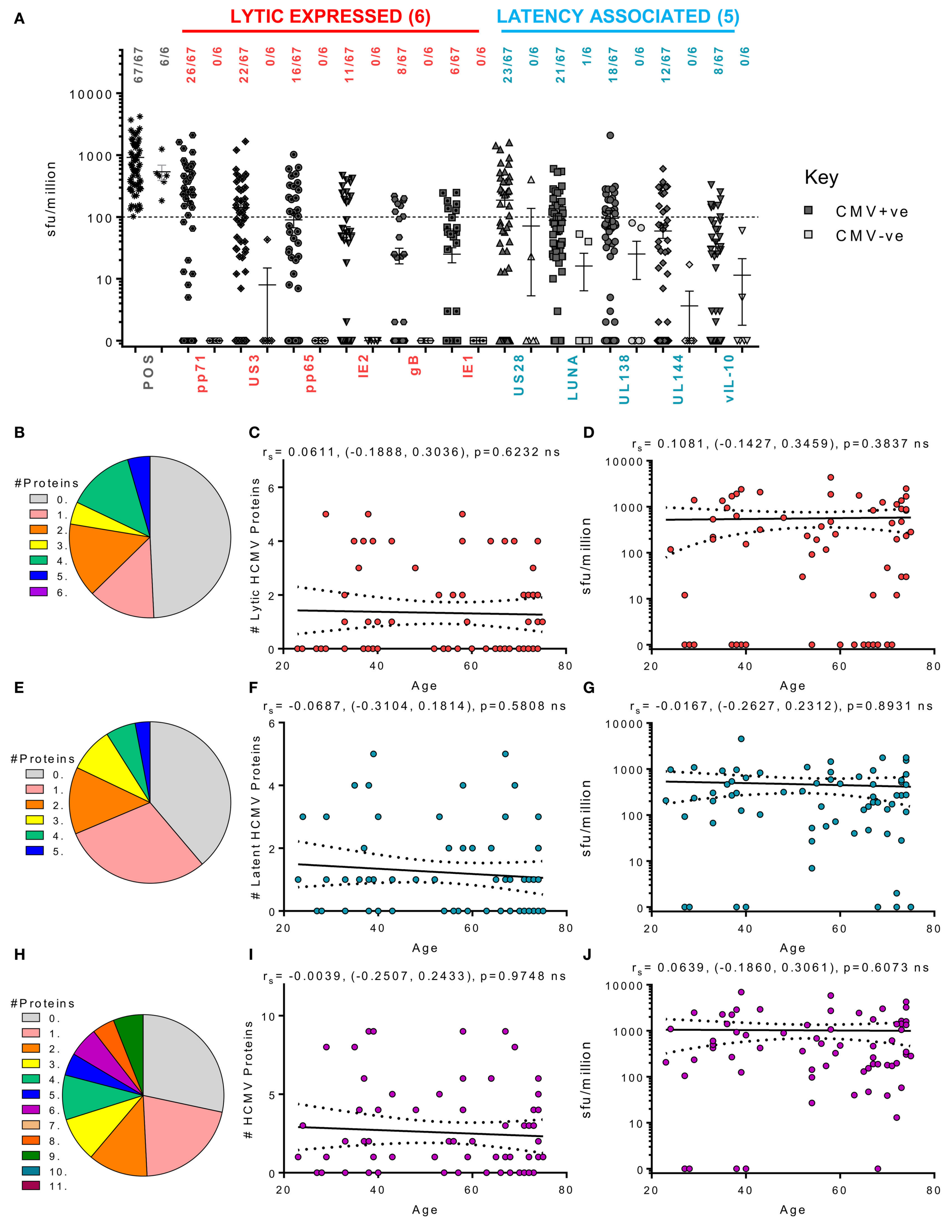

FIGURE 5 | Continued

FIGURE 5 | Continued
Magnitude and breadth of $CD4^+$ T cell IL-10 response to human cytomegalovirus (HCMV) proteins. The IL-10-secreting $CD4^+$ T cell response to 6 HCMV proteins only expressed during lytic infection: pp65, IE2, pp71, IE1, gB, and US3 (red) and 5 HCMV latency-associated proteins: UL144, US28, vIL-10, LUNA, and UL138 (blue) were measured in a cohort of 67 HCMV seropositive and 6 seronegative donors. The production of IL-10 was measured using an IL-10 FluoroSpot method; with the results converted to spot forming units/million cells (sfu/million) with background counts then subtracted. The response to the HCMV proteins and the positive control by all 73 donors are summarized **(A)** with HCMV seropositive donors (dark) and HCMV seronegative donors (light) both illustrated. The positive response threshold cutoff of 100 sfu/million (dashed line) and the proportion of donors responding to each HCMV protein is indicated. The proportion of the 67 seropositive donors producing a positive IL-10 response to 1 or more of the 6 lytic expressed proteins **(B)**, 5 latency-associated proteins **(E)** or all 11 HCMV proteins **(H)** are summarized as pie charts with the key to segment color for each graph shown. Graphs illustrating the breadth of HCMV seropositive donors IL-10 response to HCMV proteins correlated with age are illustrated for lytic expressed **(C)**, latency associated **(F)**, and all 11 proteins **(I)**; also shown is the summed IL-10 response to lytic **(D)**, latent **(G)**, and all proteins **(J)** correlated with age. Spearman rank correlation [Spearman r_s (95% confidence intervals) and *p* values] results are indicated on each graph.

DISCUSSION

The aims of this study were to determine whether HCMV-specific $CD4^+$ T cells secreting cIL-10 increase with age and long-term viral carriage and to determine whether there are changes in breadth and frequency of the IFNγ-secreting T cell response to HCMV infection in healthy older donors. We also wanted to measure the latent viral load of HCMV DNA in a large donor cohort for the first time and assess whether donors aged over 65 years manifested changes in immune cell numbers indicative of immunosenescence. Using an age cross-sectional study methodology, we recruited a donor cohort spanning six decades (23–78 years) and measured virological and immunological parameters. The donors were recruited by the Cambridge Bioresource from their Biobank of volunteers who live predominantly in areas local to Cambridge and the East Anglian Region of the UK. Donors were recruited based on HCMV serostatus and by excluding donors suffering from immune altering illnesses or under treatment for these conditions, such that all participants could be safely considered to be generally healthy.

We analyzed the $CD4^+$ and $CD8^+$ T cell compartments in peripheral blood and observed a loss of naïve $CD4^+$ and $CD8^+$ T cell numbers as well as a corresponding loss of total $CD4^+$ and $CD8^+$ T cell numbers with increasing age. The age-related loss of naïve T cells numbers is a well-established phenomenon due to the involution of the thymus and decreased T cell output (66) and has been observed in most studies of aging populations (24). In our study, there was no accumulation of memory T cell populations (measured in absolute numbers) within this cohort, which has also been observed in other studies when using absolute numbers (52, 67). However, when expressed as a percentage of the $CD8^+$ T cell compartment, there was a significant age-related accumulation of differentiated T_{EMRA} ($CD27^-CD45RA^+$) and Late stage ($CD27^-CD28^-$) memory cell populations as has been previously reported (24). It is likely that the increase in percentage (relative frequency) of differentiated memory T cell populations previously reported in aged cohorts was due to the decrease in the absolute size of the overall $CD8^+$ T cell compartment, which results in an increase in the proportion of memory cells even if the absolute numbers do not increase (1, 52, 67).

Previous investigations into the impact of HCMV persistence on immunosenescence in older people have reported a range of immune parameters and HCMV-specific markers altering with age. These include the IRP, defined by a collection of markers which, taken together, were suggested to be indicative of increased mortality in the elderly and which included an inversion of the CD4:CD8 ratio, expansion of $CD8^+$ $CD28^{null}$ and $CD8^+$ T_{EMRA} memory T cells and HCMV seropositivity (8, 9, 12, 13). There have also been reports of HCMV-specific IgG levels increasing in older donors (54, 68, 69) as well as accumulation of HCMV-specific T cells with age [summarized in Ref. (24)]. Similarly, it has been suggested that there is an age-related increase in levels of HCMV DNA in blood (37), urine (36), and an increase in latent viral genome copy number in $CD14^+$ cells of donors aged over 70 years (54). Overall, as our donor cohort exhibited a normal aging immune phenotype, we examined the impact of HCMV seropositivity on T cell memory phenotype within the study group. There were no significant differences in naïve T cell numbers between aged HCMV seropositive compared to aged HCMV seronegative donors in our cohort and we only observed an inverted CD4:CD8 ratio in 10% of the seropositive donor cohort; donors exhibiting this phenotype were distributed throughout the age categories. We did see an increase in the numbers of differentiated T cells in HCMV seropositive donors of all ages compared to seronegatives, confirming that our study participants have a similar T cell phenotype to that observed in many previous studies of HCMV infection (24). There was, however, no association between increasing donor age and higher levels of HCMV IgG nor was there an increase in the breadth and frequency of the HCMV-specific T cell IFNγ response or $CD4^+$ cIL-10 response to the 11 HCMV proteins examined within the study group. We also did not detect increased copies of latent HCMV genome in $CD14^+$ monocytes of our older donors. The separate impact of HCMV infection from aging on the differentiation of T cells has been observed in other population studies (19, 21) and the kidney transplant primary infection model and reports from primary infection has shown a rapid acquisition of a more differentiated T cell phenotype in the months following initial infection (30, 70–72). Furthermore, we observed a significant association between high latent viral loads and higher frequency HCMV-specific $CD8^+$ T cell responses, which was again irrespective of donor age. These observations alongside the increased numbers of differentiated memory T cells suggest that, within this healthy donor cohort, it is HCMV infection, rather than the age of the donor, which leads to increased differentiation of the T cell population and expansion of HCMV-specific T cells.

Work on donor cohorts from different geographical locations have reported different findings from the original Swedish

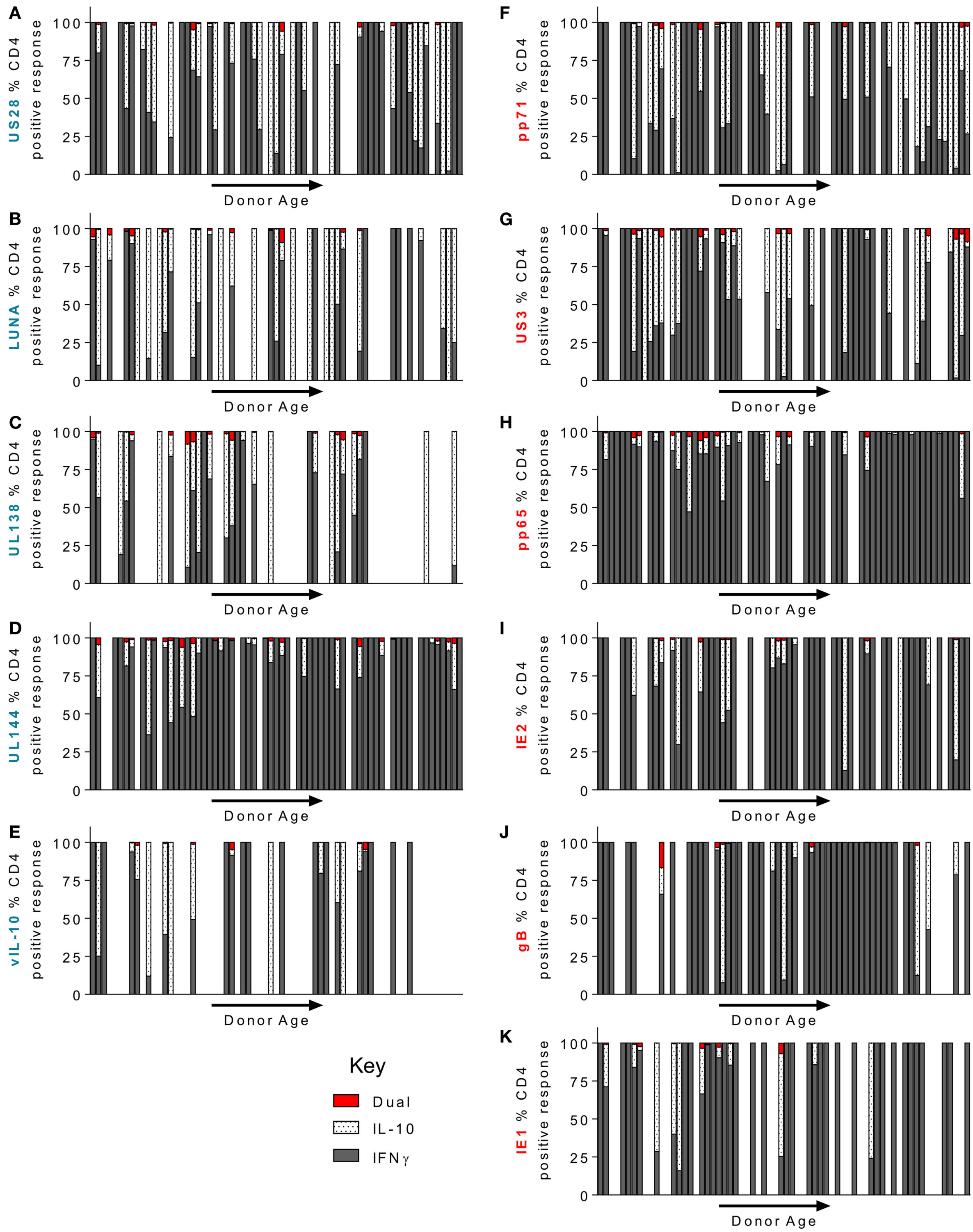

FIGURE 6 | CD4+ T cell donor responses to human cytomegalovirus (HCMV) LUNA, UL138, pp71, US3, and US28 proteins were more frequently IL-10 biased. The frequency of CD4+ T cells that secrete IFNγ or IL-10 or both in response to stimulation by HCMV proteins was measured simultaneously using a dual IFNγ/IL-10 FluoroSpot assay. 67 HCMV seropositive donors were analyzed, only donors with above threshold responses for either IFNγ or IL-10 (100 sfu/million) to each protein are shown. The IFNγ (dark gray), IL-10 (white spotted), and dual cytokine (red) responses of the donor cohort to US28 **(A)**, LUNA **(B)**, UL138 **(C)**, UL144 **(D)**, vIL-10 **(E)**, pp71 **(F)** US3 **(G)**, pp65 **(H)**, IE2 **(I)**, gB **(J)**, and IE1 **(K)** are shown as a percentage of the total CD4+ T cell (IFNγ+ IL-10) response of each donor, the donors are arranged along the *x*-axis in increasing age order. The lytic expressed proteins axis label is in red **(F–K)** and the latency-associated protein responses are labeled in blue **(A–E)**.

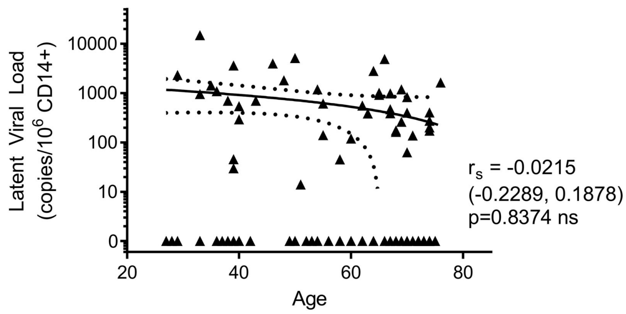

FIGURE 7 | There was no effect of donor age on the magnitude of latent human cytomegalovirus (HCMV) load in CD14⁺ monocytes. The DNA of purified CD14⁺ monocytes was extracted and HCMV viral load detected using droplet digital PCR analysis. No HCMV was detected in 14 HCMV seronegative donors tested. The HCMV viral load (copies/10^6 CD14⁺ cells) results from 94 HCMV seropositive donors are shown correlated with donor age. Spearman rank correlation [Spearman r_s (95% confidence intervals) and *p* values] analysis is indicated on the graph.

studies which described the IRP (8, 9, 12, 13), these have included a lack of "inflation" of HCMV-specific T cells with age despite high HCMV seroprevalence in the aged donor groups (73) and the association of a naïve T cell phenotype in HCMV seropositive old people with increased morbidity in Belgium (74). HCMV seroprevalence varies depending on geographical location and socio-economic status (6, 75); in the developed world between 30 and 70% of populations are HCMV seropositive, with acquisition of the virus increasing with age (76). In contrast, in developing countries, seroprevalence can be higher than 90% with acquisition of the virus commonly occurring in early childhood (30, 76). Consequently, the disparate observations reported as consequences of HCMV infection in different aged donor cohorts may be a result of geography as well as other biological parameters such as exposure to infectious diseases, vaccination history, and the current health of the participants. It has also been shown in other studies of very old cohorts that increased HCMV IgG levels and differentiated $CD4^+$ T cells are associated with elderly individuals in poor health (27), and there are also a number of studies associating HCMV seropositivity and higher HCMV IgG titers with poor outcomes from cardiovascular disease (14, 15, 17, 18). Our view is that, in some cohorts that have been studied, aged donors suffering from, e.g., heart disease, cancer or neurodegenerative disorders may not control virus efficiently leading to increased HCMV IgG levels or HCMV DNAemia and concomitant increased numbers of differentiated memory T cell populations and an inverted CD4:CD8 ratio, thereby confounding some studies.

One of our aims was to address the production of cIL-10 by HCMV-specific $CD4^+$ T cells within a large donor cohort in order to assess how prevalent the production of this suppressive cytokine is by HCMV antigen-specific T cells and whether this response increases in older donors. Evidence from mouse models of MCMV infection have shown that production of cIL-10 can result in reduced viral clearance and a reduction in production of IFNγ by MCMV-specific T cells (49, 50). This could provide an explanation for the observation that, despite a functional immune response preventing overt HCMV mediated disease, older donors have detectable HCMV DNA in blood and urine (36, 37). In some HCMV studies, increases in inducible regulatory $CD4^+$ T cells have been reported in older people with this being associated with vascular pathology in these individuals (47). Similarly, it has also been suggested that the HCMV-specific $CD4^+$ $CD28^-CD27^-$T cell population, reported as expanded in HCMV seropositive older people (77), contains a T regulatory population characterized by FoxP3 and $CD25^{hi}$ expression (45). As already discussed, there was no accumulation of the cIL-10 $CD4^+$ T cell response with increasing donor age in this cohort; we were also interested to see if there was a shift in the bias of the responding $CD4^+$ T cells to individual HCMV proteins from IFNγ to IL-10 or *vice versa*. The results confirmed our previous observation that the production of cIL-10 by $CD4^+$ T cells is more likely to be in response to latency-associated proteins (53); in this cohort, almost 50 and 40% of donors produced a majority cIL-10 response to stimulation by the LUNA and UL138 peptide pools, respectively, regardless of donor age. Similarly, other latency-associated proteins included in this study, US28 and vIL-10, also showed a number of donors biased toward cIL-10 production, which is in contrast to the response toward many of the lytically expressed proteins included in this study.

The use of the ddPCR protocol (54) has enabled better quantification of the levels of latent HCMV genomes in the $CD14^+$ cell compartment. We were able to detect and quantify latent HCMV genomes in 45.7% of examined HCMV seropositive donors comparing favorably to the 36% detection rate in HCMV positive donors described recently by ddPCR (54). Our ability to quantify latent HCMV load in our donor cohort led to a particularly interesting observation with respect to HCMV-specific T cell response. As already noted, high copy numbers of latent HCMV detected in $CD14^+$ monocytes significantly correlated with an increase in the breadth and magnitude of the HCMV-specific $CD8^+$ T cell response measured by IFNγ secretion. From this result, we hypothesize that higher viral genome copy number was a result of an accumulation of reactivation events over the time, resulting in viral replication and reseeding of the latent $CD34^+$ cellular pool; consequently, this production of viral proteins stimulates and activates HCMV-specific memory T cell response leading to an increase in frequency of these cells. The virus most likely employs its immune evasion functions to create a window of opportunity to allow reactivation from latency and the production of new virions despite the presence of a primed antiviral immune response (5). In older donors, uncontrolled reactivation of HCMV subsequently causing either disease or other medical complications has not been observed, and HCMV DNA has not been routinely detected in the blood (36, 78), apart from in a Japanese cohort study, but the DNA positive detection rate was only 4.3% of donors aged 60–69 years (37). However, there is evidence that older people may not control virus replication as adequately as the young, as HCMV DNA has been detected in other bodily fluids in the old (36). Within this study, our exclusion criteria may have precluded recruitment of donors who had less effective control of virus replication resulting in low-level virus

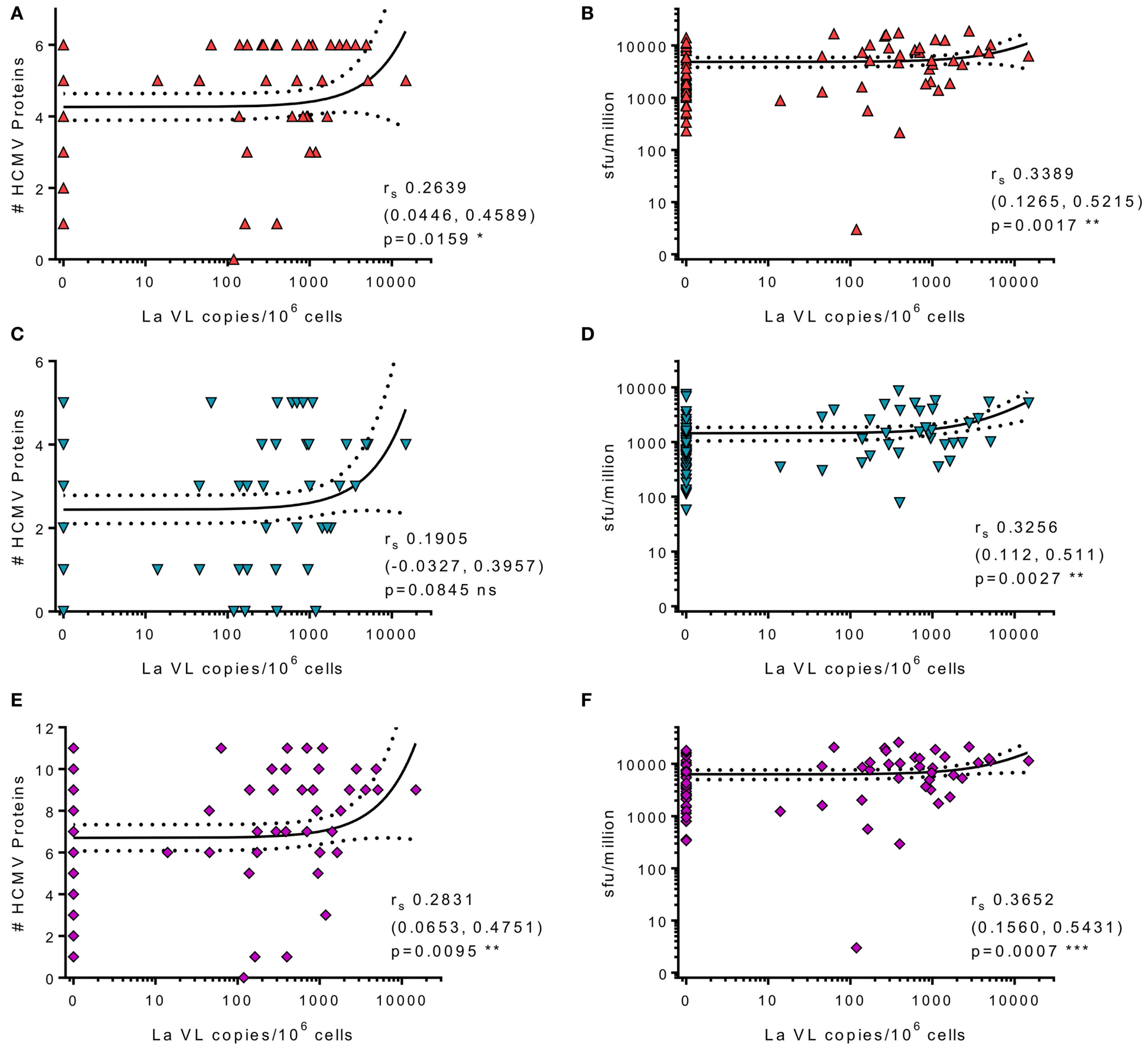

FIGURE 8 | High levels of latent human cytomegalovirus (HCMV) load in CD14+ monocytes correlates with increased frequency and breadth of HCMV-specific IFNγ CD8+ T cell responses. The HCMV viral load (copies/10^6 CD14+ cells) from 83 HCMV seropositive donors was correlated with CD8+ HCMV-specific T cell responses. Graphs illustrating the breadth (positive response) of individual donors CD8+ IFNγ response to the 6 lytic expressed (red) **(A)**, 5 latency-associated (blue) **(C)**, and all 11 HCMV proteins (purple) **(E)** correlated with CD14+ cells HCMV viral load are shown. The magnitude of the CD8+ IFNγ response summed for all protein groups is correlated with HCMV viral load for lytic (red) **(B)**, latent (blue) **(D)**, and all proteins (purple) **(F)**. Spearman rank correlation [Spearman r_s (95% confidence intervals) and *p* values] results are indicated on each graph.

dissemination. In support of this conclusion, it is interesting to note that a single aged male donor with detectable HCMV DNA in whole blood did have an inverted CD4:CD8 ratio as well as an above average number of highly differentiated memory CD8+ T cell populations; they also had limited HCMV-specific T cell responses to our 11 candidate HCMV proteins (Figure S4 in Supplementary Material).

We have demonstrated that, in an East Anglian-based donor cohort which has a typical healthy aging profile, older HCMV seropositive donors do not exhibit the hallmark features of the IRP, differences in the breadth, and magnitude of their HCMV-specific IFNγ production, or that latent viral load was affected by age. Importantly, though we did see a significant relationship between high latent viral load and increased breadth and magnitude of the functional HCMV-specific CD8+ T cell responses, latent viral load did not correlate with increased numbers of differentiated memory T cell populations or HCMV-specific IgG. This, we believe, reflects the importance of including measurement of viral load in studies on the impact of HCMV infection in older donors as opposed to inferring the impact of the virus from measuring a variety of other immune parameters as has previously occurred. In a previous study in a Birmingham based old aged cohort, the authors observed an increase in HCMV-specific T cell responses alongside, an increase in latent viral carriage in donors aged over 70 years (54). While the authors do not present data correlating latent viral load with the frequency of HCMV-specific T cells, we think it possible in light of our findings, that in this older cohort study, the increase in HCMV-specific T cell responses in older donors could be associated with increased latent viral carriage.

Detection of low-level HCMV viremia in the blood of the old would be a strong indicator of a diminution of immune control; however, the results from our study group and others (36, 78) suggests this is rarely observed, probably because it would represent a significant loss of control. However, the presence of virus in other bodily fluids, e.g., saliva or urine could also indicate loss of immune control. It should be considered that chronic low-level persistent HCMV replication and an associated inflammatory environment could be important in particular old patients groups; there is epidemiological evidence that HCMV comorbidity plays a role in exacerbating cardiovascular disease (14, 15, 17, 18) and also with increasing impaired physical function and ill health (27, 29, 74, 79). Future investigations into the impact of HCMV infection in older people should also monitor latent viral carriage of the virus alongside measuring whether low-level viremia is present in the blood and other bodily fluids, e.g., urine or saliva; in order to improve our understanding of the impact of HCMV infection in the elderly.

ETHICS STATEMENT

Ethical approval was obtained from University of Cambridge Human Biology Research Ethics Committee. Informed written consent was obtained from all donors in accordance with the Declaration of Helsinki (HBREC.2014.07).

AUTHOR CONTRIBUTIONS

SJ, MW, EP, and JS designed the project and experiments. SJ, GS, GO, and EP carried out the experiments. SJ and MW wrote the manuscript. SJ carried out statistical analysis and prepared figures. SJ and MW submitted this paper. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00733/full#supplementary-material.

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REVIEW
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Impact of Aging and Cytomegalovirus on Immunological Response to Influenza Vaccination and Infection

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The number of people over the age of 60 is expected to double by 2050 according to the WHO. This emphasizes the need to ensure optimized resilience to health stressors in late life. In older adults, influenza is one of the leading causes of catastrophic disability (defined as the loss of independence in daily living and self-care activities). Influenza vaccination is generally perceived to be less protective in older adults, with some studies suggesting that the humoral immune response to the vaccine is further impaired in cytomegalovirus (CMV)-seropositive older people. CMV is a β-herpes virus infection that is generally asymptomatic in healthy individuals. The majority of older adults possess serum antibodies against the virus indicating latent infection. Age-related changes in T-cell-mediated immunity are augmented by CMV infection and may be associated with more serious complications of influenza infection. This review focuses on the impact of aging and CMV on immune cell function, the response to influenza infection and vaccination, and how the current understanding of aging and CMV can be used to design a more effective influenza vaccine for older adults. It is anticipated that efforts in this field will address the public health need for improved protection against influenza in older adults, particularly with regard to the serious complications leading to loss of independence.

Keywords: cytomegalovirus, influenza, vaccination, infection, older adult, aging, elderly, cytotoxic T-lymphocyte

INTRODUCTION

Cytomegalovirus (CMV) is a β-herpes virus that infects fibroblasts, epithelial, endothelial, stromal, smooth muscle cells, but most importantly, monocytes and dendritic cells (DCs) (1). Depending on the country and its state of development, 25–90% of the worldwide population is CMV seropositive (2, 3) with prevalence higher in older adults (4). Once infected with CMV, the immune system is unable

Abbreviations: AID, activation-induced cytidine deaminase; APC, antigen-presenting cell; BCR, B-cell receptor; CMV, cytomegalovirus; CTL, cytotoxic T-lymphocyte; DC, dendritic cell; EBV, Epstein–Barr virus; GrB, granzyme B; HA, hemagglutinin; M1, matrix 1; MHC, major histocompatibility complex; NA, neuraminidase; NP, nucleoprotein; pH1N1, pandemic H1N1; pT_{FH}, peripheral T-follicular helper cell; TCR, T-cell receptor; Treg, regulatory T-cell.

to eliminate the virus, resulting in persistent latent infection. While the contribution of CMV infection to features of immune senescence are well recognized (5, 6), the translation to predicting outcomes in older adults has been much more challenging. Earlier reports of the association between CMV seropositivity and prevalent frailty in community-dwelling older women (7, 8) (frailty determined based on a five component measure: unintentional weight loss, weak grip strength, exhaustion, slow walking speed, and low level of activity) have not always been replicated in more recent longitudinal studies of CMV seropositivity as a predictor of frailty as measured by grip strength (9). However, many other studies have reported an association between CMV seropositivity and frailty (5, 6, 8, 10, 11), and increased mortality (12–15), but these findings are not consistent across all age groups and under all conditions. For example, in the BELFRAIL study, CMV seropositivity was not associated with an increased risk for all-cause mortality in a cohort of very old people. This may have been the result of a survival effect, whereby CMV-seropositive subjects with high anti-CMV titers die at a younger age compared with other individuals. This may reflect CMV reactivation being more common in the end stages of life (15). In terms of the impact of CMV on immune function, CMV seropositivity has been linked to poor $CD4^+$ T-cell responses to influenza internal proteins (16), while other studies have found no association between CMV pp65-reactive $CD8^+$ T-cells and poor $CD8^+$ T-cell responses to influenza internal proteins (17). Although CMV seropositivity in older adults has never been directly correlated with poor vaccine-mediated protection in older adults, high levels of CMV-reactive $CD4^+$ T-cells have been associated with an increased risk of viral respiratory illness in elderly nursing home residents (18) and predict increased morbidity and mortality.

Cytomegalovirus and aging of the immune system are associated with oligoclonal expansions of $CD8^+$ T-cells, possibly due to an increase in the frequency and magnitude of reactivation of CMV in older compared to young adults (19). At the same time, CMV IgG titers and viral load increase markedly with age (20). These findings suggest that while older adults are able to contain CMV, they do so at the cost of investing ever-increasing resources to control this single pathogen, with the result that immune responses to other challenges may be reduced (21).

Influenza is a single-stranded negative-sense RNA virus that is transmitted through the air by coughing and sneezing and infects epithelial cells, usually in the nose, throat, and lungs. The virus has a major impact on the aging population; ≥90% of annual influenza-related deaths occur in individuals ≥65 years of age (22). Often, influenza itself is not the cause of death, but rather it predisposes older adults to develop secondary bacterial infections and exacerbations of preexisting medical conditions (23, 24). Furthermore, older adults represent the majority of individuals hospitalized with influenza illness (25), which raises concern as hospitalization itself is often followed by a decline in the ability to perform activities of daily living for individuals in this age group (26). Additionally, influenza-related hospitalizations have a significant economic and social impact (27). Although antiviral drugs against influenza are available, vaccination continues to be the most effective method to control infection (28). Prevention of influenza illness through vaccination aids in reducing the burden on the health-care system and maintaining the quality of life of older adults. Hospitalization rates for influenza remain high (25) in spite of evidence that vaccination campaigns can reduce such events (29). Furthermore, multiple impairments associated with CMV and aging appear to lessen the effectiveness of influenza vaccination and reduce the ability to respond to influenza infection to prevent serious complications (30, 31). Recently, it has been shown that influenza vaccination provides good protection against influenza-related hospitalization, but vaccine effectiveness declines as frailty (using the Frailty Index) increases in older adults (32).

THE ROLE OF ANTIGEN-PRESENTING CELLS (APCs) IN INFLUENZA INFECTION AND VACCINATION

Macrophages and DCs play an important role in directing the immune response to the site of infection. These cells act as APCs and modulate the innate and adaptive immune response. Macrophages initiate the inflammatory responses, while activation of DCs is required for the induction of adaptive immunity.

There are two major categories of macrophages: M1 macrophages, which are induced by Th1 cytokines (IFN-γ and TNF-α); and M2 macrophages, induced by Th2 cytokines (including IL-4 and IL-13). M1 macrophages are characterized by the production of IL-1, IL-12, and TNF-α. In addition, M1 macrophages drive Th1 responses. Aging is characterized by an elevation in baseline inflammatory factors in blood, contributing to a skewed M1/M2 macrophage distribution (32, 33). Specifically, monocytes (macrophage precursors) obtained from older adults prior to influenza vaccination exhibit impaired function with decreased TNF-α and IL-6 secretion, but intact IL-10 responses (34). The dysregulation of IL-10 production from monocytes suggests its potential role in impaired influenza vaccine responses in older adults (34). This dysregulation of TNF-α and IL-6 vs. IL-10 response to influenza vaccination has been linked to the downregulation of the expression of the costimulatory molecules CD80 and CD86 by activated monocytes as a predictor of the antibody response to influenza vaccination (35). A link between CMV infection and dysregulation of DC function may be provided by the finding that CMV itself encodes an IL-10 ortholog, which is known to be expressed during latent infection of myeloid precursor cells (36). CMV-IL-10 inhibits DC function by hindering their maturation and functionality (37) and hence may also play a role in poorer responses to vaccination. Furthermore, plasmacytoid DCs from the elderly are also impaired and produce less TNF-α/IFN-γ in response to TLR7 and TLR9 stimulation (38), which has been associated with poor antibody response to influenza vaccination as well.

THE ROLE OF B-CELLS IN INFLUENZA INFECTION AND VACCINATION

As noted above, CMV may act as an environmental amplifier of immunosenescence resulting in the accumulation of large amounts of late-differentiated CMV-specific effector T-cells

(39, 40) and possibly contributing to inflammation. CMV seropositivity is also associated with intrinsically mediated increased levels of inflammatory cytokines in B-cells and diminished B-cell function that predicts poor antibody responses to influenza vaccination (41).

The B-cell response to vaccination decreases with age (42–44) and the compromised effector function of B-cells in the elderly results in lower antibody production and poor Ig class switching (45). While the intrinsic deficits found in B-cells as a result of aging are limited, they are mostly associated with lower levels of long-lived plasmablasts (46–48) and memory B-cells (46, 48, 49). Studies have shown that the age-related decrease in antibody response to influenza vaccination is correlated with extrinsic factors, including impaired T-cell help (39, 40), poor DC function (38), and high IL-10 production by monocytes/macrophages (34) as discussed above.

Influenza vaccines function by generating a B-cell and follicular helper T-cell (T_{FH}) response, which in turn results in the proliferation of vaccine antigen-specific B-cells (50, 51). It is believed that IgA and IgM specific for viral hemagglutinin (HA) protect against the establishment of initial infection though neutralization of the virus, while IgG antibodies against nucleoprotein (NP) neutralize the virus if infection becomes established (52, 53).

B-cell defects associated with aging include reduced activation-induced cytidine deaminase (AID). AID is an enzyme required for class switch recombination as well as somatic hypermutation (54) and has been found to correlate with IgG production (55). Prior to vaccination, AID mRNA levels and switched memory B-cell frequencies in response to CpG stimulation correlate with the serum antibody response and are thus predictors of the response to both vaccination and infection. In this context, older adults who are seropositive for CMV show a reduction in both AID and switched memory B-cells relative to CMV seronegatives, and a correspondingly diminished antibody response to influenza A/H1N1 strains.

Another biomarker of B-cell functionality is intracellular TNF-α, which correlates with serum TNF-α levels, which are elevated in the elderly, and more so in CMV seropositives. TNF-α places B-cells in a status of preactivation, which impairs functionality (41). Although the exact mechanism involved in reduced B-cell function by CMV is not known, it may involve a TNF-α feedback loop. Specifically, CMV induces increased production of TNF-α in B-cells *via* NF-κB induction (56). This results in a systemic elevation of TNF-α levels and contributes to CMV-associated B-cell activation, systemic inflammation, and reduced function in these older individuals (41). The important role of TNF-α is further illustrated in B-cell cultures in which TNF-α is neutralized, resulting in improved antibody class switching in elderly individuals (57).

Ambiguity of the Role of CMV and Aging on the Antibody Response to Influenza Vaccination

Some reports indicate that CMV seropositivity may be associated with better antibody response to vaccination in younger adults (58). This is different in studies involving older adults, where CMV seropositivity has been variably found to be associated with beneficial (59), negative (41, 58–62), or negligible effects (58, 63). The overall impact of CMV infection on influenza vaccine responsiveness remains controversial, as it is depends on many variables. Different studies performed with different seasonal vaccines, tested in different populations, at different times, are difficult to compare directly. As such, there have been no studies that have directly linked CMV seropositivity with increased risk of influenza illness in vaccinated older adults.

In addition to lack of consensus on the impact of aging and CMV seropositivity on antibody responses to influenza vaccination, investigating this issue is further complicated by differing responses to strains of the virus. Vaccine efficacy in the elderly against H3N2 is particularly poor compared to H1N1 or B strains (64, 65). One explanation for an apparent lack of responsiveness in the elderly may reside in the manner in which antibody responses are quantified, which is dependent on the immunological history of the individual. Thus, older adults who already have a high-antibody titer prior to vaccination may be classified as non-responders if they do not further increase an already-protective titer. More importantly, comparisons of the antibody response to influenza vaccination in young and older adults have been confounded by the effects of age and exposure history related to prior vaccination (66). In addition, these differing observations may be explained in the context of original antigenic sin, which supports the notion that vaccination re-stimulates immunological memory of past exposure to a similar strain, and may explain the relative protection of older adults against the pandemic H1N1 (pH1N1) strains (67). The theory of vaccine re-stimulation has not been explored in the context of CMV, but highlights the importance of identifying which subtype of influenza is being studied and a consensus as regards to the definition of vaccine "responder."

Contradictory observations of influenza strain-specific titers post-vaccination between CMV-seropositive and -negative individuals have also been identified. Specifically, CMV^+ subjects were found to have higher antibody titer to H1N1 (58, 59, 61), while others have observed the opposite (41). Similarly, in some studies, no association was observed between CMV status and H3N2-directed antibodies (63), while others have reported lower H3N2 antibody responses in such subjects (62). Those identifying an improved response to vaccination have hypothesized that CMV infection is accompanied by a higher level of a low-grade chronic inflammation that in turn provides an ongoing stimulation to the immune system in older (68) and younger adults (58).

It should be noted that studies in this area have used different measures of the antibody response to vaccination as a correlate of protection. Specifically, some have reported peak antibody response, while others measured antibody persistence. Although peak antibody titers after vaccination depend mainly on short-lived plasma B-cells, antibody persistence depends on memory B-cells and long-lived plasma cells. As such, antibody persistence may be a more meaningful measure of clinical protection. Some apparent discrepancies in the literature could derive from such different measures.

Other possible reasons for the discrepancies reported in the literature may be related to confounding factors such as medications, as illustrated in a recent study by Reed et al. These investigators identified a poorer antibody response (quantified based on antibody persistence) to vaccination in CMV-seropositive older adults, but only if they were taking β-adrenergic-blocking drugs (69). β-adrenergic blockers, a class of drug commonly used for blood pressure control, may also influence immune responses (70) and could therefore represent a confounding factor resulting in non-consensus of previous studies of antibody responses in older adults. However, it should also be noted that the use of β-blockers may simply reflect non-specifically generally poorer health or could represent an association with other health conditions relating to immunosenescence. The potential impact of drug treatment on vaccine response is further illustrated in the case of statins and antibody responses to influenza vaccination in older adults (71) as well as vaccine efficacy (72). Statins are known to influence immune responses *via* multiple different mechanisms (73), indicating a need to investigate the relationship further, especially since this class of drug is used by a growing number of older adults (74, 75).

Molecular Genetics As a Tool to Investigate B-Cell Function in the Context of Aging and CMV

Sequencing of the immunoglobulin heavy chain has been conducted to study B-cell receptor (BCR) repertories. It was found that V (variable), D (diversity), and J (joining) usage is consistent between age groups, although mutations in V genes are associated with CMV seropositivity. Furthermore, mutations in IgM and IgG sequences are higher in the elderly, and more so in those who are CMV seropositive (76). This suggests that repeated antigen exposure with aging and CMV reactivation induces B-cell proliferation and IgG gene mutations.

In a groundbreaking study, de Bourcy et al. used next-generation sequencing technology to study BCR diversity. They showed that while BCR repertoires become more specialized over the lifespan, they also demonstrate decreased capacity for plasticity or adaptability. Relative to the young, older adults have a smaller naïve repertoire and lower intra-lineage diversity, resulting in a reduced ability to mount a diverse response to novel antigens (77). This suggests that annual updates of the strains of influenza contained in the vaccine are less likely to induce antibody responses to new viral variants in older adults.

The association of leukocyte telomere shortening with some aspects of aging has been well documented. In a seminal study of the correlation between telomere length of B-cells and humoral immune responses to influenza vaccination in adults over the age of 70, it was shown that those with longer telomeres (6.3 kb) had superior antibody responses (based on fold-increase of influenza-specific antibody titers) relative to those with shorter telomeres (5.6 kb) (17). While the mechanism involved is still not well understood, telomere length could be used as a marker for immune function and vaccine responsiveness.

THE ROLE OF T-CELLS IN INFLUENZA INFECTION AND VACCINATION

The antibody response to influenza virus plays a vital role in protection against influenza infection, but epitope-specific T-cells are also critical (78–80). Although assessment of antibody responses to influenza vaccines is mainly used as a measure of efficacy, studies continue to show that humoral immunity by itself does not provide sterilizing immunity against infection in older adults, and that T-cell responses are critically important when antibody-mediated protection fails (81). Furthermore, T-cell responses are cross-reactive within the strains of influenza A or influenza B, allowing for broad protection against drifted strains of influenza (82).

Decreased output of naïve T-cells resulting from thymic involution after puberty results in a reduced ability to respond to novel antigens (83) and has been linked to poor response to influenza vaccination in the elderly (84). Studies have shown that telomere length of T-cells specific for CMV are longer on average than those specific for influenza and may suggest that CMV continues to recruit cells from the naïve T-cell pool over time (85).

Aging is correlated with a loss of naïve $CD8^+$ T-cells, more so than naïve $CD4^+$ T-cells. This loss in naïve cells is not associated with CMV seropositivity (86). The loss of naïve $CD4^+$ T-cells is associated with an increase in effector and effector memory $CD4^+$ T-cells and is observed essentially only in CMV-seropositive individuals (86). These findings illustrate the distinct, and sometimes additive effect of aging and CMV in different T-cell populations.

Helper T-Cells

Influenza infection induces HA-specific $CD4^+$ T-helper cells (87), resulting in a diverse antibody response (88). Th2 responses stimulate antibody production that is driven by the production of cytokines, including IL-4, IL-5, IL-10, IL-13, IL-31, and IL-33 by mast cells and eosinophils, which are responsible for a Th2 response, thereby leading to the activation of B-cell clones and production of influenza-specific IgG1 and IgE (89, 90). The regulatory T-cell (Treg) response results in the expression of IL-10 and TGF-β, which further hinders a Th1 response (91).

Aging is associated with an increasing acquisition of a Th2 bias, specifically with an increase in $CD4^+CD294^+$ (Th2) cells (92). Furthermore, some studies have reported a decline in the total number of $CD8^+$ T-cells and increases in T-helper cells reflected in a lower Th1:Th2 ratio (92). IL-10 and other cytokines produced by Th2 or Treg have been associated with reduced cytotoxic T-lymphocyte (CTL) activity in older adults (93) and against *ex vivo* influenza virus challenge (94). Although Th2-associated cytokines do not help in the recovery from influenza infection (95), these cytokines continue to be expressed at high levels at the site of influenza infection and may be a factor in inflammation and lung damage associated with infection (78).

Role of T_{FH} Cells during Influenza Infection and Vaccination in the Elderly

IL-12 production by activated DCs induces naïve $CD4^+$ T-cells to differentiate into IL-21-producing T_{FH} cells (96, 97). Elevated IL-21 is positively correlated with CMV seropositivity (98) as it is believed to be required for the maintenance of latent infection (99–101) as well as clearance of acute viral infections (102, 103).

T_{FH} cells are a separate type of helper T-cells that are known to promote germinal center formation, B-cell survival, proliferation, class switching, plasma cell differentiation, and somatic hypermutation (104–108) and are hence found in germinal centers (GC T_{FH}) and in peripheral blood (pT_{FH}). Studies have found a direct relationship between the frequency of activated pT_{FH} cells following vaccination and influenza vaccine-induced antibody responses (109–111). It has also been shown that pT_{FH} cells isolated post-influenza vaccination are better able than other $CD4^+$ T-cell subsets to support B-cell differentiation and to stimulate influenza-specific antibody secretion (110).

A study of older women found a high frequency of activated T_{FH} cells ($CD38^+HLA\text{-}DR^+Ki\text{-}67^+$). The presence of activated pT_{FH} cells in older women prior to influenza vaccination was negatively correlated with antibody titers post-vaccination and suggests that activated pT_{FH} cells are less capable of providing help to B-cells when faced with new antigens (112).

Cytotoxic T-Lymphocytes

Aging creates specific challenges to effective CTL activity against infection: (a) T-cell receptor (TCR) diversity reduction, (b) reduced effector function of cells, (c) cell type frequency changes, and (d) general inflammation.

Cytomegalovirus elicits $CD4^+$ and $CD8^+$ T-cell responses (113, 114), which play an important role in maintaining latency of CMV infection (115). The immune response necessary to maintain latency has two major consequences: driving T-cells to a late-differentiated state associated with immunosenescence (6, 14, 116) and memory inflation (117). The latter develops from chronic antigen exposure as a result of CMV infection (118, 119), but interestingly Epstein–Barr virus does not have the same impact on inflation (120). CMV-specific memory T-cells account for 0.1–40% of the total memory population in the periphery (113, 114). The high frequency of CMV-specific T-cells develop during the first year after infection and either gradually increases or remains constant long term (121–123). Over a lifetime, a large group of T-cells recognizing CMV epitopes emerges, the majority of which may be dysfunctional (124). This contributes to the concept of a restricted "immunological space," whereby the T-cell populations consisting of dysfunctional clonally expanded and anergic cells are targeted toward a small number of epitopes (125). In addition, aging results in a decrease of the TCR repertoire, which is associated with a poor response to influenza vaccination (126–129). Furthermore, the repertoire may become oligoclonal due to extended lifespan and homeostatic turnover of naïve T-cells (130).

On infection, viral epitopes bind to the major histocompatibility complex (MHC) molecules of APCs, and through the interaction with TCRs activate naïve or memory T-cells to become effector CTLs (131, 132). The majority of the $CD8^+$ T-cell epitopes derived from influenza virus are contained within the NP and matrix 1 (M1) proteins. As a result of the homology of these internal proteins and highly conserved epitopes across the different subtypes (A/H1N1 and A/H3N2), the $CD8^+$ T-cell response to influenza is cross-reactive among all of the strains of influenza A. Activation of T-cells leads to their migration to the infection site where they recognize influenza virus-infected cells and eliminate them *via* lytic activity. While CTL killing of influenza-infected cells can be mediated through Fas- (132), and TRAIL- (133) associated pathways, the dominant mechanism appears to be perforin-mediated killing (134). Recent studies suggest that perforin-mediated killing through granzyme B (GrB) apoptotic pathways is the most critical for viral clearance (135).

Role of GrB in Protection against Influenza Infection

A correlation between low GrB prior to H3N2 infection, fever, and lack of seroconversion is indicative of the association of cell-mediated immunity and illness severity (136). GrB levels increase in response to H3N2 infection independently of serological responses (136), with a deficiency in the production of GrB and IFN-γ in $CD8^+$ T-cells observed in vaccinated older adults (137, 138).

Granzyme B has been associated with clinical protection from influenza (139) and is produced by both $CD4^+$ and $CD8^+$ T-cells (140). Late-differentiated T-cells ($CD45RA^+GrB^+$ $Perforin^-$) particularly $CD8^+$ subsets are abundant (with as many as 50% of these cells producing GrB in the resting state) and are associated with poor $CD8^+$ T-cell cytolytic activity following influenza vaccination (137, 141). Of note, the cytolytic activity of $CD8^+$ T-cells dramatically declines by 10 weeks post-vaccination, and while this occurs to a lesser degree in $CD4^+$ T-cells, their cytolytic potential is relatively minor compared to $CD8^+$ T-cells (137). However, this discordant change in $CD4^+$ and $CD8^+$ vaccine-specific T-cells suggests that $CD4^+$ CTLs in older adults could be targeted to promote cell-mediated immune protection *via* vaccination.

$CD4^+$ T-cells in the lung expressing GrB and perforin have cytolytic activity against influenza in mice (142, 143). Using influenza M1 peptide stimulation, it has been shown that the proportion of subjects mounting a $CD4^+$ T-cell response was lower in CMV-seropositive than seronegative individuals (16).

Granzyme B and perforin-expressing $CD4^+$ T-cells also produce IFN-γ, suggesting a Th1 lineage (143, 144). Many $CD4^+$ T-cells responding to influenza in the lung produce IL-10, largely in cells also producing IFN-γ (78, 145). This IL-10 production by influenza-specific $CD4^+$ T-cells results in reduced protection (146) by suppressing cytokine production in Th17 cells (78, 145), but also plays an important role in limiting immunopathology (78).

Cytomegalovirus-seropositive older adults have higher levels of GrB in resting T-cells, the majority of which have a late-differentiated T-cell phenotype ($CD45RA^+$) or are $CD28^-$ (147). The accumulation of GrB in putatively terminally differentiated $CD8^+$ T-cells in the absence of perforin *in vivo* (147) suggests that GrB may be released into and accumulate in the extracellular space, resulting in inflammation and tissue damage (148–150). It has also been shown that *ex vivo* live influenza virus challenge results in a lower GrB response in CMV-seropositive compared to -seronegative older adults (63), further suggesting an impairment of CTL response to influenza mediated by CMV.

Impact of Reduced $CD28^+$ T-Cells in Elderly and CMV Seropositivity

CD28 has an important function as a costimulator in the activation of T-cells and influences their susceptibility to apoptosis

(151). It is required for optimal T-cell activation, but its level of expression by CD8+ T-cells decreases with age (152, 153). This cell phenotype has been associated with a poor response to influenza vaccination (39, 40, 154), and it has been suggested that it has some similarities with replicative senescence (155). It has also been shown that CMV infection contributes to the accumulation of these cells (156, 157). In addition to CD8+CD28− cells impacting vaccine response, late-stage differentiated CD4+ T-cells, lacking CCR7, CD27, and CD28 and re-expressing CD45RA are also found in CMV-seropositive subjects and correlated with poor vaccination response (158). A low frequency of CD45RA re-expressing late-differentiated CD4+ T-cells are found in CMV-seropositive individuals, independent of age (159–161), with the majority of these cells being CMV specific (158, 160).

CD4:CD8 Ratio As a Biomarker of CTL Function

CD4:CD8 T-cell ratios contribute to immune risk profiles with a ratio of less than 1 being predictive of 2-year mortality in some studies (162, 163). CMV infection has been found to be associated with a CD4:CD8 of <1 (13, 164). These findings are consistent with the notion that the clonal expansions of CD8+ T-cells observed in the elderly (165–167) are to a large extent CMV-specific (124) and associated with mortality (168). Changes in the ratio were found to be the result of an increase in CD8+ T-cell populations, specifically, CD27−, CD28−, CD56+, and CD57+, CD45RA+, and CD45RA+/RO+ cells (13), markers that indicate reduced effector functionality and provides support for the finding of poor humoral response to influenza vaccination in those with a low CD4:CD8 ratio (169). Furthermore, the majority of the CMV-specific CD8+ T-cells in the elderly have reduced functionality, supported by the finding that the fraction of cells producing IFN-γ in response to peptide stimulation in the elderly was significantly lower than in the young (124). Elderly with a CD4:CD8 ratio <1 had about 10% of total CD8+ T-cells specific for a single CMV epitope (170), but in the group with a CD4:CD8 ratio >1, the frequency was similar to the middle-aged group (124).

ALTERATIONS TO VACCINE DESIGN

In addition to the aging immune system and CMV seropositivity both potentially hindering the immune response to influenza vaccination, other challenges to effective vaccine design are also in play. Challenges faced particularly in influenza vaccine development lie in the high level of strain divergence from season to season resulting from error-prone replication of the influenza virus *via* RNA-dependent RNA polymerase, recombination, and genetic drift (171). While the plasticity of the virus is sustained when variability accumulates in the HA and neuraminidase (NA) proteins, internal proteins are not as capable of maintaining functionality in the face of mutation accumulation, resulting in much less variation in the matrix and NP (172). The current vaccine strategy is designed to stimulate the development of antibodies against the HA and NA proteins, resulting in the ongoing need for annual vaccine modification to account for viral strain variation, which may be obviated to some extent through vaccines designed to also target internal proteins.

While antibody titers are the generally accepted standard for the testing of influenza vaccination protection, it has been suggested that it is a poor measure if used alone to assess protection in the elderly (173). The limitations of antibody titers are apparent when examining the strain-specific differences in the elderly. For example, vaccine efficacy against H3N2 in the elderly is particularly poor compared to H1N1 or B strains (64, 65), while concurrently, others have found higher antibody titers against the H3N2 strain post-vaccination in this group (174–176).

The focus on humoral immune protection against influenza has its limitations, as circulating strains may not match the vaccine (177). Current vaccines do not induce sufficient cross-reactive CD8+ T-cells to provide protection against non-homologous influenza A virus challenge (178), but this would be an advantageous characteristic of future vaccine candidates.

Dosage

Studies comparing dosage of trivalent inactivated influenza virus vaccines in older adults have found that people who received high dose vaccinations had significantly higher antibody titers for all three strains (to varying degrees) than those who received standard dose vaccinations (179, 180). High dose vaccine recipients had a greater frequency of pT_{FH} cells post-vaccination than those receiving a standard dose. Specifically, the expression of CD278 (also referred to as inducible T-cell costimulator: ICOS) on the pT_{FH} cells was elevated (181), suggesting an increase in their ability to provide B-cell help. Furthermore, as mentioned previously, the frequency of pT_{FH} cells was a predictor of seroconversion for all three vaccine strains (181). It has also been shown that the longevity of the antibody response is not influenced by vaccine dose (182). An assessment of the impact of vaccine dose on the cellular immune response has been limited to analysis of IFN-γ response, which found no significant difference (182, 183). However, this important issue requires further study.

While the high dose vaccine has been shown to deliver better clinical protection in older adults (184), further investigation as to the mechanism is required. Furthermore, the impact of vaccine dose in more vulnerable older adults, including those who are CMV seropositive also requires investigation.

Adjuvants

The elderly might benefit from the increased application of adjuvanted vaccines. For example, glucopyranosyl lipid adjuvant (GLA) is a TLR4 agonist that has been found to be safe and well tolerated (185). GLA, when combined with Fluzone, a split virus vaccine, showed enhanced antibody response as well as a shift to a Th1 cytokine profile in mice (185). *In vivo*, GLA produces a shift toward a Th1 cell-mediated response to influenza challenge by reducing IL-10 expression along with an increase in GrB activity (186). It has also been shown *in vitro* that GLA can induce the maturation of human DCs with an associated release of Th1-inducing cytokine and chemokine constellation (187).

Older adults seropositive for CMV have been found to have lower levels of activated DCs than young CMV+ adults, as it is believed that the older group has a greater control of infection due to higher CMV-specific antibodies. Due to a lower preactivated state, the DCs of older adults, regardless of CMV status, were also relatively more responsive to TLR4 antagonist (in this case LPS) and were able to produce TNF and IL-6 at the same level, and same cell frequency as younger adults who were CMV seronegative (188). Thus, the use of a TLR4 agonist in influenza vaccines for the CMV-seropositive elderly may be a potential adjuvant to improve vaccine efficacy in this group.

Vaccine Antigen

Conserved proteins, NP and M1, share high levels of homology among influenza strains (189, 190) and contain immunodominant MHC Class I and II epitopes (191). At the present time, influenza vaccines are designed to have specific quantities of HA protein, but the quantity of the internal proteins are believed to be low and are not quantified as part of vaccine formulation quality control (192). Evidence suggests that the inclusion of NP and M1 proteins in influenza vaccines would develop a Th1 memory and provide more effective protection in older adults.

Clinical studies of mRNA-based vaccines demonstrated their ability to elicit functional antibodies and T-cell responses (193, 194). Unlike previously considered DNA-based vaccines, there is no concern regarding genome integration, or the need to design nuclear transport mechanisms for mRNA vaccines. RNA-based vaccines allow for cell entry, viral protein translation, and broad immune responses, while also acting as an adjuvant (TLR7/8) (195). Two different forms of RNA-based vaccines are currently being developed against influenza: non-amplifying mRNA (196) and self-amplifying mRNA molecules (197, 198). A modified vaccinia virus Ankara-based vaccine against influenza has been developed which consists of non-replicating RNA encoding both NP and M1 proteins (199). This vaccine was found to be safe and able to stimulate T-cell responses in older adults similar in magnitude to young adults (200). Amplifying mRNA (SAM1 technology) is based on non-viral delivery of antigen-encoding RNA by modified alphavirus single-stranded RNA, which allows for viral RNA replication and greater viral protein translation (193, 194, 197). In a comparison of replicating and non-replicating vaccines, it was found that self-replicating vaccines elicit significantly stronger cellular and humoral immune responses, which was suggested to be the result of greater antigen presentation (201). These findings suggest that self-replicating influenza RNA vaccines may be used to overcome the effects of immune senescence and CMV seropositivity.

Vaccination Schedule

A study mapping the prevalence of influenza-specific antibodies in children found that by the age of 6, all children had seroconverted and thus had immunological memory for at least one influenza virus strain (202). Over time, this memory is boosted and diversified by subsequent infections with drifted influenza virus strains. In turn, this memory response (both humoral and cellular) can act to protect against infection with similar stain variants, referred to as cross-protection. Cross-protection was shown in the pH1N1 virus identified during the 2009–2010 and 2013–2014 influenza outbreaks which resulted in high rates of morbidity and mortality. Analysis of the CD8+ T-cells specific for the 2009 pH1N1 virus identified epitopes shared with the 1918 pH1N1 strain, as well as stains circulating prior to 1945 (203). These findings corroborate the observation that the severity of the influenza illness in the over-65 age group infected with pH1N1 was considerably lower than other influenza seasons (204), suggesting cross-protection. Furthermore, it was found that the majority of severe cases of pH1N1 infection occurred in young adults (205). This has led to suggestions that vaccines may only be effective due to their ability to act as a booster to memory CD8+ T-cells remaining from previous infections, rather than creating a memory response to new targets (137). Furthermore, evidence suggests that T-cells generated in youth can remain protective over decades (206, 207), while those derived later in life are severely impaired (208, 209). Thus, it could be postulated that the most effective way to ensure protection *via* vaccination in older adults would be to design a vaccine strategy targeted at youth.

Vaccine effectiveness against influenza declines with time after vaccination over the winter season and is most evident in older adults, with efficacy lasting 140 days for H3N2 and 200 days for influenza B (210). Interestingly, vaccine effectiveness appeared to be maintained for over 200 days for subtype H1N1 (210). Influenza vaccines are only administered once a year but the impact of a double dose or booster vaccines is being studied to determine whether efficacy and level of protection can be improved. At present, a clinical trial of vaccines administered bi-yearly in older adults is underway (http://ClinicalTrials.gov: NCT02655874). The benefit of two-dose influenza vaccines has been shown under somewhat different circumstances in studies of solid organ transplant recipients where a second influenza vaccine dose after 5 weeks resulted in higher rates of seroconversion and seroprotection (defined as titer ≥1:40) (211). Frail older adults who were non-responders to an initial dose of influenza vaccine showed a decline in antibody titers to the A/H3N2 strain following a booster dose of influenza vaccine and derived no clinical benefit from this booster strategy (212). These results are consistent with our earlier studies showing a significant IL-10 response to a booster dose vaccination and a decline in the antibody and GrB response to influenza challenge following vaccination, compared to those older adults who received a single dose of influenza vaccine (213). Studies of H5N1 have shown that a two-dose approach using heterosubtypic H5 antigen results in a greater magnitude of T-cell cytokine response with heterosubtypic protection, but this does not seem to be the case in older adults (214). As such, the impact of a two-dose influenza vaccine is not yet clear and requires further investigation to determine effectiveness.

Reducing the Impact of Immunosenescence through Anti-CMV Strategies

One possible method to improve influenza vaccine efficacy might be an anti-CMV strategy *via* vaccination or treatment. While the majority of CMV treatment strategies have been developed for the prevention of congenital CMV infection or for the immunocompromised, there is potential for its application in the wider population.

The challenge in CMV vaccine design lays in the extensive genetic diversity of CMV strains due to recombination (215, 216). Several multi-component subunit (217), recombinant live-attenuated (218), and DNA (219) vaccine candidates aimed at achieving broad cross-neutralizing humoral and cellular immune responses are currently under investigation. One clear caveat for the CMV vaccine strategy to be effective is the need for its administration at a young age, and the ability for the vaccine to impart long-lasting immunity. Drug treatments may be a more

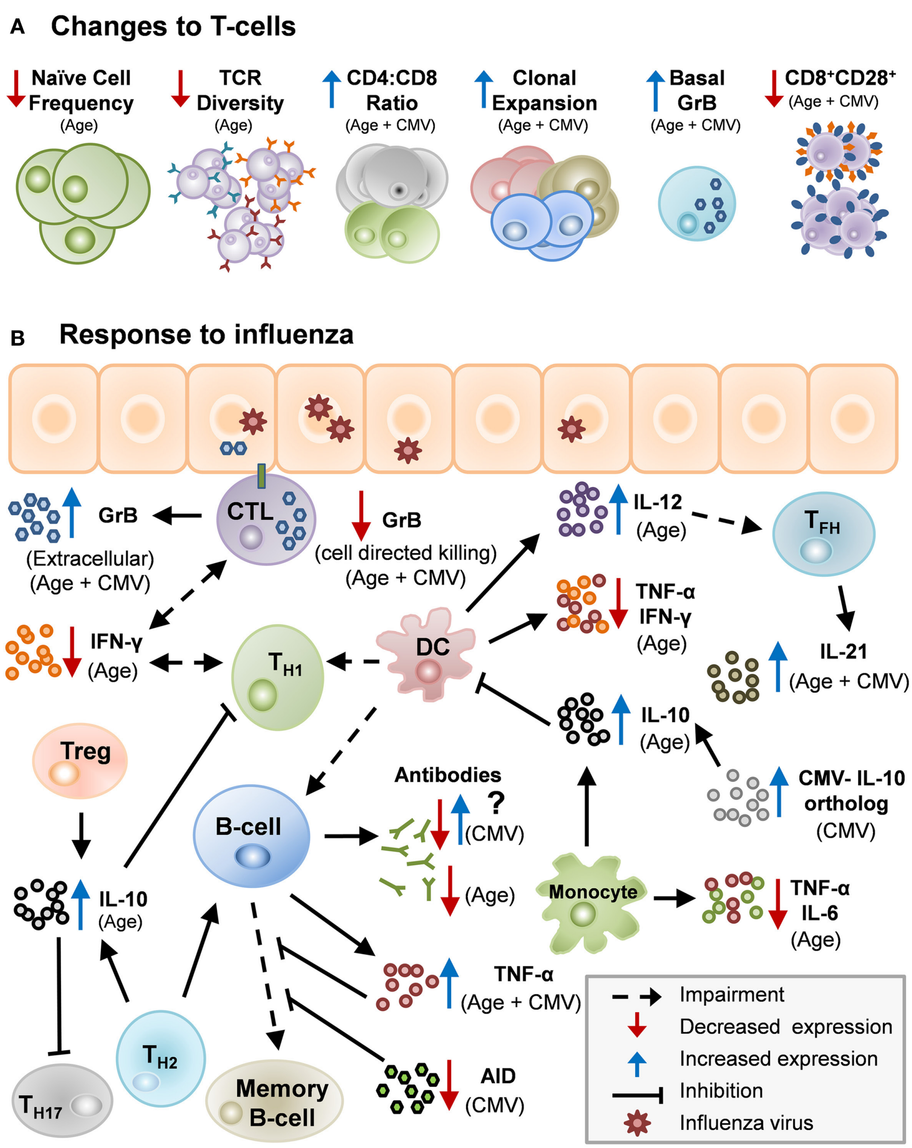

FIGURE 1 | Potential mechanisms by which age and cytomegalovirus (CMV) may cause changes in human immunity. **(A)** Age and CMV can act in unison to impair aspects of the immune system: lower numbers of naïve T-cells, decrease in T-cell receptor (TCR) repertoire, heightened CD4:CD8 ratio, clonal expansion, increased levels of basal granzyme B (GrB) in resting T-cells and decrease in CD8+CD28+ cells. **(B)** There are several potential mechanisms by which age and CMV may cause changes in human immunity. Influenza infection stimulates Th1/Th2 although impaired in older adults due to diminished antigen-presenting cell function in the elderly. Th1 response with IFN-γ activating memory cytotoxic T-lymphocyte (CTL) which clears virus from the lungs. Age-related changes drive a Th2/regulatory T-cell (Treg) response to infection, and IL-10 production suppresses the CTL response. CMV infection further impairs the response to influenza infection by contributing to age-related impairments and by other mechanisms.

TABLE 1 | Summary of the impact of cytomegalovirus (CMV) in the elderly and resulting influence on the response to influenza infection and vaccination.

Impact of CMV	Impact on immune system	Impact on influenza infection/vaccination	Reference
CMV-encoded IL-10 ortholog	Inhibits dendritic cell function by hindering maturation and functionality	Potentially poor antigen-presenting cell capacity during infection or response to vaccination	(37)
Elevated IL-21	Greater frequency of activated pT_{FH}	Associated with improved antibody response to influenza vaccination	(109–112)
Reduction in activation-induced cytidine deaminase	Impaired class switch recombination and somatic hypermutation	Diminished antibody response to influenza vaccination	(54, 55)
Increased TNF-α in B-cells	Causes B-cell activation, systemic inflammation, and reduced function	Poor antibody class switching	(41, 56, 57)
Lower GrB response		Potential impairment of cytotoxic T-lymphocyte response to influenza infection	(63)
Loss of naïve $CD4^+$ and $CD8^+$ T-cells	Associated with an increase in effector and effector memory $CD4^+$ and $CD8^+$ T-cells	Potentially reducing the ability to develop a response to new virus antigens	(86)
Elevated number of $CD8^+CD28^-$ and $CD4^+CD28^-$ cells	Reduced ability for cell activation	Associated with poor response to vaccination	(156–160)

practical approach in the short-term including those with anti-CMV activity *in vitro* currently being tested in clinical trials: a protein kinase inhibitor with specific activity against CMV (220); a CMV terminase inhibitor (221); and a broad spectrum antiviral agent (220).

CONCLUSION

Aging, along with CMV seropositivity, impacts the immune response to influenza infection and vaccination as a result of many interacting mechanisms (**Table 1**; **Figure 1**). With the increased risk of influenza-associated morbidity and mortality in the over-65 population, it is critical to take these impairments into consideration when developing the next generation of influenza vaccines.

AUTHOR CONTRIBUTIONS

SM developed the initial outline and draft of the manuscript. GP, GK, and JM revised the manuscript critically for important intellectual content. All the authors read and approved the final manuscript.

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Impact of Age, Caloric Restriction, and Influenza Infection on Mouse Gut Microbiome: An Exploratory Study of the Role of Age-Related Microbiome Changes on Influenza Responses

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Immunosenescence refers to age-related declines in the capacity to respond to infections such as influenza (flu). Caloric restriction represents a known strategy to slow many aging processes, including those involving the immune system. More recently, some changes in the microbiome have been described with aging, while the gut microbiome appears to influence responses to flu vaccination and infection. With these considerations in mind, we used a well-established mouse model of flu infection to explore the impact of flu infection, aging, and caloric restriction on the gut microbiome. Young, middle-aged, and aged caloric restricted (CR) and ad lib fed (AL) mice were examined after a sublethal flu infection. All mice lost 10–20% body weight and, as expected for these early time points, losses were similar at different ages and between diet groups. Cytokine and chemokine levels were also similar with the notable exception of IL-1α, which rose more than fivefold in aged AL mouse serum, while it remained unchanged in aged CR serum. Fecal microbiome phyla abundance profiles were similar in young, middle-aged, and aged AL mice at baseline and at 4 days post flu infection, while increases in Proteobacteria were evident at 7 days post flu infection in all three age groups. CR mice, compared to AL mice in each age group, had increased abundance of Proteobacteria and Verrucomicrobia at all time points. Interestingly, principal coordinate analysis determined that diet exerts a greater effect on the microbiome than age or flu infection. Percentage body weight loss correlated with the relative abundance of Proteobacteria regardless of age, suggesting flu pathogenicity is related to Proteobacteria abundance. Further, several microbial Operational Taxonomic Units from the Bacteroidetes phyla correlated with serum chemokine/cytokines regardless of both diet and age suggesting an interplay between flu-induced systemic inflammation and gut microbiota. These exploratory studies highlight the impact of caloric restriction on fecal microbiome in both young and aged animals, as well as the many complex relationships between flu responses and gut microbiota. Thus, these preliminary studies provide the necessary groundwork to examine how gut microbiota alterations may be leveraged to influence declining immune responses with aging.

Keywords: aging, influenza, gut microbiome, caloric restriction, cytokines

INTRODUCTION

Aging is a complex process that has dramatic impacts on most systems in the body (1). This is especially true of the immune system where significant age-related changes are observed in both innate and adaptive immune responses. One of the most prominent manifestations of aging is an increase in susceptibility to infections. In fact, influenza infection is one of the top killers of elderly people in the world, with the oldest being most at risk (2, 3). In aging mouse models, the clearance of influenza virus is slower and T cell responses are reduced and delayed when compared to young mice, which closely mirrors what happens during influenza infection of elderly humans (4–7). In addition, our recent study demonstrated that there are lingering inflammatory cytokines such as IL-6, IL-1α, and G-CSF in the bronchiolar lavage fluid (BAL) of aged mice during influenza infection. We have also shown that there is an increasing level of albumin in the BAL of aged mice, which is indicative of lung damage during the later stages of infection (8). These results indicate that the inability to efficiently clear virus in aged lungs corresponds to extended inflammation and lung damage.

One of the most consistent ways to delay aging in mice is *via* caloric restriction (CR). Indeed, CR was first shown to extend the lifespan of rodents in the 1930s (9) and has since been observed across multiple species, ranging from invertebrates to rodents to even some non-human primate studies (10–13); however, it is important to note that this is not observed in all studies (14–19) suggesting some mechanisms of longevity with CR may not be conserved among species and that details of implementation likely effect outcomes. Despite these discrepancies, and more importantly, along with extending lifespan, CR has been shown to delay age-related deficits in multiple physiological systems in mice (20–25), seemingly targeting the process of aging itself. From a translational perspective, human CR studies assessing longevity are nearly impossible, and short term studies evaluating other healthspan measures are difficult to control and criticized for practicality (please refer to Ref. (26) for a recent review of human CR trials). Regardless of these limitations, results from both population studies (27), as well as the Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy (28–30) and Caloric Restriction with Optimal Nutrition (31, 32) studies show benefits in some areas, albeit not in all aspects of CR in rodent trials, suggesting CR does hold value within human aging research. Moreover, the benefits to multiple different systems evident in rodent studies makes it an attractive avenue to pursue. It is important to note that CR in these cases is not malnutrition (although feed is generally reduced by 40% caloric content, it is fortified with micronutrients to prevent deficiencies), and it is normally introduced after mice have reached maturation. There are many hypotheses for the mechanism of action of CR, including modulation of glucose–insulin homeostasis, growth hormone axis, autophagy changes, and alteration of inflammatory pathways; however, it is likely many of these changes may act in concert to delay aging. Nonetheless, CR has consistently shown improvement in multiple facets of aging, including delaying immunosenescence (33–37). Most notably, CR attenuates the shift from naive to memory phenotype observed in T cells with aging (38) and maintains the proliferative capacity of T cells (39, 40). Despite these positive effects on the aged immune system, the effects of CR on immune responses to influenza with aging is not clear. Original studies by Effros et al. (41) demonstrated that CR could enhance the immune response in aged mice ameliorating age-related declines in T cell proliferation and antibody production in response to intraperitoneal immunization to influenza. Conversely, work from Gardner and colleagues (42–45) demonstrated increased severity of infection in aged CR mice with increased viral titers and mortality in response to intranasal influenza infection attributed to impaired NK cell responses (43, 44) and/or reduced energy reserves and lethal weight loss (46). However, in these studies, high doses of influenza were utilized with mortality even observed in young mice; thus, it is unclear how CR modulates the immune response to more sublethal doses of influenza.

More recently, the importance of the microbiome in regulating immune responses has been elucidated. The gut microbiota have regulatory effects on not only intestinal immunity but also systemic immune responses (47) and systemic T cell subset populations can be skewed by different microbiota predominance (48–51). Further, pulmonary immunity is directly affected by the gut microbiota with regards to both allergic airway (52) and infectious disease (53, 54) responses. Importantly, the gut microbiota plays a major role in mediating flu infection-related immune responses and is particularly crucial for respiratory tract DCs migration, T cell priming, cytokine secretion, and overall viral clearance (53). Dysbiosis of the gut microbiota induced by antibiotic administration during flu infection influences helper T cell responses and can negatively impact flu outcomes and recovery (55). In addition, influenza infection itself induces gut microbiota dysbiosis through type I interferons (IFN-I) favoring Proteobacteria overgrowth (56). Thus, the relationship between gut microbiota and influenza infection seems complex and integrated.

Furthering this line of thought, the gut microbiome also changes with age [recently reviewed in Ref. (57, 58)]. More specifically, there seems to be a general decrease in microbiota diversity with aging (59, 60), as well as an increase in Proteobacteria abundance and lower levels of Firmicutes (59–63). Also, a shift toward a more Bacteroidetes dominated microbiome was associated with frailty (61). It is unknown how these changes to gut microbiota with aging may influence immune responses. CR also impacts the microbiome with greater levels of Lactobacillus and other potential probiotics associated with longevity (64). But importantly, the influence of gut bacteria microbiota changes on age-related pathologies has yet to be determined. It is possible that gut microbiota changes with CR may be a potential mechanism of longevity and/or related to some of the “antiaging” effects evident in many murine studies.

It is known that different components of nutrition can affect the aged immune system [reviewed elsewhere (65)] and that prebiotic/probiotic supplementation may decrease the severity of infection in the elderly (66–69). Moreover, small-scale studies have indicated that specific probiotics and prebiotics may improve flu vaccine response in hospitalized elderly (70–73) suggesting that age-related alterations in gut microbiota may precipitate reduced flu responses in the elderly, and that this dysbiosis can be treated to improve immune responses. Indeed, depletion of specific gut microbiota through antibiotic treatment can negatively affect both DC (53) and T cell (53, 55) influenza immune responses, while the gut microbiota itself is affected by influenza infection through type I interferons (IFN-I) (56), thus the relationship between gut microbiota and influenza infection is bidirectional and warrants further investigation. Here, in this exploratory study, we sought to examine how CR, a known modulator of aging and gut microbiota, can influence influenza-induced gut microbiota changes and immune responses during acute influenza infection in young, middle, and aged C57BL/6 mice. We hypothesized that CR would protect aged mice from age-related gut microbiota changes and thus mitigate influenza-induced changes to gut microbiota and improve immune responses.

MATERIALS AND METHODS

Mice

Young (5–6 months old), middle (9–10 months old), and aged (19–21 months old) caloric restricted (CR) and *ad libitum* (AL) C57BL/6 male mice from the National Institute on Aging caloric restricted rodent colony were obtained at least 6 weeks prior to experimentation to allow appropriate acclimation to our facility. CR mice were fed the NIH31 fortified diet with CR was initiated at 14 weeks of age at 10% restriction, increased to 25% restriction at 15 weeks, and to 40% restriction at 16 weeks where it was maintained throughout the life of the animal. AL mice were fed the NIH31 diet for their entire life. All mice were singly housed in a climate controlled environment with 12:12 light:dark cycle and water was provided *ad libitum*. For all analyses, 2–3 mice per group were analyzed. Due to the closing of the NIA caloric restricted rodent colony, we were unable to obtain more mice for experiments and thus the results presented are preliminary insights into the ability of CR to modulate gut microbiota and influenza responses with aging. All procedures were approved by the University of Connecticut School of Medicine Institutional Animal Care and Use Committee and carried out in accordance with these regulations. All mice underwent gross pathological examination at time of sacrifice and animals with obvious pathology were excluded from the study.

Viral Infection and Analysis

To infect with Influenza virus A/PR/8/34 (PR8), 400 EID_{50} were given intranasally in 40 µl to isoflurane anesthetized animals. Mice were sacrificed 7 days post infection. Lungs were harvested and the viral burden in mRNA from digested whole lung tissue was determined by real-time PCR measuring influenza *polymerase acidic protein* gene (PA) copy number (74, 75). BAL was collected by flushing lungs with 1 ml saline. BAL and serum were assayed for cytokine and chemokine content using the Luminex Mouse Cytokine/Chemokine 32-plex panel (EMD Millipore, Billerica, MA, USA).

Stool Collection and Microbiome Analysis

Fecal samples were collected between 6:00 a.m. and 7:00 a.m. in the morning each day beginning 3 days prior to infection (day −3) and stored at −80°C immediately after collection for microbiome analysis. A total number of 187 samples were collected. Total DNA was extracted from fecal samples by using Power Soil DNA Extraction kit (Mo Bio Laboratories, Carlsbad, CA, USA) per manufacturer's protocol. Bacterial 16S ribosomal RNA gene was amplified by using the 27F/534R primer set (27F 5′-AGAGTTTGATCCTGGCTCAG-3′, 534R 5′-ATTACCGCGGCTGCTGG-3′). PCR reactions were performed using phusion high-fidelity PCR Mastermix (Invitrogen, Carlsbad, CA, USA) with the following condition: 95°C for 2 min (1 cycle), 95°C for 20 s/56°C for 30 s/72°C for 1 min (30 cycles). PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) according to manufacturer's protocol. Library was prepared with Illumina's instruction specifically for Miseq platform. 17 samples failed the DNA extraction and sequencing library preparation. Full microbiome data are available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA393321.

Statistical Analysis

Weight loss and cytokine/chemokine parameters were analyzed *via* two-way ANOVA with Bonferroni *post hoc* corrections (GraphPad Prism, GraphPad Software, Inc., La Jolla, CA, USA). Raw sequencing reads were assembled using FLASH (75). Chimeric sequences were removed using USEARCH (76). Operational taxonomic units (OTUs) were generated at ≥97% sequence similarity. Taxonomic assignment of OTUs was performed by comparing sequences to the RDP database (Confidence threshold = 50%) (77). The R package "phyloseq" (78) was used for alpha diversity and beta dissimilarity analysis. Relationship between microbiota phyla and influenza-induced weight loss and serum/BAL cytokine/chemokine concentration were analyzed *via* Spearman's correlation using GraphPad software for Mac 6.0 (GraphPad Prism, La Jolla, CA, USA) and R package "corrplot" (79). Corrplot: visualization of a correlation matrix. R package version 0.77.

RESULTS

The goal of this study was to explore the interaction of aging, diet, and influenza infection with the gut microbiome. We aimed to gain preliminary insight into how aging may impact influenza-induced microbiome changes, as well as how age-related microbiome changes may impact influenza responses. The first part of the study examined how age and CR impact the response to flu, while the second part examined the effect on the composition of gut microbiome. For these studies, we employed a sublethal infection dose and examined immune parameters at 7 days postinfection. At this time point, influenza-induced weight loss becomes more evident, however, is not different between ages (80) or CR and AL groups (unpublished experiments from the Haynes lab). Thus, percent weight loss should not be a confounding factor between groups and should not put CR mice at greater risk to succumb to infection (45, 46). While this limits the observed differences between groups due to infection, it allows us to assess early time points where the gut microbiome may play a role. Additionally, since it is known that weight loss itself affects gut microbiota (81–83), this control was necessary to gain preliminary insight into influenza-induced alterations.

Effect of Age and CR on the Response to Influenza Infection

Weight loss was monitored throughout the experiment. **Figure 1A** shows that there were no significant differences in percent weight loss in any of the groups regardless of age or diet. In addition, **Figure 1B** shows that on day 7 postinfection, there was no significant difference in the amount of flu virus in the lungs in each group. These correlate well with our previous studies showing that the main age-related differences in response to sublethal influenza infection are not seen during the peak of the infection during the first week, but become apparent during the resolution phase in week two following flu infection (8).

The BAL fluid from each mouse was analyzed for cytokine and chemokine contents. As shown in **Figure 1C**, there were no significant differences in cytokines important for a protective immune response to influenza infection including GCSF, IFNγ, IL-1α, IL-6, and TNF. In addition, there were no significant differences in chemokines that recruit protective immune cells to the lungs including CCL3, CCL4, CCL5, CXCL1, and CXCL10. A similar pattern was also observed when the serum from these groups was analyzed (**Figure 1D**) except for a significant increase in IL-1α in the aged AL group that was not seen in aged CR mice. Thus, both locally in the BAL and systemically in the serum, there are few measurable differences between the response to infection in young, middle aged, and aged groups and between AL and CR groups at this relatively early time point following flu infection.

Effect of Age and CR on the Gut Microbiome during Influenza Infection

It is known that gut microbiome is affected by influenza viral infection (56). To fully understand the dynamics of this process and its implication, stool samples were collected prior to and during influenza infection. Analysis was done by reconstruction of the gut bacterial microbiome by amplicon sequencing to access the composition of the microbiota population. **Figure 2A** shows the relative abundance of nine bacterial phyla in each experimental group. While there are no major age-related differences apparent in phylum level, there are differences due to influenza infection and diet. In each age category, the distribution of phyla is changed by day 7 postinfection in both AL and CR groups, characterized by an increase in Proteobacteria and Verrucomicrobia. Furthermore, Proteobacteria and Verrucomicrobia are more abundant in the CR groups when compared to AL in each age category. **Figure 2B** shows the Bray Curtis dissimilarity of samples indicating they segregate by diet but not by infection in each age group, this implies that diet had a greater impact on the composition of gut microbiome when compared to the impact of influenza infection.

Similar to a previous report (56), we observed increased phylum Proteobacteria in all groups at day 7 post influenza infection. We determined that regardless of age, the relative abundance of phylum Proteobacteria was positively correlated with percent body weight loss at this time in AL and CR ($r = 0.8095$, $p = 0.0218$ and $r = 0.8333$, $p = 0.0154$; respectively, **Figures 3A,B**) indicating a relationship between severity of infection and Proteobacteria abundance. To further examine the relationship between flu pathogenicity and gut microbiota abundances, we examined this relationship among all groups and major OTUs. This approach increases the overall sample size ($n = 17$) to increase the power of our correlation analysis and provide preliminary insight into potential key bacteria associated with flu responses regardless of age and diet. Ten specific OTUs correlated with percent weight loss, and interestingly, Alistipes OTU 34 and Parabacteroides OTU 9, both members of the Bacteroidetes phyla showed the strongest relationship ($r = 0.8235$, $p < 0.0001$ and $r = 0.7672$, $p = 0.0005$; respectively, **Figures 3C,D**), while another Bacteroidetes, Hallella OUT_11 was the next strongest relationship, however, was negatively correlated with percent weight loss ($r = -0.5907$, $p = 0.0216$, **Figure 3E**); highlighting differential relationships within phyla. Next, we examined the relationship between serum cytokine/chemokines and major OTUs observed among all groups to determine how influenza-induced inflammation relates to bacterial abundances (**Figure 4**). We observed 22 significant correlations between relative bacterial abundance and inflammatory mediators in the serum. Of note, CXCL1 was positively correlated with unclassified Lachnospiraceae OTU_12, Alistipes OTU 26, and Bacteroides OTU_29, CCL2 was positively correlated with Parabacteroides OTU_9, Alistipes OTU_34, and Unclassified Clostridiales OTU_25, and TNFα was positively correlated with unclassified alpha-Proteobacteria OTU_2, Butyrivibrio OTU_28, Unclassified Clostridiales OTU_30, and lachnospiraceae incertae sedis OTU_37. Conversely, CXCL10 was negatively correlated with Unclassified Porphyromonadaceae OTU_1 and Parasutterella OTU_18, and CCL5 is negatively correlated with Barnesiella OTU_3, Butyrivibrio OTU_28, Unclassified Porphyromonadaceae OTU_27, Allobaculum OTU_35, and Anaerostipes OTU_40. Among those correlations we observed, the three strongest positive correlations were CXCL1 with Bacteroides OTU_29, CCL2 with Parabacteroides OTU_9, and CCL2 with Alistipes OTU_34. The three strongest negative correlation were CCL3 with Prevotella OTU_8, CCL5 with Unclassified Porphyromonadaceae OTU_27, and CCL5 with Butyrivibrio OTU_19. Thus, systemic immune responses were related to gut microbiota alterations. Interestingly, aside from Butyrivibrio, the strongest correlations, both positive and negative, were Bacteroidetes. Though it is not possible to conclude any causal relations between these bacteria taxa and the correlated host response, these findings provide insight into future research in manipulating bacterial gut microbiome to facilitate antiviral immune responses.

DISCUSSION

In this exploratory study, we sought to obtain preliminary information as to how CR, a known modulator of aging and gut

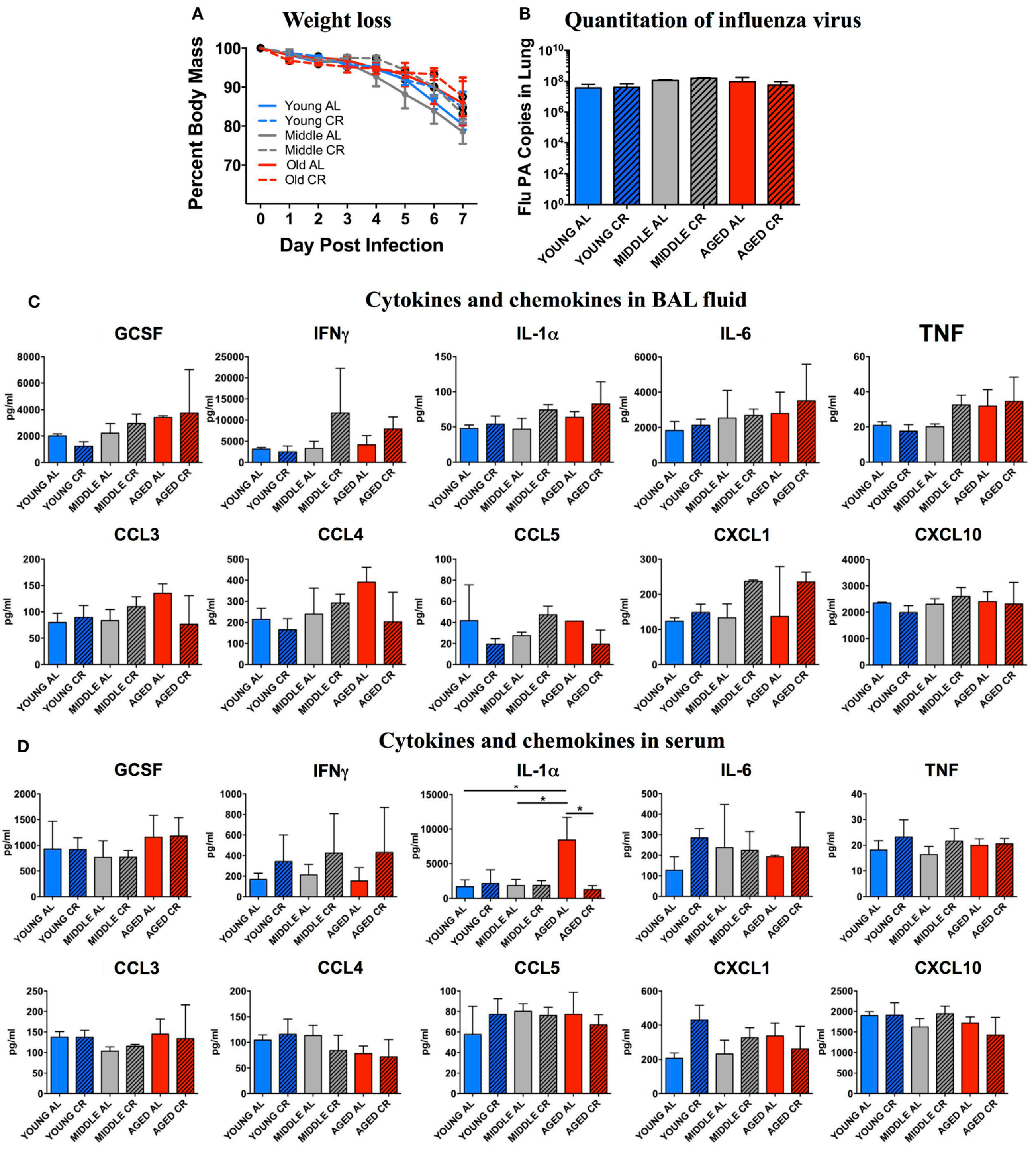

FIGURE 1 | Response to influenza infection. Young, middle-aged, and aged C57BL6 mice on an *ad libitum* (AL) or caloric-restricted (CR) diet were anesthetized and infected with 400 EID50 of influenza virus. **(A)** Each day, mice were weighed and the percent of the starting weight is shown. On day 7 postinfection, mice were sacrificed. **(B)** mRNA was isolated from the lungs and influenza virus was quantitated by real-time PCR measuring influenza *polymerase acidic protein* gene (*PA*) copy number. **(C)** Bronchiolar lavage fluid and **(D)** serum was collected and subjected to multiplex analysis to determine levels of cytokines and chemokines. Data were analyzed *via* two-way ANOVA with Bonferroni *post hoc* corrections; $^{*}p < 0.05$.

microbiota, could influence influenza-induced gut microbiota changes and immune responses during acute influenza infection in young, middle aged, and aged mice. Different outcomes of influenza infection, due to the influence of aging and CR, could be mediated by modulation of the gut microbiota by these factors with subsequent effects of infection due to the

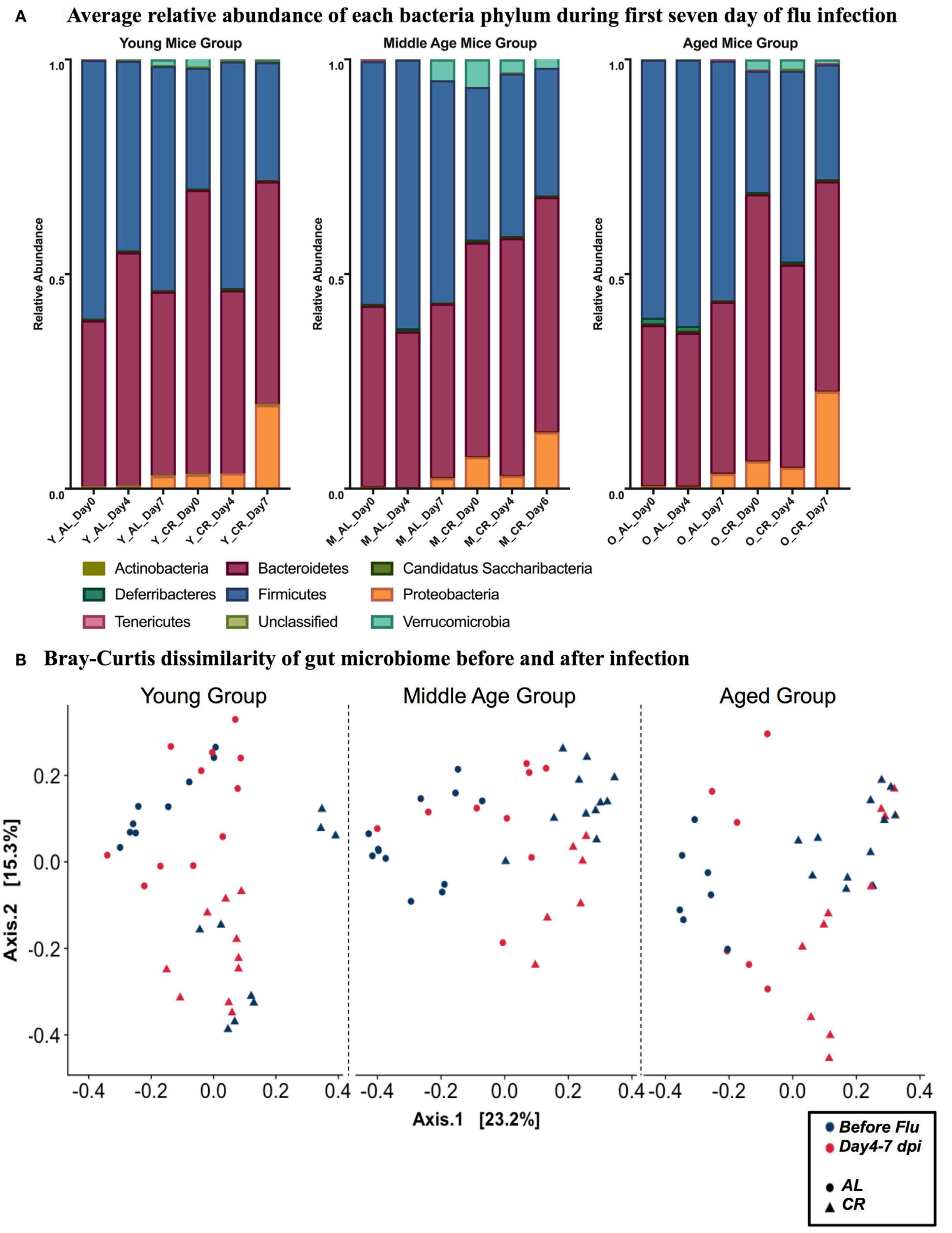

FIGURE 2 | **(A)** Relative abundance of nine phyla at 0, 4, and 7 day postinfection (dpi) in young, middle, and aged *ad libitum* (AL) or caloric-restricted (CR) mice. For middle-aged CR group, we do not have data at 7 dpi, so 6 dpi is shown here instead. Relative abundance of bacteria is shown as a fraction. **(B)** PCoA plot of Bray Curtis dissimilarity prior to (naïve, −3–0 dpi) and after (postinfection, 4–7 dpi) flu infection.

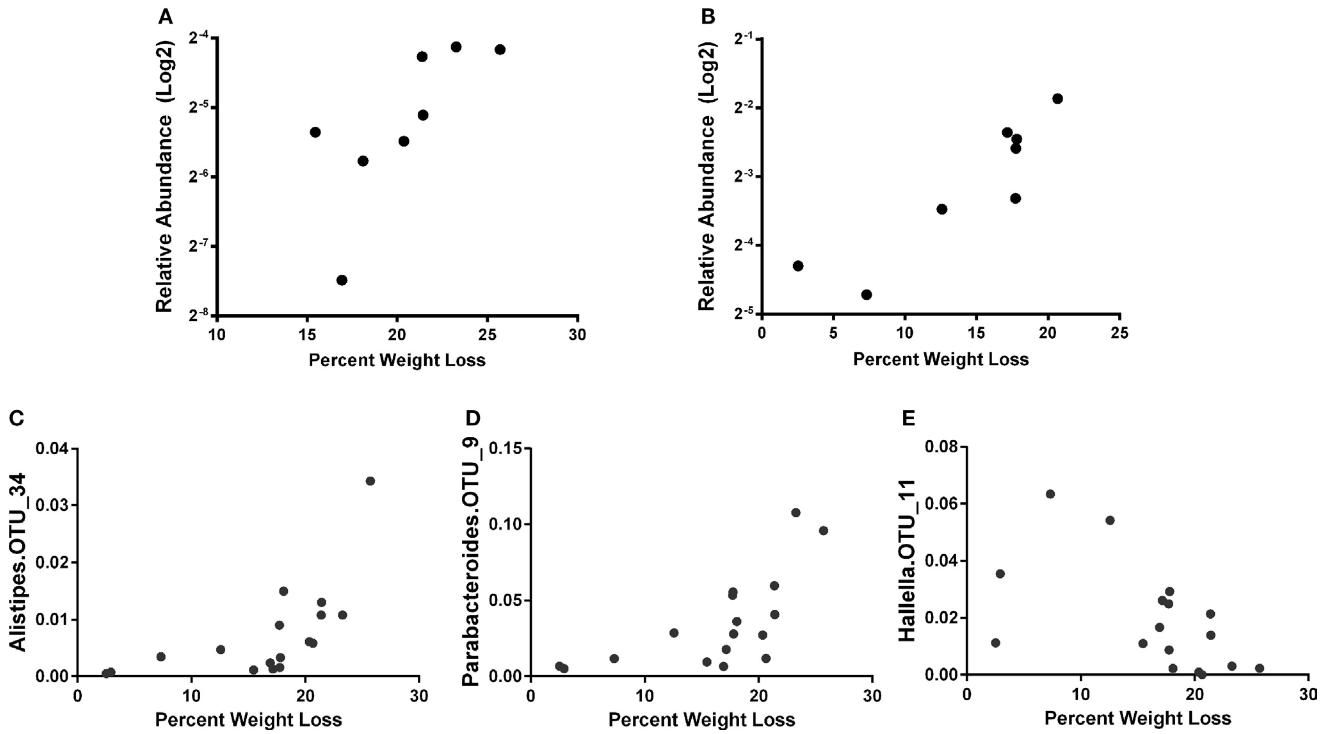

FIGURE 3 | The percent weight loss for AL **(A)** and caloric restricted **(B)** at 7 days post infection (dpi) is plotted against the relative abundance of phylum Proteobacteria (after log2 transform). Correlation of relative abundance of Alistipes OTU_34 **(C)**, Parabacteroides OTU_9 **(D)**, and Hallella OTU_11 **(E)** with percent weight loss among all groups is shown. The bacteria abundance is the average of 5, 6, and 7 dpi. Spearman's correlation with $p < 0.05$.

different microbial communities. We observed that CR affected the gut microbial communities (**Figure 2**), as found in previous studies, and that age also has an effect (the different patterns in **Figure 2B**). However, no obvious effect of infection was observed (**Figure 2**). Unlike previous studies (41, 45), we did not observe significant differences in weight loss or viral burden following influenza infection in CR aged animals. However, our study differed in that we utilized a sublethal dose of influenza and sacrificed mice at a relatively early point of infection, and both of these could contribute to a lessening of the effect of infection on the microbiome.

Although there was not a large effect on microbial community structure following infection, we did observe an increase in the proportion of Proteobacteria, which was more significant in CR mice but independent of age (**Figure 2A**). The increase in the proportion of Proteobacteria was correlated with weight loss (**Figure 3**), taken as a marker of infection severity. This again was independent of age. Further, multiple OTUs correlated with weight loss from the bacteroidetes phyla. This connection raises the possibility that a change in the microbiome has a connection with infection outcome, as hypothesized above; however, the relationship does not seem to be straight forward and members of the same phyla have differential relationships. Future research can examine if elimination or transfer of these specific bacteria can impact flu responses. Indeed, others have shown antibiotic treatment is detrimental to flu immune responses, specifically oral neomycin eliminated Gram-positive bacteria and impaired immune responses (53). Here, we identify Gram-negative bacteria that may also be crucial for immune responses.

The PR8 strain of influenza virus used for these studies will not directly infect gut tissue of B6 mice (84), raising the question of how a respiratory infection can affect the gut microbiome. The mucosal surfaces in lung and gut are considered a common mucosal surface that share immunological signals (85), so inflammation from one site is likely to affect the other site. Since Proteobacteria are generally observed during gut inflammation (86), the more severe infection in the lung, the greater the effect on the gut, and this would result in the observed correlation between Proteobacteria (a measure of inflammation in the gut) and weight loss (a measure of infection). Finally, we note that overgrowth of Proteobacteria can be impaired by blocking the type I interferon (IFN-I) signal (56), suggesting this gut microbiota change correlated with lung inflammation is mediated by an IFN-I-related immune response.

The gut is believed to be the largest immunological compartment in the body (87) and thus signaling from the gut microbiome may play an important role in viral infections. For example, mouse mammary tumor virus (88) and Enteric virus (89) require intestinal bacterial flora to establish effective infection. Lymphocytic choriomeningitis virus and influenza virus (90), conversely, will trigger a more effective immune response if intestinal bacterial flora is present. Also, germ-free mice (91), mice treated with an antibiotic cocktail (53), or TLR5 KO mice (with impaired function in sensing bacterial flagellin) (92) will not generate adequate

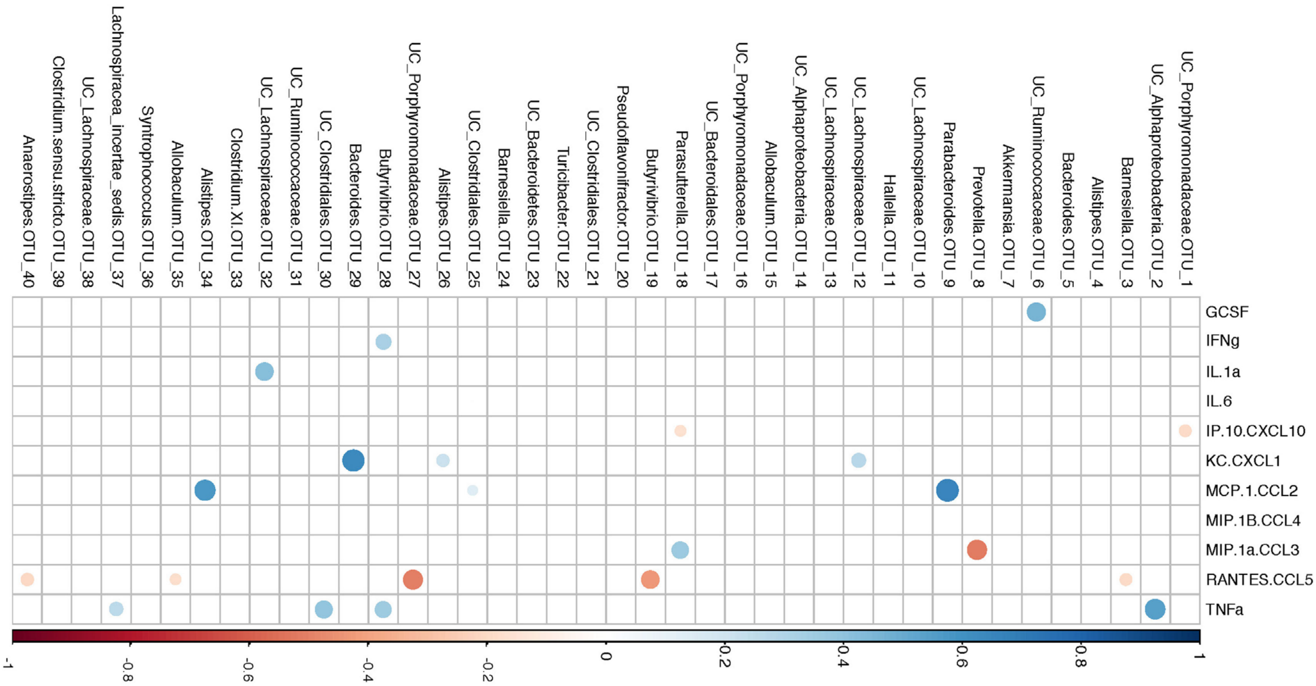

FIGURE 4 | Correlation of operational taxonomic units (OTUs) relative abundance with serum cytokines at 7 day postinfection (dpi). OTUs relative abundance is the average of 5, 6, and 7 dpi. Color intensity and size are proportional to the spearman correlation coefficient ($p < 0.05$). Positive correlations are displayed as blue and negative correlations are displayed in red. UC: unclassified according to RDP database (confidence threshold = 50%).

immune response to influenza viral infection. Thus, the microbiome helps protect during flu infection. Although here we show a correlation between Proteobacteria abundance and infection, each member of the microbiota may signal the immune system in a different manner (93). For example, a previous report (53) showed that a host with antibiotic treatment to largely deplete Lachnospiraceae would not generate a good antibody response against trivalent inactivated influenza vaccine. Similarly, we noticed that higher relative abundance of genus Lachnospiraceae OTU_12 and OTU_32 are correlated with higher amount of CXCL1 and IL-1α, respectively. More generally, we show here that cytokine production associated with flu infection in our study correlates differently for each of 22 OTUs (**Figure 4**). This suggests that members of the microbiome regulate the immune response in different ways.

These preliminary findings contribute to the understanding of dynamics and complexity of gut bacterial microbiota and influenza infection. We are the first to show the early changes (Day 4 post influenza infection) of gut bacterial microbiota composition in both young, middle, and aged mice on both AL or CR diet. Interestingly, though CR had a great impact on gut microbiota, it did not seem to affect flu-induced immune responses or flu-induced alterations in the gut microbiota at these early time points. It is possible that these alterations, such as increased proteobacteria compared to AL mice, may have effects at later time points in flu responses and recovery. More research is necessary to determine if CR modulation of gut microbiota with aging is beneficial to flu immune responses. Cytokine production associated with influenza infection in our study correlates differently with each OTUs. Although the data we present do not allow causal relations between bacteria and cytokine production to be determined, they do provide hypotheses for virus-bacterial interactions through the immune system. There is evidence that the genus *Lactobacillus* can improve the immune response to the PR8 strain of influenza (94, 95) and respiratory syncytial virus (96) infection. Here, we find multiple members of the Bacteroidetes phyla to be correlated with immune parameters and flu pathogenicity. Thus, while influenza infection promotes Proteobacteria overgrowth through IFN-I (56), members of the Bacteroidetes phyla are also affected and likely in turn affect immune parameters, both positively and negatively. Interestingly, a Bacteroidetes dominated microbiome was associated with increased frailty among the elderly (61), perhaps suggesting a microbiota link associated with the increased disability observed following influenza infection in the elderly (97). Future research should explore manipulation of bacterial species from this phyla to modulate flu immune responses. Thus, we believe this exploratory study can provide some additional guidance to the use of microbiota to facilitate virus-specific immune responses, especially for elderly whose immune responses are known to be deficient.

ETHICS STATEMENT

This study was carried out in accordance with all federal, state, and institutional laws, policies, and guidelines. The protocol was approved by the UConn Health Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

JB. and LH designed the study; JB carried out the influenza infection and analysis of responses; XZ and GW performed the microbiome analysis; JB, LH, GK, XZ, and GW participated in preparation of the figures and manuscript.

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Increased IL-15 Production and Accumulation of Highly Differentiated CD8⁺ Effector/Memory T Cells in the Bone Marrow of Persons with Cytomegalovirus

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Cytomegalovirus (CMV) has been described as a contributor to immunosenescence, thus exacerbating age-related diseases. In persons with latent CMV infection, the CD8⁺ T cell compartment is irreversibly changed, leading to the accumulation of highly differentiated virus-specific CD8⁺ T cells in the peripheral blood. The bone marrow (BM) has been shown to play a major role in the long-term survival of antigen-experienced T cells. Effector CD8⁺ T cells are preferentially maintained by the cytokine IL-15, the expression of which increases in old age. However, the impact of CMV on the phenotype of effector CD8⁺ T cells and on the production of T cell survival molecules in the BM is not yet known. We now show, using BM samples obtained from persons who underwent hip replacement surgery because of osteoarthrosis, that senescent CD8⁺ T_{EMRA} cells with a bright expression of CD45RA and a high responsiveness to IL-15 accumulate in the BM of CMV-infected persons. A negative correlation was found between CMV antibody (Ab) titers in the serum and the expression of CD28 and IL-7Rα in CD8⁺ T_{EMRA}^{bright} cells. Increased IL-15 mRNA levels were observed in the BM of CMV⁺ compared to CMV⁻ persons, being particularly high in old seropositive individuals. In summary, our results indicate that a BM environment rich in IL-15 may play an important role in the maintenance of highly differentiated CD8⁺ T cells generated after CMV infection.

Keywords: bone marrow, cytomegalovirus, aging, immunosenescence, senescence

INTRODUCTION

Aging is associated with a decline of immune function, a condition known as immunosenescence, which reduces the capability to fight infections, thus contributing to age-related diseases. Due to thymic involution, the generation of new naïve T cells diminishes with age (1, 2). In parallel, more differentiated T cells accumulate in the elderly, particularly in the bone marrow (BM) (3, 4). Recently, the important role of the BM in producing the T cell survival factors IL-15 and IL-7, which are

Abbreviations: Ab, antibody; BM, bone marrow; BMMCs, BM mononuclear cells; CMV, cytomegalovirus; KLRG-1, killer cell lectin-like receptor G1; PB, peripheral blood; PBMCs, PB mononuclear cells; ROS, reactive oxygen species; SLECs, short-lived effector cells; T_{EM}, effector memory T cells; T_{EMRA}, terminally differentiated effector memory cells re-expressing CD45RA.

necessary for the long-term maintenance of effector/memory T cells, has been documented (5–8). In particular, IL-15 has been shown to be important for the preservation of highly differentiated CD8+ effector T cells (9–11). In old age, a proinflammatory BM environment promotes the accumulation of IL-15, which may lead to increased numbers of highly differentiated T cells as a consequence (12).

A major contributor to immunosenescence is cytomegalovirus (CMV), a lifelong-persisting herpes virus present in 60–100% of adult individuals depending on the cohort (13, 14). CMV infection has been linked to increased CRP levels in the blood and diseases with an inflammatory component such as cardiovascular disease and cancer (15–18). Even in healthy carriers, CMV-specific T cells expand over time leading to memory inflation (19–21). Although inflation of CD4+ T cells has also been observed, virus-specific effector/memory CD8+ T cells accumulate in the peripheral blood (PB) at higher frequencies (19, 22, 23). In addition, CMV seropositivity has been associated with an inverted CD4:CD8 ratio in the blood in old age (24). In the elderly, the majority of effector/memory CMV-specific CD8+ T cells has been shown to re-express CD45RA, acquiring the typical feature of terminally differentiated cells (25–27). Although the CD8+ T cell phenotype in CMV+ persons has been extensively described in the PB, the impact of CMV on BM CD8+ T cells has only been partially investigated so far.

In the present study, the phenotypes of effector CD8+ T cell subsets in the BM of CMV− and CMV+ persons were compared. A population of CD8+ T_{EMRA} cells with a bright expression of CD45RA, low levels of CD28, and expressing the senescence marker killer cell lectin-like receptor G1 (KLRG-1) expanded in CMV+ persons. While the responsiveness of these BM CD8+ T^{bright}_{EMRA} cells to IL-15 was high, the expression of IL-7Rα was reduced. In addition, CMV antibody (Ab) titers in the serum correlated negatively with the expression of CD28 and IL-7Rα in CD8+ T^{bright}_{EMRA} cells and positively with a ratio between CD122 (IL-2/IL-15Rβ) and IL-7Rα+ cells. Increased IL-15 mRNA expression and more interactions between CD8+ T cells and IL-15-producing cells were found in the BM of CMV+ persons. Our results show that, in CMV+ persons, IL-15 may contribute to the accumulation and the survival of senescent CD8+ T_{EMRA} cells in the BM.

MATERIALS AND METHODS

Study Subjects

Samples were obtained from systemically healthy individuals who did not receive immunomodulatory drugs or suffer from diseases known to influence the immune system, such as autoimmune diseases and cancer. None of them was frail or had symptoms of cognitive impairments. In all patients, the indication for surgery was osteoarthrosis. Further information about the donors included in the study is summarized in **Table 1**.

Sample Collection and Preparation

Hip replacement surgery was performed and bone from the femur shaft was harvested. A biopsy of *substantia spongiosa osseum*, which would otherwise have been discarded, was used to isolate BM mononuclear cells (BMMCs). BM biopsies were fragmented, washed once with complete RPMI medium (RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin; Invitrogen) and treated with purified collagenase (CLSPA, Worthington Biochemical; 20 U/ml in complete RPMI medium) for 1 h at 37°C. BM biopsies were then centrifuged and BMMCs purified by density gradient centrifugation (Ficoll-Hypaque). Purification of PB mononuclear cells (PBMCs) from heparinized blood was also performed by density gradient centrifugation.

TABLE 1 | Demographic data of the donors included in the study, divided into cytomegalovirus (CMV)− and CMV+ groups.

	CMV−	CMV+
N	33	37
Sex	14F, 19M	20F, 17M
Age range (years)	43–87	32–87
Mean age (years)	66 ± 10	70 ± 11
Body weight	76.2 ± 18	82.3 ± 11
Hip fracture (%)	0	0

Isolation of RNA and Quantitative RT-PCR

RNA was isolated from purified BMMCs using the RNeasy Plus mini kit (Qiagen). First-strand cDNA synthesis was performed using a Reverse Transcription system (Promega). Quantitative RT-PCR experiments were performed using the LightCycler 480 System (Roche Diagnostics), 2X SYBR Green 1 Master (Roche Diagnostics), and β-actin as housekeeping gene for relative quantification of effector/memory cell survival factors. Sequence-specific oligonucleotide primers were designed using Primer3 software (25) and synthesized by MWG Biotech (Ebersberg, Germany). The following primers were used: IL-15FW 5′-ATTTTGGGCTGTTTCAGTGC-3′, IL-15RW 5′-TTACTTTGCAACTGGGGTGA-3′, IL-7FW 5′-GTAGCAATTGCCTGAATAATG-3′, IL-7RW 5′-GTTGTGCCTTCTGAAACT-3′.

Flow Cytometric Analysis

Immunofluorescence surface staining was performed by adding a panel of directly conjugated Abs to BMMCs. After surface staining, cells were permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen), and incubated with intracellular Abs. Labeled cells were measured using a FACSCanto II (BD Biosciences). Data were analyzed using Flowjo software. The following labeled Abs were used: IL-7Rα-PE (hIL-7Rα-M21), CD8-PeCy7 (RPA-T8), CCR7-FITC (150503), and CD28 BV421 (CD28.2) purchased from BD, CD122 (IL-2/IL-15Rβ)-APC (TU-27), CD45RA-PerCp (HI100), and KLRG-1-PeCy7 (2F1/KLRG1) purchased from Biolegend, CD3-APC-eFluor 780 (SK7) purchased from eBioscience.

Responsiveness to BM CD8+ T Cell Survival Factors

The responsiveness of CD8+ T cell subsets to IL-15 and IL-7 was measured by quantifying the cells expressing the receptors CD122 (IL-2/IL-15Rβ) and IL-7Rα, respectively (26–28).

Immunofluorescence Analysis of BM Biopsies

Immunofluorescence analysis of BM sections was performed, as described by Herndler-Brandstetter et al. (8). Formalin-fixed, paraffin-embedded 4-μm BM sections were deparaffinized in xylene and re-hydratated in ethanol. The slides were boiled in 0.01 M citrate buffer (pH 6) for 16 min in the microwave for epitope retrieval and allowed to cool for about 1 h at room temperature. Slides were blocked with 3% skim milk in TBS/Tween for 20 min at room temperature. Rabbit anti-IL-15 (1:200; ab55276; Abcam) and mouse anti-CD8 (1:50; C8/144B; Dako) Abs were incubated overnight at 4°C. After washing, the slides were incubated for 1 h at 4°C with biotinylated swine anti-rabbit Ab (1:300; E0431; Dako) and a goat anti-mouse Alexa Fluor 546 Ab (1:300; A11018; Molecular Probes). Following washing steps with TBS/0.1% Tween, the BM sections were stained with a streptavidine-Alexa Fluor 488 Ab (1:500; S11223; Molecular Probes) for 30 min at 4°C. The stained slides were analyzed using confocal microscopy with an m-Radiance confocal scanning system (Bio-Rad) attached to a Zeiss Axiophot microscope (Carl Zeiss). For the quantitative analysis of $CD8^+$ T cells in close contact with IL-15–producing cells in the BM, pictures from different areas of the BM sections were analyzed. In total, 800–1,000 $CD8^+$ T cells were analyzed from each donor to calculate the percentage of contact with IL-15^+ cells.

Determination of CMV Seropositivity

Antibodies against CMV were determined in the serum of the donors included in the study using a commercially available ELISA Kit (Siemens).

Statistical Analysis

The data obtained in the study follow a non-parametric distribution. Statistical significance was assessed by Spearman correlation analysis, Mann–Whitney test and Wilcoxon matched pairs test. A *p*-value of less than 0.05 was considered as significant.

Study Approval

The study was approved by the Ethics Committees of the "Klinikum Wels-Grieskirchen" (Austria). Written informed consent was received from participants prior to their inclusion in the study.

RESULTS

CD8 T_{EMRA}^{bright} Cells, which Are KLRG-1^+ and Frequently Lack CD28, Increase in the BM of CMV^+ Persons

$CD8^+$ T cells with a $CD45RA^+$ $CCR7^-$ T_{EMRA} phenotype have been shown to accumulate in the blood after CMV infection (29, 30). To assess whether T_{EMRA} cells are also enriched in the BM from CMV^+ persons, we measured the levels of BM $CD8^+$ T_{EMRA} cells in CMV^- and CMV^+ persons (**Figure 1**). $CD8^+$ T_{EMRA} cells were gated, as indicated in **Figure 1A**. Within the $CD8^+$ $CCR7^-$ T cell population, a subpopulation with an intermediate $\left(T_{EMRA}^{dim}\right)$ and one with a bright $\left(T_{EMRA}^{bright}\right)$ expression of CD45RA and a subset, which does not express CD45RA (T_{EM}) were defined. Higher percentages of both T_{EMRA}^{dim} and T_{EMRA}^{bright} cells were found in BMMCs compared to PBMCs (T_{EMRA}^{dim} $p = 0.001$; T_{EMRA}^{bright} $p = 0.04$, data not shown). While the size of the $CD8^+$ T_{EM} and the $CD8^+$ T_{EMRA}^{dim} populations in the BM was similar in CMV^- and CMV^+ persons, the percentage of $CD8^+$ T_{EMRA}^{bright} cells was higher in CMV^+ persons (**Figure 1B**). No differences were observed in the numbers of $CD8^+$ T_{EM}, T_{EMRA}^{dim}, and T_{EMRA}^{bright} cells when we compared younger (≤70 years) and older (>70 years) donors in both the CMV^- and the CMV^+ group (n.s., data not shown).

$CD8^+$ T_{EMRA} cells have been described to downregulate CD28 and to express senescence markers in the PB (31). $CD8^+$ $CD28^-$ T cells have also been observed to accumulate in the BM in old age (12, 32). To assess whether CMV affects the phenotype of $CD8^+$ subsets in the BM, we analyzed $CD8^+$ $CD28^-$ T cells in the BM of CMV^- and CMV^+ persons (**Figure 1C**). Increased frequencies of $CD8^+$ $CD28^-$ T_{EMRA}^{bright} cells were found in CMV^+ compared with CMV^- persons, while no differences between the two groups were observed in $CD8^+$ T_{EM} and $CD8^+$ T_{EMRA}^{dim} cells. While $CD8^+$ $CD28^-$ T_{EMRA}^{bright} T cells correlated positively with age in CMV^- persons, no age-related changes were observed for $CD28^-$ T_{EMRA}^{bright} cells in CMV^+ persons and for T_{EM} and T_{EMRA}^{dim} cells in both the CMV^+ and the CMV^- group (Figure S1 in Supplementary Material).

We then analyzed the expression of the senescence marker KLRG-1 (33) in BM $CD8^+$ T cell subsets and compared the results in CMV^+ and CMV^- persons (**Figure 1D**). The percentage of KLRG-1-expressing cells was relatively low in T_{EM}, high in T_{EMRA}^{dim}, and was even higher in T_{EMRA}^{bright} cells. No differences were observed when the CMV serostatus was considered or when younger (≤70 years) and older (>70 years) donors were compared (data not shown). These data suggest that $CD8^+$ T_{EMRA} cells with a high expression of CD45RA, which frequently lack CD28 and express KLRG-1, increase in the BM of CMV^+ persons.

$CD8^+$ T_{EMRA}^{bright} Cells with a High Expression of CD122 and a Reduced Expression of IL-7Rα Increase in the BM of CMV^+ Persons

IL-7 and IL-15 influence the survival and turnover of $CD8^+$ T cells (34). They are of particular relevance for the interaction of T cells with stromal cells in the BM (8, 35). We, therefore, decided to study the expression of CD122 and IL-7Rα in $CD8^+$ T cells. Specifically, we compared these parameters in the T_{EM}, T_{EMRA}^{dim}, and T_{EMRA}^{bright} subsets from CMV^- and CMV^+ persons (**Figures 2** and **3**). The "typical" phenotype of a $CD8^+$ T_{EMRA}^{bright} cell in CMV^- and CMV^+ donors is shown in **Figure 2**. In the whole cohort (CMV^+ plus CMV^- persons), CD122 was expressed on a great majority of cells, in all subsets, but on almost every cell in the $CD8^+$ T_{EMRA}^{bright} population (Wilcoxon matched pairs test, $p < 0.001$ T_{EMRA}^{bright} vs. T_{EMRA}^{dim}, **Figure 3A**). No differences were found between CMV^- and CMV^+ persons. Interestingly, when relating the expression of CD122 with age, in $CD8^+$ T cell subsets from CMV^- and CMV^+ persons, we found positive correlations in CMV^+ persons, whereas negative correlations were seen among T_{EM} and

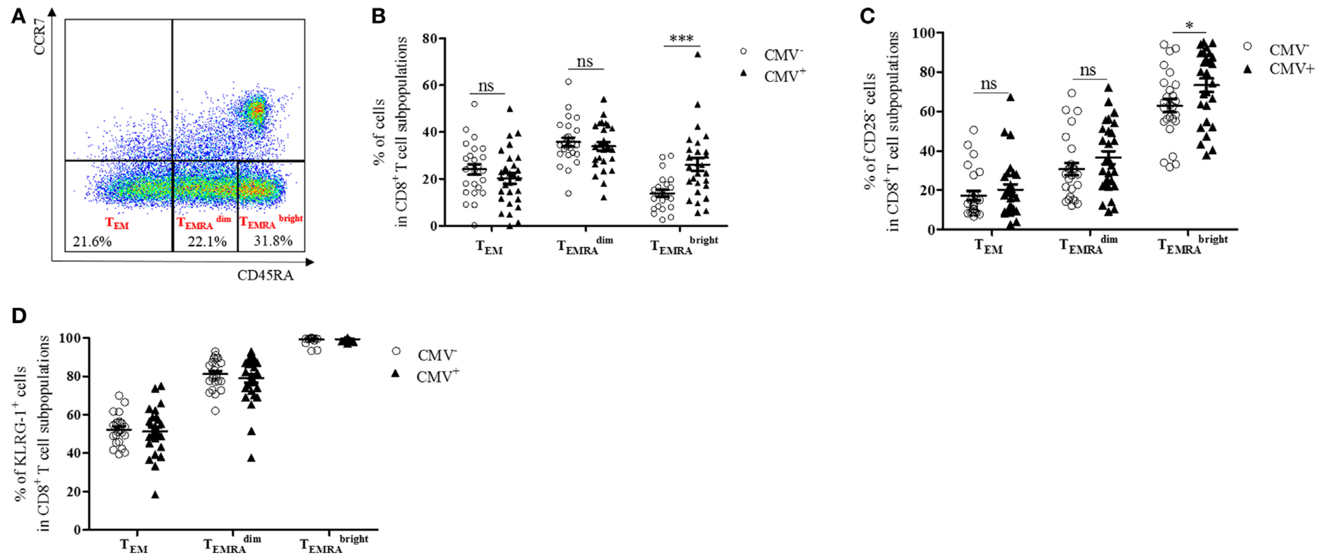

FIGURE 1 | CD8+ T_{EMRA}^{bright} cells with a senescent phenotype, which typically lack CD28 increase in the bone marrow of cytomegalovirus (CMV)+ persons. **(A)** FACS plot showing gating strategies for T_{EM}, T_{EMRA}^{dim}, and T_{EMRA}^{bright} subsets in CD8+ T cells. In CCR7− CD8+ T cells, a population of CD45RA− (T_{EM}) cells, one with an intermediate (T_{EMRA}^{dim}) and one with a high expression of CD45RA (T_{EMRA}^{bright}) were defined. **(B)** Percentages of T_{EM}, T_{EMRA}^{dim}, and T_{EMRA}^{bright} subsets in CD8+ T cells (=100%) in CMV− (dots) and CMV+ (triangles) persons. Mann–Whitney test, ***$p < 0.001$. **(C)** Percentages of CD28− T cells in CD8+ T_{EM}, T_{EMRA}^{dim}, and T_{EMRA}^{bright} subsets in CMV− and CMV+ persons. Mann–Whitney test, *$p = 0.05$. **(D)** Percentages of KLRG-1+ cells in CD8+ T_{EM}, T_{EMRA}^{dim}, and T_{EMRA}^{bright} cells in CMV− and CMV+ persons. $N = 26$ (CMV− group) and $N = 28$ (CMV+ group).

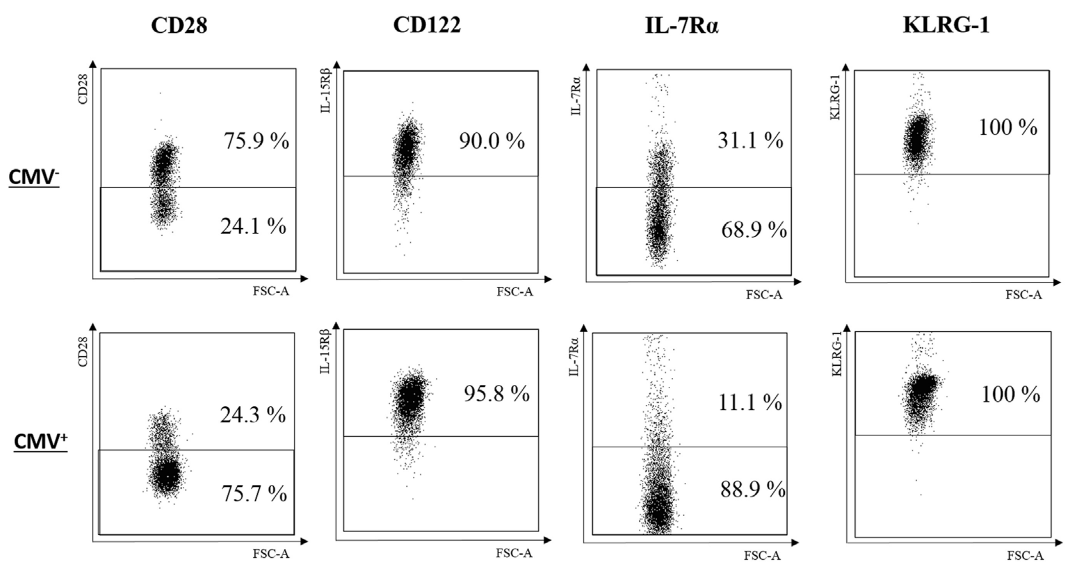

FIGURE 2 | Typical phenotype of CD8+ T_{EMRA}^{bright} cells in cytomegalovirus (CMV)− and CMV+ persons. FACS plots for CD28, CD122, IL-7Rα, and KLRG-1 from one representative CMV− (69 years) and one CMV+ (72 years) person are shown.

T_{EMRA}^{dim} subsets (Figures S2A,B in Supplementary Material). No significant correlations with age were found in CD8+ T_{EMRA}^{bright} cells (Figure S2C in Supplementary Material). Lower percentages of IL-7Rα+ cells were observed in both CD8+ T_{EMRA}^{dim} and T_{EMRA}^{bright} cells from CMV+ persons when compared to their CMV− counterparts (**Figure 3B**). In CMV− persons, there was a positive correlation between IL-7Rα and age in CD8+ T_{EM} cells, while negative correlations were seen between CD8+ T_{EMRA}^{dim} and T_{EMRA}^{bright} cells in CMV+ persons (Figures S2D–F in Supplementary Material).

We then compared the expression of CD122 and IL-7Rα in CD8+ CD28+ and CD8+ CD28− T_{EMRA}^{bright} cells (**Figures 3C,D**). Higher percentages of CD122+ cells were found in CD8+

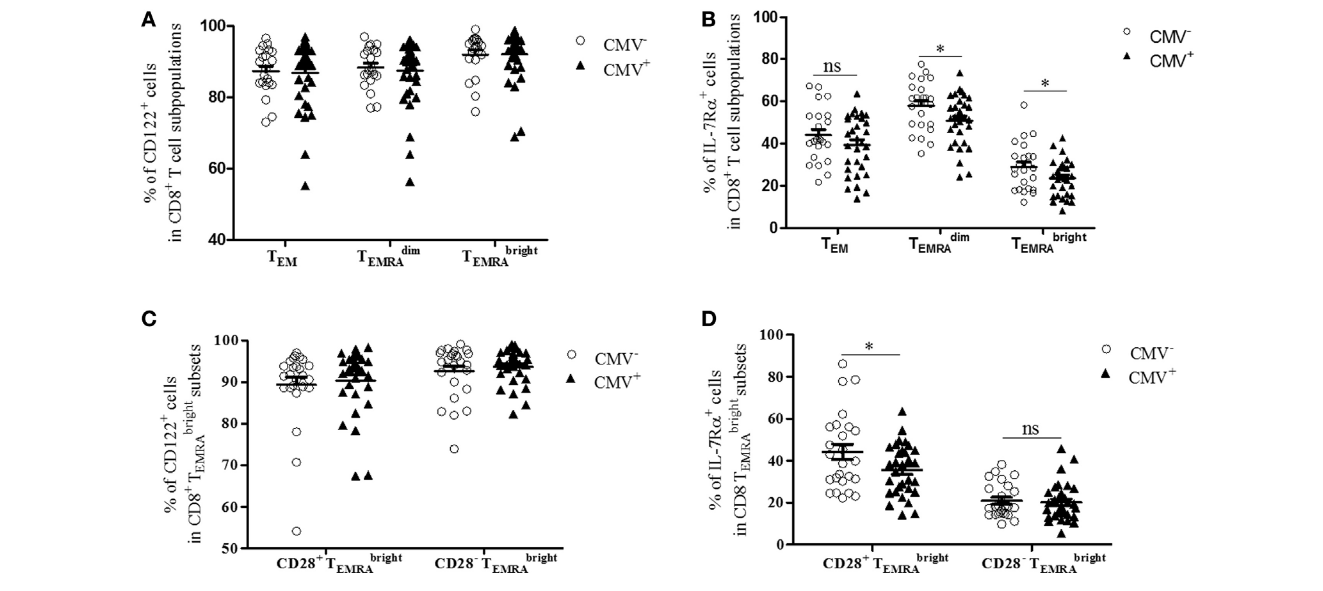

FIGURE 3 | Expression of CD122 and IL-7Rα in bone marrow CD8+ T cell subsets from cytomegalovirus (CMV)− and CMV+ persons. Percentages of **(A)** CD122+ and **(B)** IL-7Rα+ cells in CD8+ T_{EM}, T_{EMRA}^{dim}, and T_{EMRA}^{bright} subpopulations in CMV− and CMV+ persons. Mann–Whitney test, *$p < 0.05$. $N = 32$ (CMV− group) and $N = 37$ (CMV+ group). Wilcoxon-matched pairs test, CD8+ T_{EMRA}^{dim} vs. CD8+ T_{EMRA}^{bright} cells in the whole cohort (CMV+ plus CMV−) ***$p < 0.001$ (significance not indicated in the Figure). Percentages of **(C)** CD122+ and **(D)** IL-7Rα+ cells in CD8+ CD28+ and CD8+ CD28− T_{EMRA}^{bright} cells from CMV− and CMV+ persons. Mann–Whitney test, *$p < 0.05$. $N = 32$ (CMV− group) and $N = 37$ (CMV+ group).

CD28− T_{EMRA}^{bright} cells compared to their CD8+ CD28+ counterparts (Wilcoxon matched pairs test, $p = 0.01$ in CMV− and $p = 0.002$ in CMV+ persons; **Figure 3C**). No differences were seen between CMV− and CMV+ persons. In contrast, the percentage of IL-7Rα+ cells was lower in CD8+ CD28− T_{EMRA}^{bright} cells compared to CD8+ CD28+ T_{EMRA}^{bright} cells (Wilcoxon matched pairs test, $p < 0.001$ in both CMV− and CMV+ persons; **Figure 3D**). When comparing CMV+ and CMV− samples, reduced numbers of IL-7Rα+ cells were seen in CMV+ persons within the CD8+ CD28+ T_{EMRA}^{bright} subset; however, no differences were seen within the CD8+ CD28− T_{EMRA}^{bright} population. In summary, our findings indicate that numbers of CD8+ T_{EMRA}^{bright} cells with high CD122 and/or low IL-7Rα expression increase in the BM of CMV+ persons. Thus, in CMV+ persons, the "typical" CD8+ T_{EMRA}^{bright} cell is most likely CD28−, CD122^{hi} IL-7Rα^{low}, and KLRG-1+; however, whether every cell in the T_{EMRA}^{bright} subset carries the full marker pattern is not yet known and is currently being investigated. In CMV− persons, the phenotype of CD8+ T_{EMRA}^{bright} cells differs somewhat as these cells still express reasonably high levels of both CD28 and IL-7Rα.

CMV Ab Titers Correlate Positively with CD28− Cells and Negatively with IL-7Rα+ Cells in CD8+ T_{EMRA}^{bright} Cells

Although it is not clear which role CMV-specific Abs play in latent CMV infection, high Ab titers are connected with CMV re-activation (36). We, therefore, determined whether CMV Ab titers correlated with the percentage of CD28+, CD122+, and IL-7Rα+ expression in BM CD8+ T_{EMRA}^{bright} cells. There was a positive correlation between Ab titers and the percentage of CD28− cells in the CD8+ T_{EMRA}^{bright} population, but no relationship between Ab titers and CD122-expressing cells was observed (**Figures 4A,B**). In contrast, when the percentage of IL-7Rα+ cells in the CD8+ T_{EMRA}^{bright} population was assessed in relationship to CMV Ab titers, there was a negative correlation (**Figure 4C**). In addition, a ratio between the percentages of CD122+ cells and of IL-7Rα+ cells in the CD8+ T_{EMRA}^{bright} population was calculated (% CD122+ cells/% IL-7Rα+ cells). When this ratio was correlated with CMV Ab titers, a positive correlation was found (**Figure 4D**). Thus, our data indicate that CMV Ab titers in the serum correlate with the expression of CD28 and IL-7Rα as well as with the ratio CD122+/IL-7Rα+ in CD8+ T_{EMRA}^{bright} cells.

IL-15 but Not IL-7 mRNA Expression in the BM Is Affected by CMV and More CD8+ T Cells Are in Close Proximity to IL-15-Producing Cells

In order to assess whether the expression of effector/memory T cell survival factors in the BM differs in CMV+ persons, we measured the expression of IL-15 and IL-7 at the mRNA level in BMMCs from CMV− and CMV+ persons of varying ages (**Figure 5**). Indeed, higher IL-15 mRNA levels were found when CMV+ persons were compared with their CMV− counterparts (**Figure 5A**). IL-15 mRNA was 1.9 ± 0.1-fold higher in BMMCs from CMV+ compared to CMV− persons. No difference in the expression of IL-7 mRNA was observed (**Figure 5B**). In a previous study, we demonstrated that IL-15 increased while IL-7 decreased during aging in the BM (12). We now confirm these data in CMV− and CMV+ persons (**Figures 5C,D**). Again,

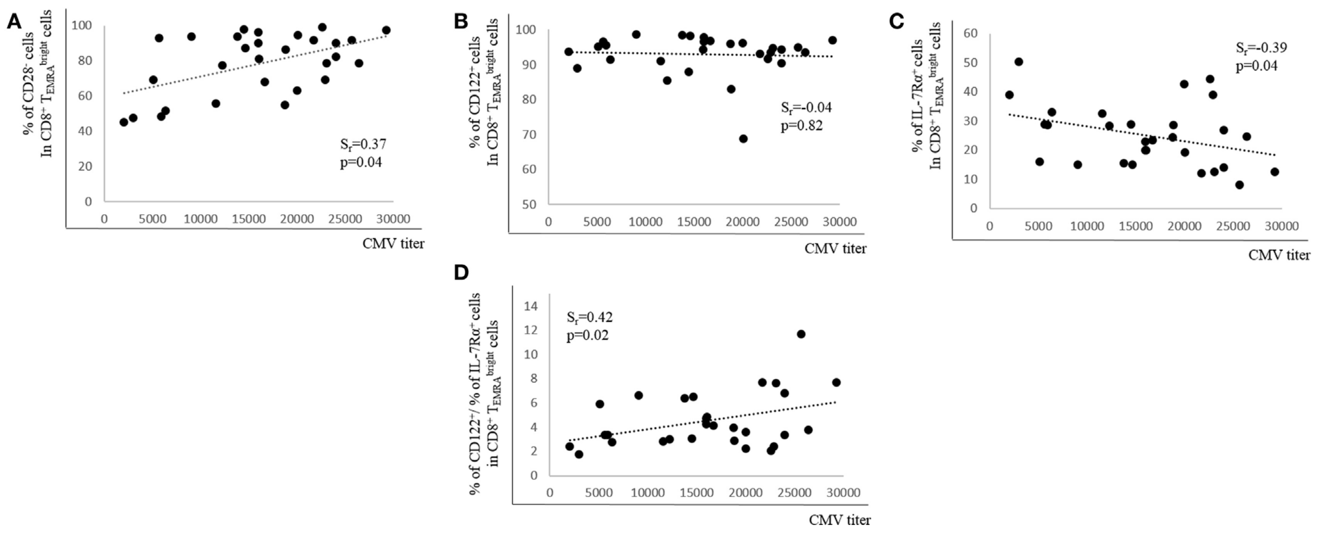

FIGURE 4 | Relationship between cytomegalovirus (CMV) antibody (Ab) titers and expression of CD28, CD122, and IL-7Rα in bone marrow CD8+ T_{EMRA}^{bright} T cells. Percentages of **(A)** CD28−, **(B)** CD122+, **(C)** IL-7Rα+ cells, and **(D)** the ratio between the percentages of CD122+ and of IL-7Rα+ cells in the CD8+ T_{EMRA}^{bright} subset in relationship to CMV Ab titers in the serum are shown. Spearman coefficient (r_s) and p values are shown in each graph (N = 29).

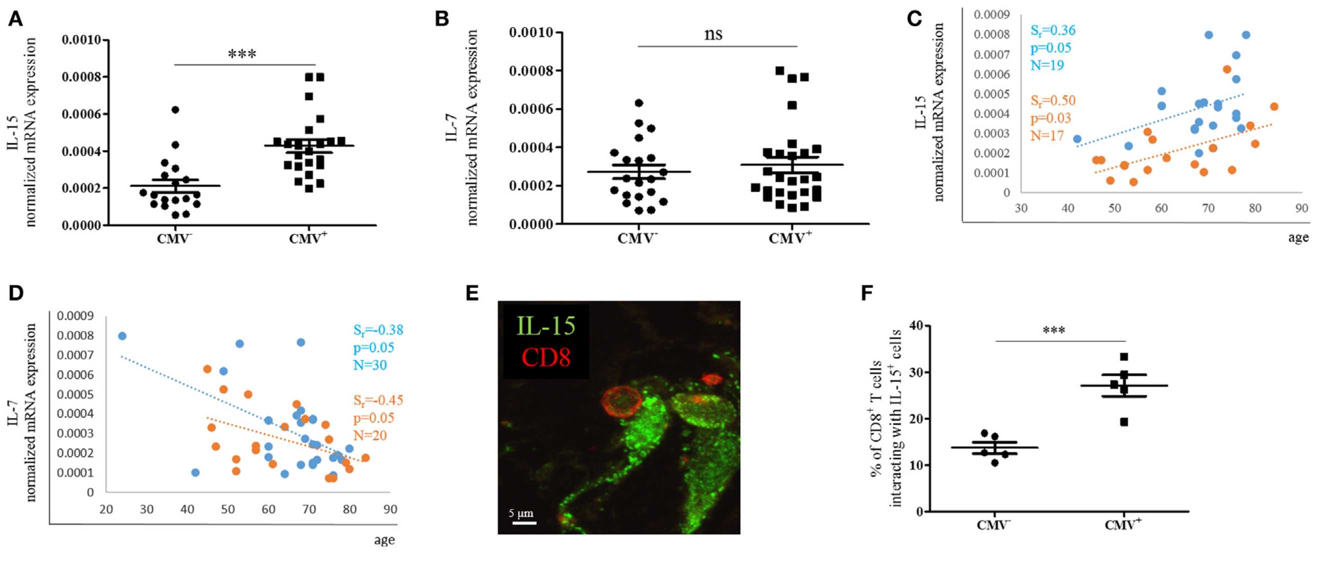

FIGURE 5 | mRNA expression of IL-15 and IL-7 in bone marrow (BM) mononuclear cells from cytomegalovirus (CMV)− and CMV+ persons. mRNA expressions of **(A)** IL-15 and **(B)** IL-7 in the CMV− and CMV+ group. Mann–Whitney test, $p < 0.001$. The sample size is shown in the graphs in **(C,D)**. The mRNA expressions of **(C)** IL-15 and **(D)** IL-7 in CMV− (orange) and CMV+ (blue) persons in correlation with age are shown. Spearman coefficient (r_s), p-value, and sample size (N) are shown in each graph. The mRNA expression of each gene was normalized against the housekeeping gene β-actin. **(E)** Representative picture obtained after immunofluorescence staining of BM sections showing one interaction between a CD8+ T cell (red) and an IL-15+ cell (green). **(F)** Percentages of CD8+ T cells interacting with IL-15-producing cells in the BM of CMV− and CMV+ persons. Mann–Whitney test, N = 5 for each group (***$p < 0.001$).

a positive correlation for IL-15 and a negative one for IL-7 was found with age in both groups, CMV− and CMV+ persons. Interestingly, CMV− persons clustered differently from CMV+ ones when IL-15 mRNA expression was studied in correlation with age.

Interactions with IL-15-producing cells in the BM are required for the survival of effector/memory CD8+ T cells (7, 8). To assess whether the increased expression of IL-15 in CMV+ persons affects the number of interactions between IL-15-producing cells and CD8+ T cells, we studied BM sections and quantified CD8+ T cells in close proximity to IL-15+ BM cells in CMV− and CMV+ persons (**Figure 5E**). 13.7 ± 2.7 and 27.1 ± 5.2% of CD8+ T cells, respectively, were interacting with IL-15-producing cells in CMV− and CMV+ persons (**Figure 5F**). In summary, our findings indicate that IL-15 expression increases not only with aging but also with CMV infection, and is highest in old CMV$^+$ persons. Additionally, the increased production of IL-15 in the BM may attract more CD8$^+$ T cells into the close proximity of IL-15-producing cells.

DISCUSSION

Latent CMV infection is currently believed to drive or at least exacerbate "immunosenescence" (37). Both conditions lead to a characteristic shift in the T cell repertoire with a decrease in naïve T cells and an increase in highly differentiated T cells, particularly within the CD8$^+$ T cell subset (38). Numerous studies have, therefore, focused on the phenotype and function of these "terminally" differentiated CD8$^+$ T cells (39), which are frequently CD28$^-$. As a corresponding cell type does not exist in mice, most studies have been performed in human blood and lymphoid organs have very rarely been investigated (40).

The BM has recently been recognized for its important role in the maintenance of T cell memory, and the existence of particular niches for adaptive immune cells has been suggested, such as the IL-7 niche for the maintenance of CD4$^+$ memory T cells (5). We demonstrated that, in the human BM, the production of IL-15 and the numbers of effector/memory CD8$^+$ T cells increased with age and a link with inflammation was found (12, 32). However, it is still unclear how CMV positivity affects CD8$^+$ T cells in the BM, particularly, highly differentiated effector cells. In previous studies, an increased frequency of effector/memory CD8$^+$ T cells lacking the costimulatory molecule CD28 and expressing markers of T cell activation has been found in the BM in comparison to the PB (8, 32). The effects of aging on the production of BM survival factors for effector/memory T cells have recently been described (12). Thus, we were now interested in considering whether CMV may have an impact on the phenotype of effector CD8$^+$ T cells not only in the PB but also in the BM in the context of the BM niches responsible for the maintenance of the immunological memory. Studies on CMV in old donors are frequently hampered by the fact that very few elderly persons are CMV$^-$. We have now had the chance to analyze an interesting Austrian cohort all living in Upper Austria with an unusually high prevalence of CMV$^-$ elderly donors. In addition, since only patients undergoing hip replacement surgery because of osteoarthrosis were included in the cohort, any possible effects of hip fracture on the immune system and the influence of depression frequently found in old patients with fractures could be excluded (41). Whether CMV infections are rare in this specific geographical area, or whether the low frequency of CMV infection is simply coincidence is not known. The availability of more than 30 BM samples from CMV$^-$ persons enabled us to acquire interesting data on the comparison of BM T cells from CMV$^+$ and CMV$^-$ elderly persons. Thus, we could show that T_{EMRA}^{bright} cells were more frequent in CMV$^+$ persons than in age-matched CMV$^-$ controls. CD45RA$^+$ CCR7$^-$ T cells have been shown to be a specific feature of CMV in the periphery (14), and we now know that this specific cell type can also be regarded as a marker of CMV infection in the BM. T_{EMRA} cells are frequently, but not always, CD28$^-$ and KLRG-1$^+$. It was of particular interest that CMV-specific changes of surface markers were observed in the CCR7$^-$ CD45RA^{bright}, but not in the CD45RA^{dim} population, suggesting that CMV does indeed drive T cell differentiation to its limits. T_{EMRA}^{bright} cells were also more frequent in BM than in the PB (data not shown), indicating that this cell type is specifically attracted by the BM. In this context, it is of additional interest that KLRG-1$^+$ IL-7Rα$^-$ so-called short-lived effector cells (SLECs), which are also enriched in the T_{EMRA}^{bright} subset, have been shown to be supported by IL-15 (42). As IL-15 production increases in the aged BM, we were interested in clarifying whether CMV infection had a similar effect. Indeed, we found that IL-15 production was higher in CMV$^+$ compared to CMV$^-$ donors (**Figure 5A**), however, both groups showed an increase in IL-15 production with age (**Figure 5C**). The highest IL-15 mRNA expression was in old CMV$^+$ donors. The increased number of interactions between IL-15-producing cells and CD8$^+$ T cells in CMV$^+$ persons further supports our concept that the BM microenvironment in old age, in combination with CMV, strongly attracts and supports CD8$^+$ T cells of a high differentiation status. The involvement of CMV in this process may be partly due to the fact that CMV is known to cause inflammation (15) and may, therefore, increase age-related inflammatory processes (43). In the BM, age-related changes such as the accumulation of reactive oxygen species (ROS) stimulate the production of IL-15, which in consequence attracts highly inflammatory T cells (12), resulting in a vicious circle, the results of which may be even more pronounced in CMV$^+$ persons.

In contrast to aging *per se*, CMV does not seem to change the BM IL-7 niche, but may still be responsible for an imbalance between the production of IL-15 and IL-7. This imbalance could lead to a preferential accumulation of highly differentiated CD8$^+$ T cells at the expense of CD4$^+$ and CD8$^+$ memory T cells and long-lived plasma cells.

In light of this possibility, our interest grew in the question whether the characteristic CD8$^+$ T cell populations in the BM were able to respond to the obvious IL-15 overload in this organ in CMV$^+$ persons. We, therefore, studied the common β-chain of the IL-15/IL-2 receptor (CD122) and found that this receptor was highly expressed in almost all CD8$^+$ T_{EMRA}^{bright} cells and was especially high in the CD28$^-$ T_{EMRA}^{bright} subset, which is particularly frequent in CMV$^+$ persons. We have previously shown that IL-15 signaling takes place in the BM (8). Our present data specifically suggest that the combination of high IL-15 production and high CD122 expression most likely leads to pronounced IL-15 effects in BM CD8$^+$ T cells of CMV$^+$ persons.

Peripheral Ab concentrations against CMV correlated positively with the percentage of CD8$^+$ CD28$^-$ T_{EMRA}^{bright} T cells in the BM, and there was a negative correlation between the peripheral Ab concentrations and IL-7Rα$^+$ on T_{EMRA}^{bright} T cells. The relationship was even more pronounced when a ratio between CD122 and IL-7Rα$^+$ was used in the correlation. It has been shown that the humoral anti-CMV response is particularly high in advanced aging associated with comorbidity and cognitive and functional impairments (44). This is of interest but would be of no relevance for our study, as none of the participants had obvious cognitive problems or overt disease. We also did not see a significant correlation between CMV Ab titers in the serum and age (data not shown). Although it is unclear what very high anti-CMV IgG Ab concentrations mean in CMV+ clinically healthy persons, they may indicate active humoral defense against re-activation of the virus. CD8+ T cell responses may be of even greater relevance during re-activation.

Our data on the relationship between highly differentiated CD8+ T cells in the BM and peripheral Ab concentrations indicate that they may both be markers of an ongoing immune response against CMV.

In summary, our data suggest that latent CMV infection leads to changes in the BM, which disturb the balance among immunoregulatory processes in the BM, in particular between stromal cell niches and T cells. CMV infection may, therefore, be considered as a risk factor for deterioration of the immunological memory in the BM, particularly in elderly individuals.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethics Committees of the "Klinikum Wels-Grieskirchen" (Austria) with written informed consent from all subjects in accordance with the Declaration of Helsinki prior to their inclusion in the study. The protocol was approved by the Ethics Committees of the "Klinikum Wels-Grieskirchen" (Austria).

AUTHOR CONTRIBUTIONS

LP and BG-L: study design, interpretation of data, critical appraisal, and final approval of the version to be published; KT: sample collection and study design; LP: method design; LP, EN, AM, BJ, and MK: experimental work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00715/full#supplementary-material.

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Differential Effect of Cytomegalovirus Infection with Age on the Expression of CD57, CD300a, and CD161 on T-Cell Subpopulations

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Immunosenescence is a progressive deterioration of the immune system with aging. It affects both innate and adaptive immunity limiting the response to pathogens and to vaccines. As chronic cytomegalovirus (CMV) infection is probably one of the major driving forces of immunosenescence, and its persistent infection results in functional and phenotypic changes to the T-cell repertoire, the aim of this study was to analyze the effect of CMV-seropositivity and aging on the expression of CD300a and CD161 inhibitory receptors, along with the expression of CD57 marker on $CD4^+$, $CD8^+$, $CD8^+CD56^+$ (NKT-Like) and $CD4^-CD8^-$ (DN) T-cell subsets. Our results showed that, regardless of the T-cell subset, $CD57^-CD161^-CD300a^+$ T-cells expand with age in CMV-seropositive individuals, whereas $CD57^-CD161^+CD300a^+$ T-cells decrease. Similarly, $CD57^+CD161^-CD300a^+$ T-cells expand with age in CMV-seropositive individuals in all subsets except in DN cells and $CD57^-CD161^+CD300a^-$ T-cells decrease in all T-cell subsets except in $CD4^+$ T-cells. Besides, in young individuals, CMV latent infection associates with the expansion of $CD57^+CD161^-CD300a^+CD4^+$, $CD57^-CD161^-CD300a^+CD4^+$, $CD57^+CD161^-CD300a^+CD8^+$, $CD57^-CD161^-CD300a^+CD8^+$, $CD57^+CD161^-CD300a^+$ NKT-like, and $CD57^+CD161^-CD300a^+$DN T-cells. Moreover, in young individuals, CD161 expression on T-cells is not affected by CMV infection. Changes of CD161 expression were only associated with age in the context of CMV latent infection. Besides, $CD300a^+CD57^+CD161^+$ and $CD300a^-CD57^+CD161^+$ phenotypes were not found in any of the T-cell subsets studied except in the DN subpopulation, indicating that in the majority of T-cells, CD161 and CD57 do not co-express. Thus, our results show that CMV latent infection impact on the immune system depends on the age of the individual, highlighting the importance of including CMV serology in any study regarding immunosenescence.

Keywords: CD57, CD300a, CD161, T-cell subsets, age and cytomegalovirus infection

INTRODUCTION

The human CD300 family has seven members, including the inhibitory receptor CD300a, which has been proposed as a possible biomarker for diagnosis and therapeutic target in several pathological situations (i.e., infectious diseases and cancer) (1–4). Human CD300a receptor is expressed on the surface of T (5, 6) and natural killer (NK) cells (7, 8). On human NK cells, the interaction between

CD300a and its ligand reduces their cytotoxic function (8). In T and B cells, the primary function of CD300a is to limit antigen receptor-mediated positive signaling (9). However, on CD8$^+$ T-cells, CD300a expression has been shown to associate with better cytotoxic function (10) and CD300a$^+$CD4$^+$ T-cells are associated with polyfunctionality and, upon stimulation, upregulate the transcription factor Eomesodermin (Eomes) (6, 11).

CD161 marker is a C-type lectin that was originally described in NK cells (12, 13). Nevertheless, CD161 is also expressed by T-cells including CD4$^+$, CD8$^+$ (12), and γδ T-cells (14). Within the CD4$^+$ subset, CD161 expression has been associated with IL-17 production. Indeed, Th17 cells can be originated from the CD161$^+$CD4$^+$ but not from their CD161$^-$CD4$^+$ counterpart (15). Of note, other IL-17-producing T-cells, such as CD8$^+$ and CD4$^-$CD8$^-$ double-negative T-cells are as well CD161$^+$ (16). Furthermore, it has been shown that CD161 expression on T-cells characterizes a transcriptional and functional T-cell phenotype that is TCR- and cell lineage-independent (17). All CD161$^+$ T-cell subsets shared a transcriptional signature and responded in a TCR-independent (innate-like) way to cytokine stimulation (IL-12 plus IL-18). However, CD161 had no regulatory effect on this response. Instead, CD161 has been shown to function as a costimulatory receptor in the context of TCR stimulation (18, 19). While the role of CD161 receptor on NK cells is agreed to be inhibitory (12, 20, 21), on T-cells, there is lack of consensus, as there is evidence of both costimulatory (20, 22) and inhibitory (18, 19) effects.

During aging, both innate and adaptive immunity are affected. Age-related changes have been described in several immune cell types including T-cells, NK cells, B-cells, macrophages, etc. Among those changes, the alterations in the number, phenotype, and functional capacity of immune cells have been associated with higher susceptibility to infectious diseases that ultimately lead to increased risk of fragility and death in those individuals (23–26). This age-associated deterioration of the immune system has been termed "immunosenescence." However, immunosenescence is not exclusively due to chronological aging of the individual and there are situations involving chronic stimulation of the immune system, such as viral infections, in which an "immunosenescence accelerated" or "early immunosenescence" is observed (27–30). In humans, infection by a common virus, cytomegalovirus (HCMV) has been shown to have profound impact on the T-cell compartment both on CD8$^+$ and CD4$^+$ T-cells (31, 32). HCMV persists after primary infection and is continuously controlled by the immune system (33, 34). Human herpes viruses, like CMV, have generally a benign/symbiotic relationship with the host (35–38). However, this benign relationship between herpesviruses and its hosts is altered with age. Indeed, CMV latent infection has been related to early immunosenescence (32, 39, 40). Particularly, CMV-seropositivity is associated with an increased risk of death and cardiovascular diseases (41–43) and is a contributor to the development of an "Immune Risk Phenotype" (IRP). This IRP is associated with early mortality in the elderly (44–46). Therefore, HCMV is considered one of the most relevant contributors to immunosenescence.

Thus, both HCMV infection and age contribute to the process of immunosenescence inducing changes on the T-cells. Understanding the mechanisms leading to immunosenescence and finding new biomarkers could open the possibility of novel therapies for the treatment of age-related diseases. In that regard, here, we study the effect of CMV latent infection and age on the expression of CD161 and CD300a receptors on CD4$^+$, CD8$^+$, CD8$^+$CD56$^+$ (NKT-like), and CD4$^-$CD8$^-$ (DN) T-cell subsets and their relation with the polyfunctionality marker CD57, which is a hallmark of CMV infection and aging in T-cells (37, 38).

MATERIALS AND METHODS

Subjects

We studied 64 healthy individuals stratified according to age and CMV serostatus (**Table 1**). Individuals in the old group and middle age group were all CMV-seropositive, as we were not able to recruit enough CMV-seronegative individuals due to the high prevalence of CMV seropositivity in Spain, which is about 80% in individuals over the age of 40 years (47) and reaches about 99% in individuals over 65 years in Andalusia (Southern Spain) where the samples were collected.

All subjects studied met the following exclusion criteria: absence of diabetes, cancer, severe renal failure, severe liver disease, endocrine disorders, autoimmune diseases, or acute infectious disease; they were not consuming drugs whose activity is known to modify the functions of the immune system. Ethical statement was approved by the Ethics Committee of the Reina Sofia University Hospital and all study participants provided informed written consent.

CMV Serology

CMV-specific IgG and IgM was determined in sera by using automated enzyme-linked immunosorbent assay (ELISA) (DRG International, Mountainside, NY, USA).

Flow Cytometry and Data Analysis

Peripheral blood from each subject was collected in lithium heparin tubes, followed by PBMCs isolation by density gradient centrifugation using Ficoll Histopage-1077 (Sigma-Aldrich, St. Louis, MO, USA). After isolation, PBMCs were cryopreserved until experiments were performed.

Cell thawing was carried out in RPMI 1640 (Sigma-Aldrich) with 10% FBS (Gibco, Life Technologies CA, USA) and cells were placed in a 96-well plate at 2×10^6 cells/ml concentration (250 µl final volume). Subsequently, cells were washed twice with PBS (4°C) and stained for the following antibodies: anti-CD3 PerCP (clone: BW 264/56, MiltenyiBiotec), anti-CD56 phosphatidylethanolamine (PE)-Cy7 (clone: B159, BD Pharmingen), anti-CD57 VioBlue (clone: TB03, MiltenyiBiotec), anti-CD300a PE (clone: E59.126, Beckman Coulter), anti-CD4 FITC

TABLE 1 | Demographics of studied individuals (n = 64).

CMV	Age (years)	No.	Group name
Negative	18–35	22	Young CMV-seronegative
Positive	18–35	15	Young CMV-seropositive
Positive	40–65	13	Middle age CMV-serpositive
Positive	>70	14	Old CMV-serpositive

(clone: M-T466, MiltenyiBiotec), anti-CD8 APC-Cy7 (clone: SK1, BD Biosciences), and anti-CD161 APC (clone: DX12, BD Pharmingen). All antibodies were titrated before use.

Samples were acquired with a nine parameters MACsQuant instrument (Miltenyi Biotech, BergischGladbach, Germany) and analyzed with FlowJo v X 10.0.7 software (TreeStar, Portland, OR, USA). First, lymphocytes were gated according to their size and granularity (FSC vs SSC), then forward scatter height versus forward scatter area to remove doublets. Within that gate (singlets), $CD3^+$ T-cells were gated, followed by identification of the different T-cell subsets by confronting CD4 vs CD8. NKT-like cells ($CD8^+CD56^+$) were then gated from $CD8^+$ T-cells (Figure S1A in Supplementary Material). The average number of events acquired for each subset was: 71161 cells for $CD4^+$ subset, 32498 cells for $CD8^+$, 5708 cells for NKT-like, and 5511 cells for DN. Individual gates (set based on fluorescence minus one controls) for $CD57^+$, $CD161^+$, and $CD300^+$ cells were gated on each of these populations (Figure S1B in Supplementary Material). FlowJo's Boolean gating options were performed to analyze the co-expression of CD57, CD161, and CD300a markers.

Statistical Analysis

Data were inspected for normal distribution using the Shapiro–Wilk test. No normality was found. According to this,

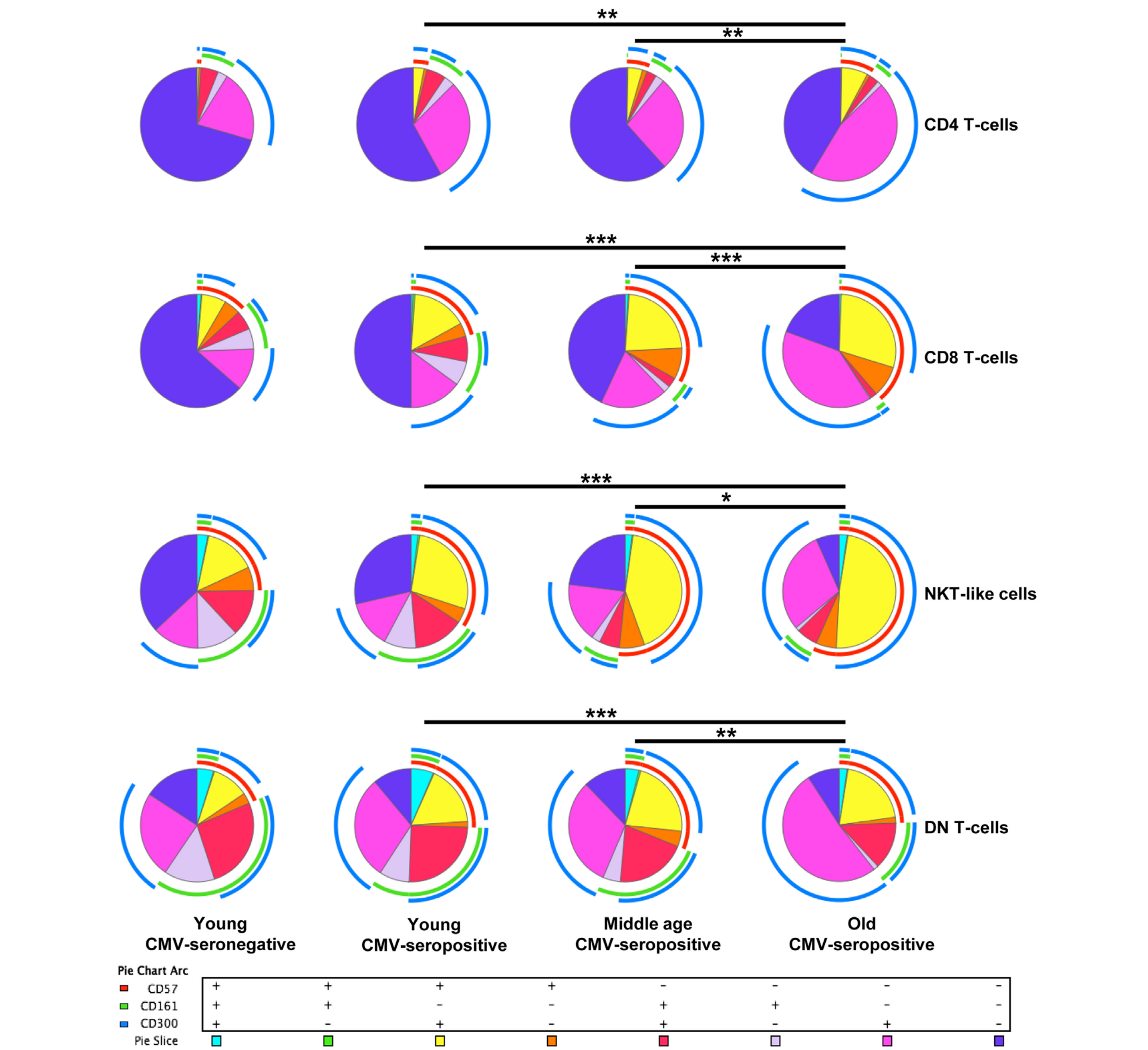

FIGURE 1 | Expression of CD57, CD161, and CD300a in T-cells. CD57, CD161, and CD300a co-expression patterns (pie charts) in $CD4^+$, $CD8^+$, NKT-like, and DN T-cells from healthy individuals (n = 64), stratified by age and CMV serostatus. Results were considered significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

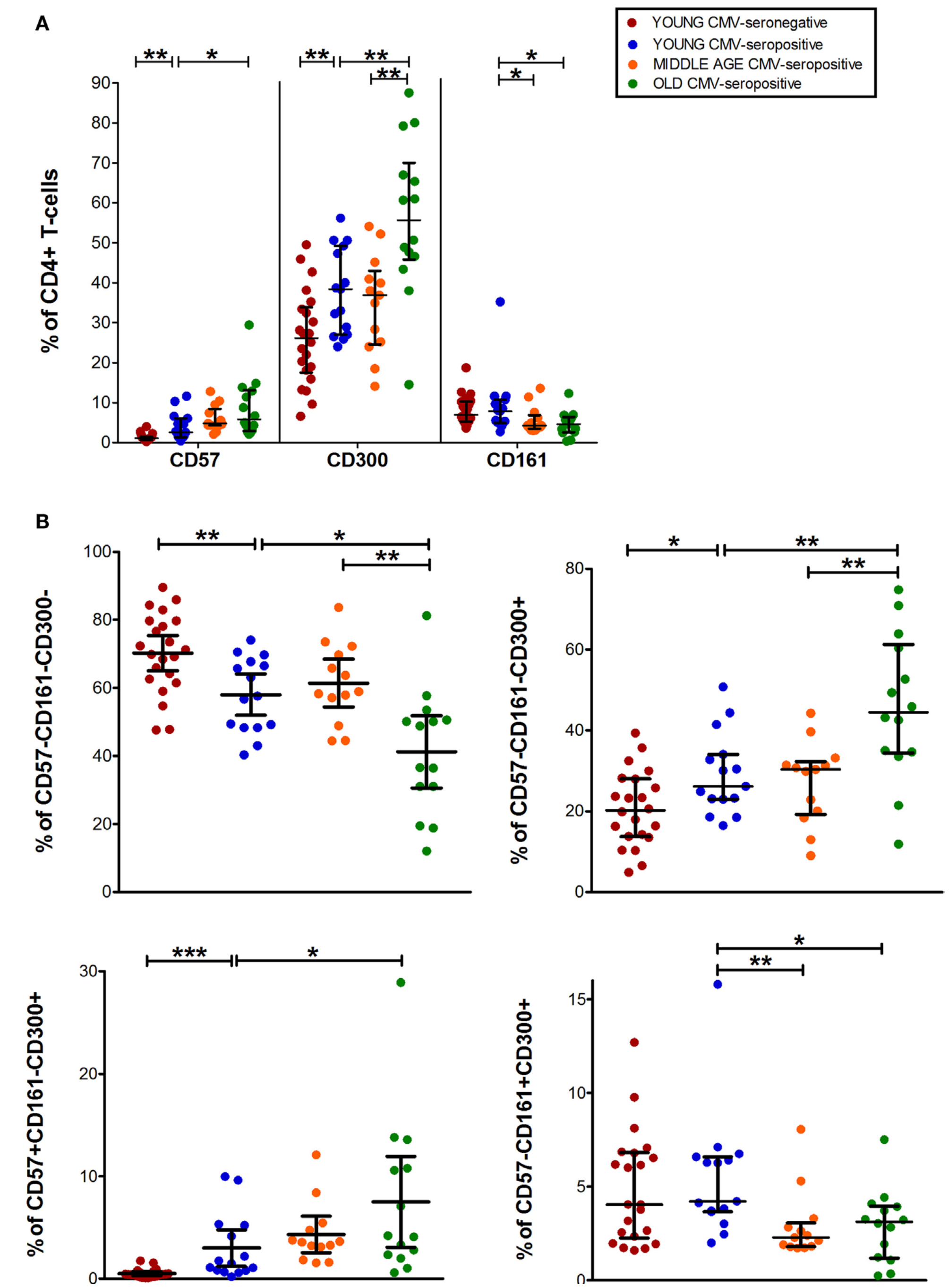

FIGURE 2 | CD57, CD300, and CD161 expression on CD4⁺ T-cells. **(A)** Total expression (percentage) of CD57, CD161, and CD300a on CD4⁺ T-cells from young CMV-seronegative ($n = 22$), young CMV-seropositive ($n = 15$), middle age CMV-seropositive ($n = 13$), and old CMV-seropositive donors ($n = 14$). **(B)** Co-expression of CD57, CD161, and CD300a on CD4⁺ T-cells. Graphs show CD4⁺ T-cell phenotypes in which we found statistical differences among the four groups studied. Vertical blacklines indicate interquartile ranges, ranging from the 25th to the 75th percentile. The median values are indicated by a horizontal black line. Results were considered significant at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Kruskal–Wallis *H* test (non-parametric test) with correction for multiple comparisons was used for direct comparison of the four groups. Those variables in which we found a statistical significant difference were then analyzed using the Mann–Whitney *U* non-parametric test for comparing data among the specific sample pairs. All statistical tests were performed with PASW Statistics v18. For scatter graphs, GraphPad Prism (version 5.0) was used. All graphs reflect only the Mann–Whitney derived *p*-values. To compare the pie charts, we used SPICE's permutation analysis (Mario Roederer, ImmunoTechnology Section, Vaccine

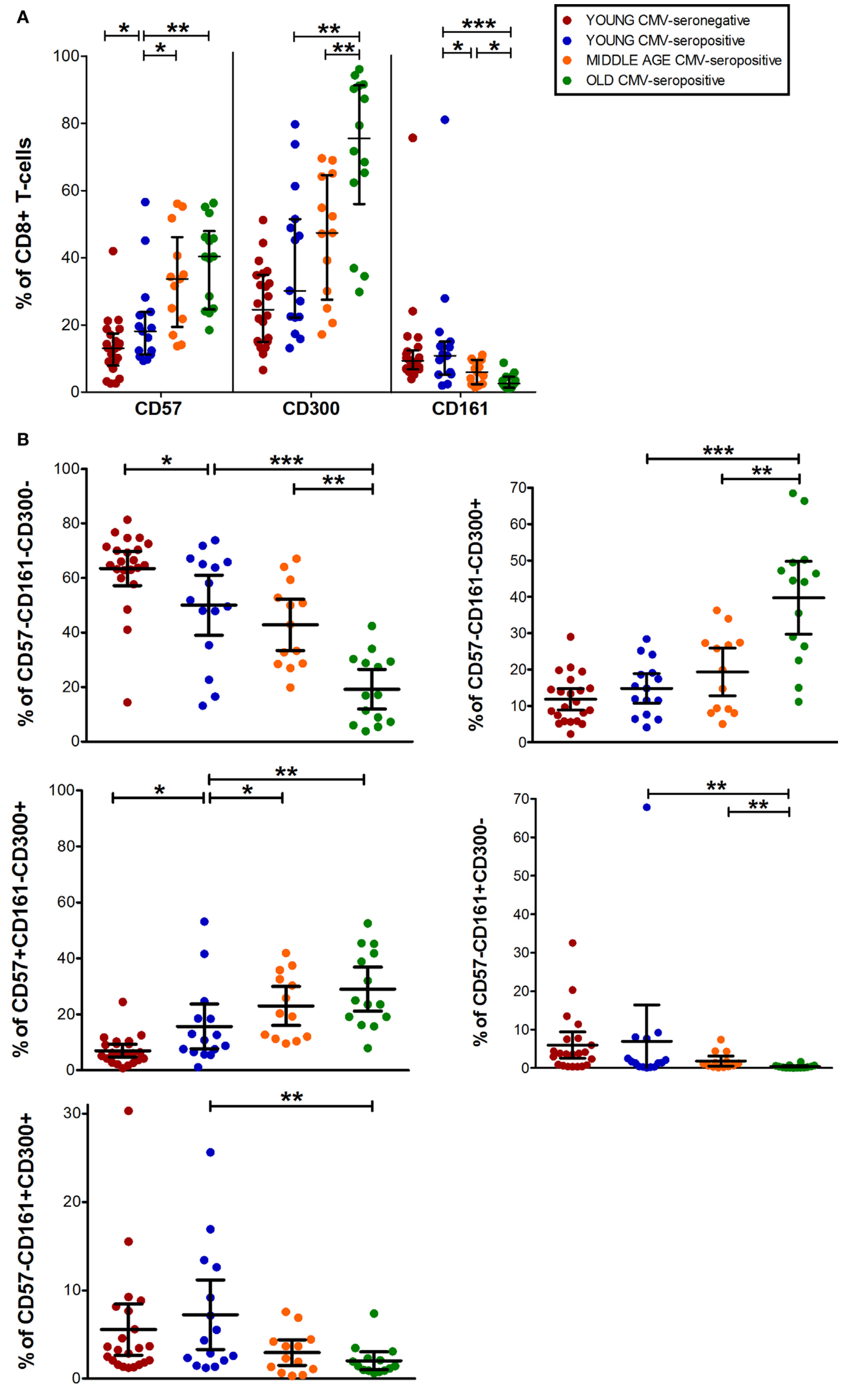

FIGURE 3 | Continued

FIGURE 3 | Continued
CD57, CD300, and CD161 expression on $CD8^+$ T-cells. **(A)** Total expression (percentage) of CD57, CD161, and CD300a markers on $CD8^+$ T-cells from young CMV-seronegative ($n = 22$), young CMV-seropositive ($n = 15$), middle age CMV-seropositive ($n = 13$), and old CMV-seropositive donors ($n = 14$). **(B)** Co-expression of CD57, CD161, and CD300a on $CD8^+$ T-cells. Graphs show the phenotype combinations $CD8^+$ T-cells in which we found statistical differences among the four groups studied. Vertical blacklines indicate interquartile ranges, ranging from the 25th to the 75th percentile. The median values are indicated by a horizontal black line. Results were considered significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Research Centre, NIH, Bethesda, MD, USA) (48), which asks how often given the samples that comprise the two pies charts, the difference observed would happen simply by chance (10,000 permutations).

RESULTS

CD57, CD161, and CD300a Expression on T-Cells

Multicolor flow cytometry was used to analyze the expression of CD57, CD161, and CD300a markers on $CD4^+$, $CD8^+$, NKT-like, and DN T-cell subpopulations from healthy individuals stratified by age and CMV-serostatus (**Table 1**).

FlowJo's Boolean analysis of CD57, CD161, and CD300a expression generated eight different possible phenotype combinations per T-cell subset. However, not all the possible combinations were biologically meaningful. Phenotype profiles for each subset were obtained using SPICE software (**Figure 1**).

$CD4^+$ T-Cells

Analysis of $CD4^+$ T-cell subset showed that $CD57^+CD4^+$ and $CD300a^+CD4^+$ T-cells increased with age in CMV-seropositive individuals and with CMV infection in young individuals (**Figure 2A**). In contrast, $CD161^+CD4^+$ T-cells percentage was decreased with age (**Figure 2A**).

Out of the eight possible Boolean phenotype combinations, we only found five within the $CD4^+$ T-cell subset, as the percentages of cells with $CD57^+CD161^+CD300a^+$, $CD57^+CD161^+CD300a^-$ and $CD57^+CD161^-CD300a^-$ phenotypes were noticeably low or null in all subjects studied (**Figure 1**).

The majority of $CD4^+$ T-cells in young and middle-age individuals did not express any of the markers studied ($CD57^-CD161^-CD300a^-$). However, in the elderly, more than 50% of the cells were $CD300a^+$ (55.67%, IQR 46.62–66.99) alone or in combination with CD161 or CD57 (**Figures 1** and **2A**, Table S1 in Supplementary Material). Our data as well showed that the percentage of triple negative ($CD57^-CD161^-CD300a^-$) $CD4^+$ T-cells was decreased by CMV infection in young individuals. The progressive reduction of $CD57^-CD161^-CD300a^-$ $CD4^+$ T-cells by CMV infection and age corresponded with an increase of $CD57^-CD161^-CD300a^+$ and $CD57^+CD161^-CD300^+$ phenotypes (**Figure 2B**). Of note, $CD57^+CD4^+$ T-cells were only present in CMV-seropositive individuals and always co-expressing CD300a (**Figures 1** and **2B**).

On the other hand, our analysis showed that CD161 is never co-expressed with CD57 in any of the groups studied (**Figure 1**). $CD57^-CD161^+CD300a^-$ and $CD57^-CD161^+CD300a^+$ $CD4^+$ T-cells decreased with age in CMV-seropositive individuals, being the percentage of $CD57^-CD161^+CD300a^-$ cells very low or null in the elderly (**Figures 1** and **2B**).

Furthermore, the phenotype profiles of $CD4^+$ T-cells changed with age in CMV-seropositive individuals, but not with CMV infection alone (pie charts representing the three makers' combinations, **Figure 1**). This shift of phenotype is mainly due to an accumulation with age of the $CD57^-CD161^-CD300a^+$ and $CD57^+CD161^-CD300a^+$ phenotypes in the CMV-seropositive individuals.

$CD8^+$ T-Cells

Data from $CD8^+$ T-cell subset showed that $CD57^+CD8^+$ T-cells increased with CMV infection alone and in combination with age. While, $CD300a^+CD8^+$ T-cells accumulate with age in CMV-seropositive individuals and $CD161^+CD8^+$ T-cells decreased progressively being very low or null in the elderly (**Figures 1** and **3A**).

The percentages of $CD57^+CD161^+CD300a^+$ and $CD57^+CD161^+CD300a^-$ $CD8^+$ T-cells were noticeably low or null in all subjects studied. The majority of $CD57^+CD8^+$ T-cells were positive for CD300a and negative for CD161 (**Figure 1**). However, in contrast to $CD4^+$ T-cells, in the $CD8^+$ subset, we found a small fraction of cells with $CD57^+CD161^-CD300a^-$ phenotype (pie slice orange, **Figure 1**), not affected by age.

In young and middle age individuals, 60–70% of the $CD8^+$ T-cells were mainly $CD57^-CD161^-CD300a^-$ (Table S1 in Supplementary Material). However, in the elderly, only 17% (IQR 7.20–29.30) of $CD8^+$ T-cells did not express any of the markers (**Figure 3B**; Table S1 in Supplementary Material). This drastic reduction observed in the elderly is due to the expansion of $CD300a^+$ cells with or without CD57 (yellow and pink pie slices, **Figure 1**). In young individuals, $CD57^-CD161^-CD300a^-$ $CD8^+$ T-cells decreased with CMV infection (**Figure 3B**) due to the expansion of $CD57^+CD161^-CD300a^+$ cells (yellow pie slice, **Figures 1** and **3B**).

Additionally, we observed that in young and middle age individuals, $CD161^+CD8^+$ T-cells were $CD300a^+$ or $CD300a^-$, whereas in the elderly, the few $CD161^+$ cells observed were all $CD300a^+$ (pie slices red and violet, **Figure 1**).

The phenotype profiles (pie charts, **Figure 1**) of $CD8^+$ T-cells changed noticeably with age in CMV-seropositive individuals, but not with CMV infection alone (**Figure 1**).

$CD8^+CD56^+$ T-Cells (NKT-Like Cells)

The expression of CD57, CD161, and CD300a markers on NKT-like cells was not affected by CMV infection alone. However, $CD57^+$ and $CD300a^+$ NKT-like cells increased with age in CMV-seropositive individuals (**Figure 4A**), while $CD161^+$ NKT-like cells decreased (**Figure 4A**).

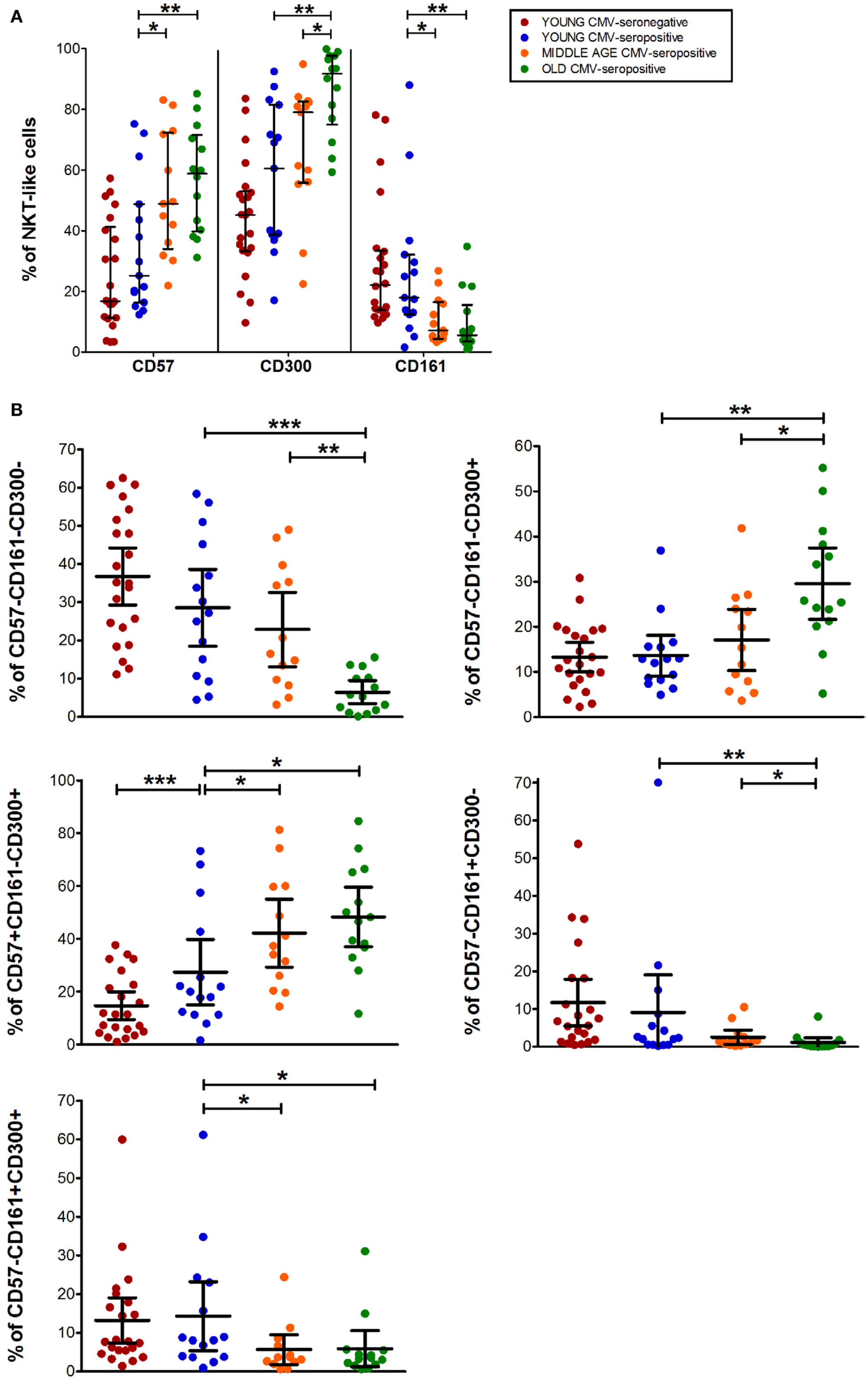

FIGURE 4 | Continued

FIGURE 4 | Continued
CD57, CD300, and CD161 expression on NTK-like cells. **(A)** Total expression (percentage) of CD57, CD161, and CD300a markers on NKT-like cells from young CMV-seronegative ($n = 22$), young CMV-seropositive ($n = 15$), middle age CMV-seropositive ($n = 13$), and old CMV-seropositive donors ($n = 14$). **(B)** Co-expression of CD57, CD161, and CD300a on NKT-Like cells. Graphs show the markers combinations in which we found statistical differences among the four groups studied. Vertical black lines indicate interquartile ranges, ranging from the 25th to the 75th percentile. The median values are indicated by a horizontal black line. Results were considered significant at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

CD57$^+$CD161$^+$CD300a$^+$ and CD57$^+$CD161$^+$CD300a$^-$ NKT-like cells were very low or null. Thus, in our hands, as CD4$^+$ and CD8$^+$ subsets, NKT-like cells did not co-express CD57 and CD161 (pie slice orange, **Figure 1**). The majority of CD57$^+$ NKT-like cells were also CD300a$^+$. However, we observed a small fraction of NKT-like cells with a CD57$^+$CD161$^-$CD300a$^-$ phenotype, not affected by CMV infection and age (**Figure 1**). Of note, in the elderly, 47.35% (IQR 36.70–65.10) of the NKT-like cells were CD57$^+$CD161$^-$CD300a$^+$ (yellow pie slice, **Figure 1**). The proportion of this phenotype is significantly lower ($p < 0.001$) in the rest of the T-cell subsets studied, particularly in the CD4$^+$ T-cells in which this phenotype frequency is quite low even in the elderly (**Figure 1**).

In the NKT-like subset, 65–95% of the cells expressed at least one of the markers studied, being the fraction of triple negative cells (CD57$^-$CD161$^-$CD300a$^-$) very low in old individuals (5.58%, IQR 1.77–10.20) (**Figures 1** and **4B**), due to the expansion of CD57$^-$CD161$^-$CD300a$^+$ and CD57$^+$CD161$^-$CD300a$^+$ phenotypes (pie slices pink and yellow, **Figure 1**). Noticeably, CD57$^+$CD161$^-$CD300a$^+$ cells were also increased in young CMV-seropositive individuals compared with CMV-seronegative (**Figure 4B**).

NKT-like CD161$^+$ phenotypes (CD57$^-$CD161$^+$CD300a$^-$ and CD57$^-$CD161$^+$CD300a$^+$) decreased with age in CMV-seropositive individuals, but not with CMV infection alone (**Figures 1** and **4B**). Remarkably, the percentage of CD57$^-$CD161$^+$CD300a$^-$ NKT-like cells was very low or null in middle age and old individuals (**Figure 4B**; Table S1 in Supplementary Material).

As in the CD4$^+$ and CD8$^+$ main populations, the phenotype profiles of NKT-like cells were not affected by CMV infection alone (**Figure 1**).

CD4$^-$CD8$^-$ T-Cells (DN T-Cells)

Data from DN T-cells (majorly γδ T-cells) flow analysis showed a similar percentage of CD57$^+$ DN T-cells among the three CMV-seropositive groups (young, middle age, and old) (**Figure 5A**). While, CD161$^+$ DN T-cell decreased gradually with age in CMV-seropositive individuals (**Figure 5A**). Furthermore, CD300a$^+$ DN T-cells increased with CMV infection in young individuals and further increased in old CMV-seropositive individuals (**Figure 5A**).

As in the other T-cell subsets studied, in DN T-cells the percentage of CD57$^+$CD161$^+$CD300a$^-$ cells is null (**Figure 1**). However, we observed a small fraction of DN T-cells co-expressing the three markers that decreases with age (**Figures 1** and **5B**).

The majority of DN T-cells in all individuals are CD300a$^+$ with or without CD161 or CD57 expression (**Figure 1**). Our analysis showed that the percentage of CD57$^-$CD161$^-$CD300a$^+$ cells increased with age in CMV-seropositive individuals, whereas the percentage of CD57$^+$CD161$^-$CD300a$^+$ increases with CMV infection in young individuals and is not affected by age (**Figure 5B**). Of note, our results showed that, similarly to CD57$^+$CD4$^+$ T-cells, the majority of CD57$^+$ DN T-cells are as well CD300$^+$ (**Figure 1**).

Besides, CD161$^+$ DN T-cells were mainly CD57$^-$ (only a small fraction co-expressed CD161 and CD57). CD57$^-$CD161$^+$CD300a$^-$ phenotype decreased with age, being the percentage of these cells null in the elderly (pie slice violet, **Figures 1** and **5B**). Whereas a reduced percentage of CD57$^-$CD161$^+$CD300a$^+$ DN T-cells was still present in old individuals (pie slice red, **Figures 1** and **5B**).

DN T-cell phenotype profiles for the makers studied (pie charts, **Figure 1**) changed with age in CMV-seropositive individuals, but not with CMV infection alone.

DISCUSSION

The combination of age and CMV latent infection has been proven to have a profound impact on the immune phenotype and function of T-cells, not only on the CD8$^+$ subset but also on CD4$^+$, NKT-like, and γδ T-cells. However, age and CMV infection do not always have similar effects and it can vary depending on the cell type.

Here, we analyzed, in different T-cell subsets, how age and CMV infection alter the expression of the inhibitory receptors CD300a and CD161 and their relation with the marker CD57, which has been shown to be a polyfunctionality maker of CD4$^+$, CD8$^+$, and NKT-like T-cells (37, 38, 49). We are aware that due to the high prevalence of CMV in our geographic area (see Materials and Methods), a limitation of our study is the lack of CMV-seronegative individuals of older ages (middle age and old groups). Thus, we can only assess the effect of aging in the context of CMV latent infection. Nevertheless, we were able to investigate the effect of CMV infection alone in young individuals.

Our analysis showed that in all T-cell subsets studied, CD57 and CD300a increase with age in CMV-seropositive individuals. Specifically, with CMV infection (young individuals), CD57 is increased only in CD4$^+$ and CD8$^+$ T-cells and CD300a in CD4$^+$ and DN subsets. No effect of CMV alone was observed on NKT-like cells. Of note, CD57$^+$CD4$^+$ T-cells are always CD300a$^+$ and were only found in CMV-seropositive individuals. In the rest of subsets (CD8$^+$, NKT-like and DN), although not all, the majority of CD57$^+$ T-cells were CD300a$^+$ as well, regardless of the age and CMV serostatus.

The expression of CD300a by several immune cell types has been associated with different pathologies, suggesting that, although the significance of CD300a on T-cell function is not completely clear, CD300a could be used as a biomarker and a target for new therapies [for review, see Ref. (50)].

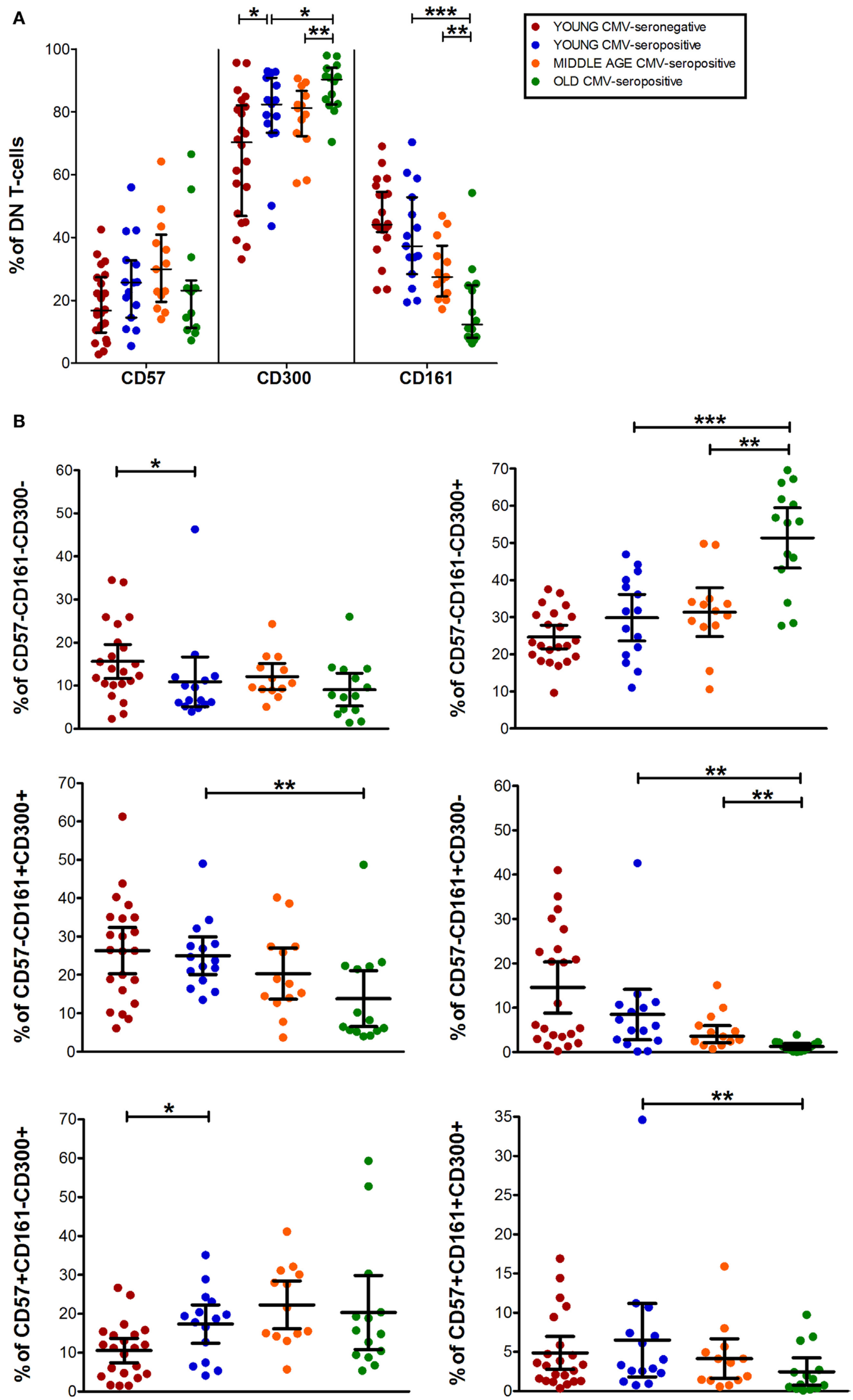

FIGURE 5 | Continued

FIGURE 5 | Continued
CD57, CD300, and CD161 expression on CD4⁻CD8⁻ T-cells (DN). **(A)** Total expression (percentage) of CD57, CD161, and CD300a markers on DN T-cells from young CMV-seronegative (n = 22), young CMV-seropositive (n = 15), middle age CMV-seropositive (n = 13), and old CMV-seropositive donors (n = 14). **(B)** Coexpression of CD57, CD161, and CD300a on DN T-cells. Graphs show DN T-cells phenotypes in which we found statistical differences among the four groups studied. Vertical black lines indicate interquartile ranges, ranging from the 25th to the 75th percentile. The median values are indicated by a horizontal black line. Results were considered significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

CD300a ligands, phosphatidylserine (PS), and phosphatidylethanolamine (PE) are associated with virus evasion. Indeed, anti-PS antibody has been shown to be a potential treatment for CMV and Pichinde virus infections (51). Additionally, HIV-specific CD8$^+$ T-cell mRNA levels of CD300a receptor have been shown to correlate with the expression of BATF transcription factor, which is highly expressed in exhausted cells. BTAF inhibits cell function by inducing the expression of inhibitory receptors such as CD300a (52). Viral envelope expression of PS and PE has been shown to be an evasion mechanism called "apoptotic mimicry" (53). However, CD300a binding to viruses expressing PS and PE in their envelopes seams to inhibit the virus endocytosis, most probably hampering the virus infection. All these data supports that CD300a has an inhibitory role and it is important for viral infections.

However, in CD8$^+$ T-cells, CD300a expression associates with higher cytotoxicity and CD300a$^+$CD8$^+$ T-cells are increased in pregnant women with chronic choriamnionitis (10). Additionally, CD300a has been shown to be a polyfunctionality marker in CD4$^+$ T-cells and CD300a$^+$CD4$^+$ T-cells upregulate Eomes transcription factor after stimulation (11). Furthermore, our group has recently shown that CD57$^+$CD4$^+$ T-cells are polyfunctional and express high levels of T-bet and Eomes transcription factors upon superantigen stimulation (38). Moreover, CD57$^+$CD8$^+$ T-cells correlate with polyfunctionality of CD8$^+$ T-cells and are expanded in young CMV-seropositive individuals (37).

Whether CD300a$^+$CD57$^-$ and CD300a$^+$CD57$^+$ T-cells display any differences regarding polyfunctionality and if there are differences in regards of T-bet and Eomes expression is currently under investigation in our laboratory. This analysis will allow us to establish if CD300a is a polyfunctional marker of T-cells *per se*, or only if co-expressed with CD57. In our hands CD57$^+$ T-cells co-expressing CD300a expand with CMV infection (in young individuals), highlighting a relevant role for both makers in the control of CMV virus by T-cells. On the other hand, CD161 receptor was hardly co-expressed with CD57 in any of the T-cell subsets studied. Particularly, co-expression of CD161 and CD57 was not observed in the elderly regardless of the T-cell subset. Furthermore, the total expression of CD161, contrarily to CD57 and CD300a, decreases with age in CMV-seropositive individuals and is not affected by CMV infection alone in young individuals. Our results support previous results from healthy children in which the expression of CD161 on T and NK cells was not affected by CMV serostatus (54). However, Almehmadi et al. suggest that NKT-like cells not expressing CD161 are increased in CMV-seropositive individuals. This discrepancy with our data can be explained by the fact that Almehmadi's cohort does not stratify the individuals by age (23–60 years), only by CMV status. Additionally, their definition of NKT-like cells differs from ours as it is based only on CD3 and CD56 expression, not including CD8 [for review of NKT-like cells nomenclatures, see Ref. (55)]. In our previous work, regarding NKT-like cell number and functionality in the context of CMV infection and age, we show that the percentage of NKT-like cells is not affected by CMV infection in young CMV individuals, but rather with the combined effect of both age and CMV latent infection (49). Similarly, the loss of CD161 by T-cells does not associate with CMV alone, but with age in the context of CMV latent infection. Indeed, the expression of CD161 in CMV-specific cytotoxic T lymphocytes is very low (56).

Besides, acute and chronic GVHD correlates with decreased levels of circulating CD161$^+$CD4$^+$ and CD161^{++}CD8$^+$ T-cells (57). Moreover, in rheumatoid arthritis patients, it has been shown an increase of CD161$^+$CD4$^+$ T-cells, but a decrease of CD161$^+$ DN T-cells that was associated with disease activity and inflammation (58, 59). As we mentioned before, T-cells-expressing CD161 are IL-17 producers. In our cohort, the percentages of CD161$^+$ T-cells are very low or null in old CMV-seropositive individuals regardless of the T-cell type. This could translate in a diminished Th17 response in the elderly.

Contrarily to what we observe in T-cells, our previous work on NK cells showed a decreased expression of CD161 on CD56dim NK cells associated with CMV seropositivity (60).

The data presented here together with our previous results highlight the importance of taking into account both age and CMV serostatus in any clinical study regarding the analysis of T-cells, as CMV latent infection has a differential effect with age on T-cell subsets. Additionally, our data support the potential use of CD57, CD300a, and CD161 as biomarkers of immunosenescence and as possible targets for novel therapies. The clinical implications of the changes found in the expression of these makers should be further investigated.

ETHICS STATEMENT

This work was approved by the Ethics Committee of the Reina Sofia University Hospital. All participants in the study provided informed written consent.

AUTHOR CONTRIBUTIONS

RS and AP designed the study. FH collected the data and performed the laboratory experiments. FH and NL-S collaborated in the laboratory analysis. AP and FH performed the statistical analysis and wrote the draft. RT, BS-C, and CC made significant technical and conceptual contributions to the manuscript. RS, RT, and AP reviewed the final version of the paper. All the authors provided intellectual content and approved the final version of the paper. RS an AP are co-senior authors and have contributed equally to this work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00649/full#supplementary-material.

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Immune Checkpoint Function of CD85j in CD8 T Cell Differentiation and Aging

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Aging is associated with an increased susceptibility to infection and a failure to control latent viruses thought to be driven, at least in part, by alterations in CD8 T cell function. The aging T cell repertoire is characterized by an accumulation of effector CD8 T cells, many of which express the negative regulatory receptor CD85j. To define the biological significance of CD85j expression on CD8 T cells and to address the question whether presence of CD85j in older individuals is beneficial or detrimental for immune function, we examined the specific attributes of CD8 T cells expressing CD85j as well as the functional role of CD85j in antigen-specific CD8 T cell responses during immune aging. Here, we show that CD85j is mainly expressed by terminally differentiated effector (TEMRAs) CD8 T cells, which increase with age, in cytomegalovirus (CMV) infection and in males. CD85j$^+$ CMV-specific cells demonstrate clonal expansion. However, TCR diversity is similar between CD85j$^+$ and CD85j$^-$ compartments, suggesting that CD85j does not directly impact the repertoire of antigen-specific cells. Further phenotypic and functional analyses revealed that CD85j identifies a specific subset of CMV-responsive CD8 T cells that coexpress a marker of senescence (CD57) but retain polyfunctional cytokine production and expression of cytotoxic mediators. Blocking CD85j binding enhanced proliferation of CMV-specific CD8 T cells upon antigen stimulation but did not alter polyfunctional cytokine production. Taken together, these data demonstrate that CD85j characterizes a population of "senescent," but not exhausted antigen-specific effector CD8 T cells and indicates that CD85j is an important checkpoint regulator controlling expansion of virus-specific T cells during aging. Inhibition of CD85j activity may be a mechanism to promote stronger CD8 T cell effector responses during immune aging.

Keywords: ILT-2, LILRB1, immunosenescence, exhaustion, NK receptors, innate-like CD8 T cells, cytomegalovirus, chronic viral infection

INTRODUCTION

Human aging is characterized by a loss of effective immune responses against viral pathogens, coinciding with an increased susceptibility to infection and a failure to control latent viruses (1, 2). Functional CD8 T cell responses are critical for protection against viral infections. However, there are considerable changes in the memory CD8 population that may contribute to reduced functionality during immune aging. These changes include an accumulation of terminal differentiated effector CD8 T cells, frequently labeled as "senescent," that exhibit reduced proliferative capacity but maintain cytotoxic and cytokine-producing functions, unlike exhausted T cells that lack both proliferative and effector responses (3, 4). Acquisition of such CD8 T cells is also a hallmark of chronic viral infections, such as cytomegalovirus (CMV), and further accelerated in CMV antibody-positive older individuals (5, 6). Moreover, recent evidence suggests that end-differentiated CD8 T cells demonstrate similar properties to innate-like T cells and gain the expression of multiple activating and inhibiting regulatory receptors including killer immunoglobulin-like receptors (7, 8).

The increased expression of the inhibitory receptor CD85j (alternatively known as ILT-2 or LILRB1) on CD8 T cells is one phenotypic hallmark of aging (9, 10). A wide range of immune cells including monocytes, B cells, dendritic cells, and a subset of NK and T cells express CD85j. However, the levels of CD85j on cell types vary widely based on cell-specific transcriptional regulation of CD85j (11). CD85j shares structural similarities with PD-1, a well-established checkpoint molecule, which include a cytoplasmic tail containing multiple immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that are able to recruit tyrosine phosphatases SHP-1 and SHP-2 and a tyrosine-based switch motif (ITSM) (12–14). CD85j recognizes a broad range of classical and non-classical MHC class I molecules, including high affinity for HLA-G (15–17). CD85j also binds with high affinity to a CMV MHC class I homolog UL18 (18, 19), is expressed by CMV-specific CD8 T cells (20), and is proposed to play a significant role in CD8 T cell responsiveness to CMV infection. Thus, CD85j may have an important function in checkpoint inhibition, maintenance of T cell homeostasis, and prevention of memory inflation with aging and CMV infection.

Previous studies suggest that CD85j can inhibit cytokine production, decrease proliferation, and reduce cytotoxicity of T cells (21, 22), which are common features of exhausted CD8 T cells. However, CD85j is often coexpressed with the senescence marker CD57 (23). Thus, it is unclear whether CD85j-expressing CD8 T cells in immune aging are truly exhausted or a subset of senescent cells. Moreover, the specific function of CD85j on CD8 T cells during chronic viral infection in older individuals is unknown. Thus, to better define the biological significance of CD85j expression on CD8 T cells and to address the question whether inhibition of CD85j in older individuals is beneficial or detrimental for immune function, we examined the specific attributes of CD8 T cells expressing CD85j as well as the functional role of CD85j in CMV-specific CD8 T cell responses during immune aging.

MATERIALS AND METHODS

Study Participants

This study included samples from three sources. We obtained data and additional peripheral blood samples from a previously published cohort of 740 healthy individuals aged 40–97 that approximately mirrored San Francisco Bay Area ethnic demographics (24). Additional healthy individuals between the age of 18 and 80 years from the same draw area were recruited. Deidentified samples from HLA-A*02$^+$ individuals of various ages and with positive CMV serology were purchased from the Stanford Blood Center (Palo Alto, CA, USA). The study was in accordance with the Declaration of Helsinki, approved by the Stanford Institutional Review Board, and all participants gave written informed consent.

Cellular Phenotyping by Flow Cytometry

For cellular phenotyping of tetramer-specific cells, we used CD3-APC/Cy7, CD8-qDot605, CD45RA-PE/Cy7, CCR7-PerCp/Cy5.5, CD28-PE, CD85j (ILT-2)-APC, and tetramer–Pacific Blue. Antibodies were purchased from BD Bioscience, Biolegend, or eBioscience. HLA-A*0201 monomers loaded with peptides for CMV pp65 (NLVPMVATV) or EBV BRLF1 (YVLDHLIVV) were tetramerized and labeled with streptavidin–Pacific Blue.

T Cell Sorting and *TRB* Sequencing

Total T cells were isolated by negative selection using human T cell RosetteSep enrichment kit (StemCell Technologies) from platelet donor apheresis lymphocytes of HLA-A2 donors who are CMV seropositive. T cells were stained with CD4, CD8, pp65 HLA-A*0201 tetramer, and CD85j antibodies. CD85j$^+$ and CD85j$^-$ pp65-HLA-A*0201 tetramer$^+$ CD8 T cells were sorted using a FACSAria (BD Bioscience) and split into two to four replicates with 4,000–5,000 cells per replicate. Total RNA was extracted from each T cell replicate using RNeasy Plus Micro kit (Qiagen), followed by generation of cDNA using SuperScript VILO master mix (Invitrogen). The amplification and sequencing of TRB gene libraries followed the protocol as previously described (25).

The sequences were mapped to human *TRB* reference sequences as described in detail previously (25, 26). Clonotypes were defined as sequences with the same *TRBV* and *TRBJ* gene segments and identical CDR3 amino acid sequences. In addition, any clonotype that was only found in one replicate library was filtered out of the analysis. The clonality index for each population was calculated using the lymphclon package (https://arxiv.org/abs/1408.1149) (25).

CyTOF

PBMCs were left unstimulated or stimulated for 18 h with CMV peptide pools in the presence of brefeldin A and monensin (BD Bioscience). For CMV-specific stimulation, two peptide super pools, each made up of overlapping peptide pools of four different antigens, were used. The immediate early (IE) pool consisted of IE-1, IE-2, US3, and UL36. The late pool consisted of pp65, UL32, UL48AB, and UL55 (gB), based on previously described work (27). Following stimulation, cells were resuspended in CyFACS buffer (1× PBS with 0.1% BSA, 2 mM EDTA, and 0.5% sodium azide) and stained with isotope-tagged antibodies before being acquired on the CyTOF. For a detailed protocol, see http://iti.stanford.edu/himc/protocols.html (CyTOF ICS protocol) and Table S1 in Supplementary Material.

Data acquired from CyTOF were initially analyzed using FlowJo v10.1 (FlowJo Inc.). CD3$^+$CD19$^-$CD8$^+$CD4$^-$ cells expressing CD107a or one of the following cytokines, IFNγ, TNFα, IL-2, GM-CSF, or MIP1β, after stimulation with IE or late pool were considered "CMV-responsive" CD8 T cells. The CMV-responsive cells for 30 individuals were concatenated, and cluster analysis was performed using X-shift (28). For final clustering, basic phenotypic (CD45RA, CCR7, CD28, CD27, and CD127) and the six preselected response factors were excluded.

In Vitro Blocking Experiments

Reagents

Peptide pools were purchased from JPT Peptide Technologies. The late antigen pp65 peptide pool was a combination of 138 peptides derived from a peptide scan (15mers with 11 amino-acid overlap) through 65 kDa phosphoprotein (pp65) (Swiss-Prot ID: P06725) of human cytomegalovirus (HHV-5). The immediate early antigen IE-1 peptide pool was a combination of 120 peptides derived from a peptide scan (15mers with 11 amino acid overlap) through 55 kDa immediate early protein 1 (IE-1) (Swiss-Prot ID: P13202) of human cytomegalovirus (HHV-5).

PBMC Assays

PBMCs were stimulated with pp65 or IE-1 peptide pools in the presence of brefeldin A. Monoclonal IgG_{2B} mouse anti-human CD85j (ILT-2) antibody (R&D Systems) or an isotype control (eBioscience) (5 µg/mL) was added prior to stimulation. For cytokine production, cells were stimulated for 13 h. For proliferation, cells were prelabeled with CFSE and stimulated for 7 days. Following stimulation, cells were resuspended in FACS buffer and stained with fluorescently tagged antibodies before being acquired on the flow cytometer. The following anti-human antibodies were used: CD3-APC/Cy7, CD8-qDot605, CD85j-APC, IFNγ-PE/Cy7, and TNFα-AF700.

Tetramer-Induced T Cell Proliferation Assay

Total T cells were isolated from PBMCs using untouched human T cells Dynabead kit (Fisher Scientific) and prelabeled with CFSE. Cells (1×10^6 per mL) were added to 96-well plates precoated with CMV_{pp65} or HIV_{gag} (SLYNTVATL) peptide-loaded HLA-A*0201 monomers (400 ng/mL) in the presence of soluble anti-CD85j or isotype control antibody (5 µg/mL). Following 7 days of stimulation, cells were resuspended in FACS buffer and stained with fluorescently tagged antibodies (same as baseline phenotyping) before being acquired on the flow cytometer.

Statistics

Statistical analyses, including Spearman correlation coefficients and non-parametric testing, were performed using GraphPad Prism v6 (GraphPad, San Diego, CA, USA). All p-values were derived using two-tailed tests and $p < 0.05$ were considered significant.

RESULTS

Correlates of CD85j Expression on CD8 T Cells in a Healthy Population

We first characterized the relationship of CD85j$^+$ cells to age and to specific CD8 T cell subsets in a cohort of 210 healthy individuals that has been previously described (24). CD85j$^+$ CD8 T cells increased with age (**Figure 1A**). Similarly, terminal differentiated effector CD8 T cells (termed "TEMRA") increased with age, with a slope and correlation coefficient similar to that of CD85j$^+$ cells (**Figure 1B**). Effector memory (EM) and naïve (N) CD8 cell frequencies also correlated with age (EM: $p = 0.007$, $r = 0.19$; N: $p < 0.0001$, $r = -0.5$) (**Figure 1B**; Figure S1A in Supplementary Material). As TEMRA and EM populations both showed similar positive correlations with age as the CD85j$^+$ population, we next asked which CD8 T cell subset, TEMRA or EM, most closely correlated with CD85j$^+$ cell frequencies. CD85j$^+$ cells positively correlated with TEMRAs (**Figure 1C**). No correlation between EM and CD85j$^+$ cell frequencies was found. Furthermore, CD85j$^+$ cells negatively correlated with naïve CD8 T cells (N: $p < 0.0001$, $r = -0.405$) (Figure S1B in Supplementary Material). We also observed increased frequencies of CD85j$^+$ cells and TEMRA cells in CMV-positive individuals and in males (Figures S1C,D in Supplementary Material).

To confirm that CD85j is expressed on TEMRA and whether increased CD85j$^+$ cells with age is a result of increased frequencies of TEMRAs with age, we analyzed CD85j expression on T cell subsets in a subcohort of 20 mid-age (40–50 years) and 20 older (>60 years) individuals. Indeed, CD85j is expressed by TEMRAs, with a small fraction of CD85j$^+$ EM T cells (**Figure 1D**). Central memory and naïve cells had very few CD85j$^+$ cells. Of note, CD85j-intermediate staining is almost exclusively observed within the effector T cell populations. Naïve and CM populations lack this population and the low CD85j staining observed in naïve and CM cells is seen in all populations of cells. With age, there was a trend for increased percentage of CD85j$^+$ TEMRA and EM, however, the difference did not reach statistical significance in our small subcohort (**Figure 1E**). We also compared the frequencies of CD85j$^+$ cells within T cell subsets by gender and CMV status within this subcohort. CMV-positive individuals had higher frequencies of CD85j$^+$ TEMRAs and CD85j$^+$ EM CD8 T cells compared with CMV-negative individuals (**Figure 1F**). Gender also has a modest effect of the frequencies of CD85j$^+$ TEMRAs, with males exhibiting slightly higher frequencies than females (**Figure 1G**). Age only had a minor effect on CD85j expression by effector subsets, not reaching significance. On the other hand, CMV infection caused a pronounced increase in CD85j$^+$ TEMRAs, regardless of age. Thus, mainly terminal differentiated effector CD8 T cells express CD85j and multiple factors including differentiation state, CMV infection, gender, and age influence the frequencies of CD85j$^+$ T cells and effector T cell subsets.

Expression of CD85j on CMV and EBV-Specific Effector CD8 T Cells

CMV$^+$ individuals expressed the highest frequencies of CD85j$^+$ TEMRAs, thus we further investigated the expression of CD85j on CMV- and EBV-specific CD8 T cells from older CMV$^+$ individuals. Directly *ex vivo*, both CMV (pp65) and EBV (BRLF1) tetramer-positive CD8 T cells were detectable (**Figure 2A**) but CMV-specific CD8 T cells were present at much higher frequencies than EBV-specific cells (median 1.2 vs. 0.29%, respectively)

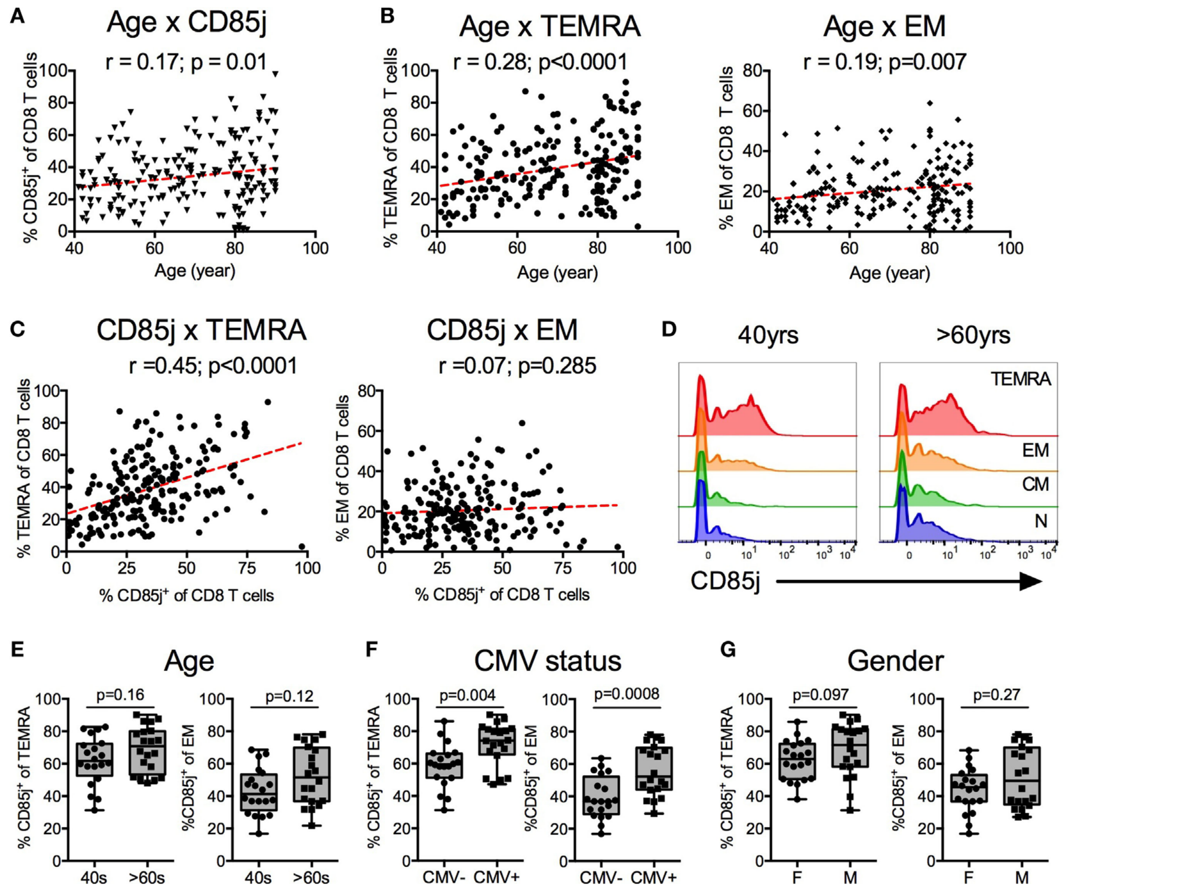

FIGURE 1 | CD85j expression on CD8 T cells and T cell subsets by age, gender, and cytomegalovirus (CMV) status. Spearman correlations between age and the percentage of **(A)** CD85j+ CD8 T cells, **(B)** TEMRA (CD45RA+CCR7−) CD8 T cells (left), and effector memory (EM; CD45RA−CCR7−) CD8 T cells (right) from 210 healthy individuals. **(C)** Spearman correlations between percentage of CD85j+ CD8 T cells and TEMRA (left) or EM (right) subsets. **(D)** Representative histograms of CD85j expression on naïve (CD45RA+CCR7+), CM (CD45RA−CCR7+), EM, and TEMRA CD8 T cell subsets in mid-age (40 years) and older (>60 years) adults. **(E–G)** Frequency of CD85j expression on TEMRA and EM CD8 T cell subsets by **(E)** age [mid-age (40 s) and older (>60 s) adults], **(F)** CMV status [CMV-negative (CMV−) and CMV-positive (CMV+) individuals], or **(G)** gender [females (F) and males (M)]. *p*-values in **(E–G)** were determined using the Mann–Whitney test.

(**Figure 2B**). Expression of CD85j was detectable on CMV- and EBV-specific CD8 T cells, in particular on CD28− effector cells (**Figure 2C**). Surprisingly, the expression of CD85j was similar on CMV- and EBV-specific cells (**Figure 2D**). Moreover, CD85j expression on CMV- and EBV-specific cells from the same individuals positively correlated (**Figure 2E**), suggesting that the probability of CD85j expression is not influenced by the nature of antigenic stimulation or the extent of clonal expansion, but by response patterns unique to individuals.

To further understand why the frequencies of CD85j+ cells varied between individuals, irrespective of whether specific for CMV or EBV, we characterized the phenotypic composition of CD85j+ CMV- and EBV-specific CD8 T cell populations. Expression of CD85j on CD8 T cell subsets was also confirmed in total CD8 T cells and CMV-specific CD8 T cells, demonstrating again robust expression on effector cells (**Figure 2F**). Both CMV- and EBV-specific populations of CD85j+ cells were predominated by effector populations (TEMRAs and EM cells), although CD85j+ CMV-specific cells had a higher median frequency of TEMRAs than EBV (**Figure 2G**). Similar to results from total CD8 T cells (**Figure 1**), the frequency of CD85j+ CMV- and EBV-specific cells positively also correlated with the frequency of TEMRA+ CMV- and EBV-specific cells (**Figure 2H**). However, individuals with high frequencies of CMV-specific CD8 T cells, which is indicative of memory inflation, exhibited increased percentage of cells expression CD85j and of the TEMRA subset (**Figures 2I,J**), suggesting that CD85j is gained during differentiation and retained during expansion of antigen-specific terminal-differentiated effector cells.

TCR Repertoire of CD85j+ and CD85j− CMV-Specific CD8 T Cells in Older Individuals

If CD85j expression is a late event in T effector cell expansion and differentiation, CD85j+ cells would be biased for larger clonal populations of antigen-specific T cells. Thus, we compared the TCR repertoires between CD85j+ and CD85j− CMV-specific (pp65 tetramer-positive) CD8 T cells. The repertoires in individual donors largely overlapped indicating that both subsets

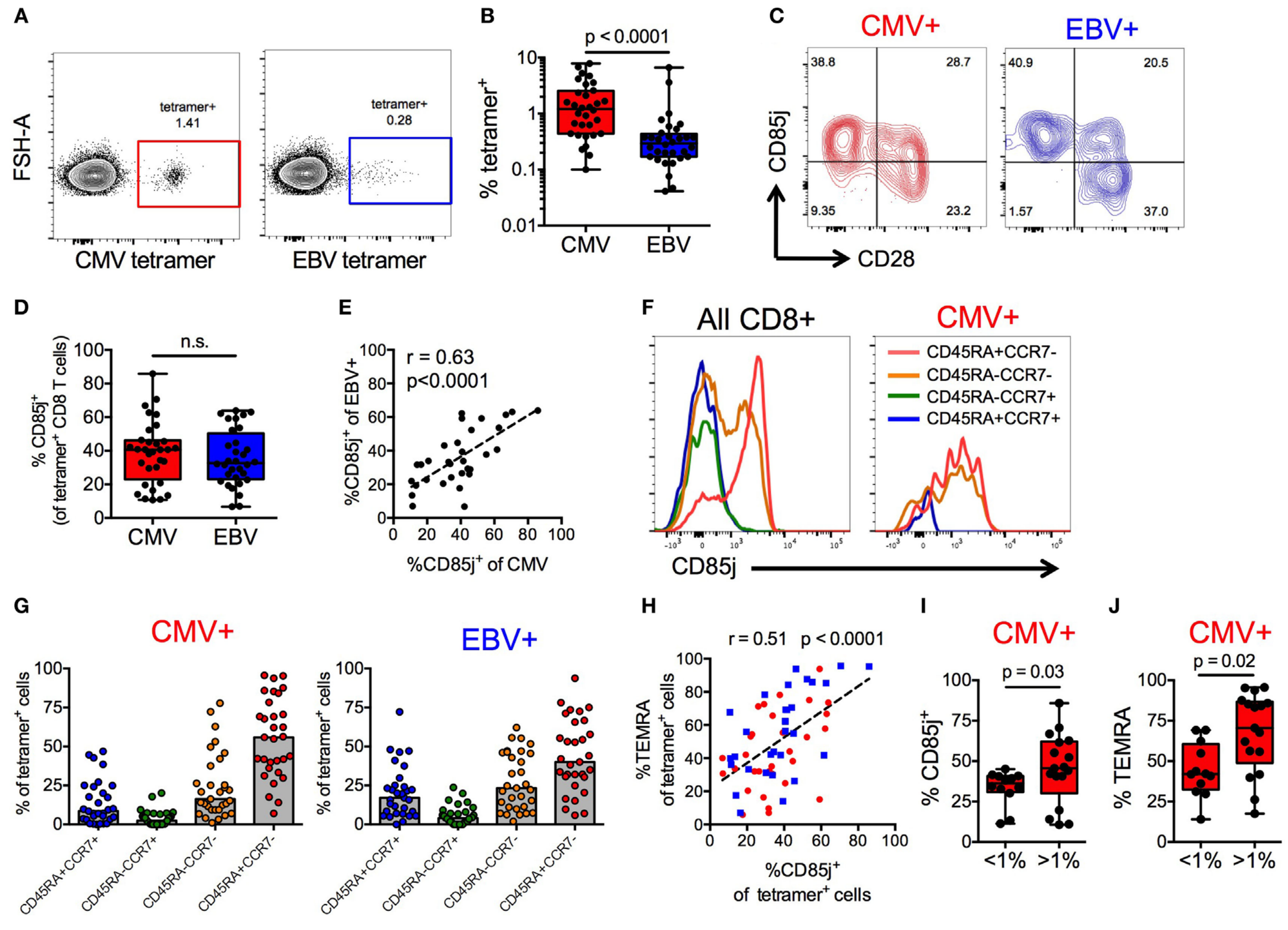

FIGURE 2 | CD85j expression on cytomegalovirus (CMV)- and EBV-specific CD8 T cells. **(A)** Representative flow plot of CMV (pp65) and EBV (BRLF1) tetramer staining on CD8 T cells from older individuals. **(B)** Frequencies of CMV- and EBV-specific CD8 T cells in total T cells from CMV+ older individuals (n = 32). **(C)** Representative flow plot of CD85j and CD28 staining on CMV- and EBV-specific CD8 T cells. **(D)** Frequencies of CD85j+ cells in CMV- and EBV-specific CD8 T cells. **(E)** Spearman correlation between the frequencies of CD85j+ cells in CMV-specific and EBV-specific CD8 T cells. **(F)** Representative histograms of CD85j expression in gated subsets of total CD8+ T cells and of CMV-specific CD8 T cells. **(G)** The frequencies of T cell subsets in CMV- and EBV-specific CD8 T cells defined as naïve (CD45RA+CCR7+), central memory (CD45RA−CCR7+), effector memory (CD45RA−CCR7−), and TEMRA (CD45RA+CCR7−). **(H)** Spearman correlation between the frequencies of CD85j+ and TEMRA+ cells within CMV- (red) and EBV- (blue) specific CD8 T cell population. Frequencies of **(I)** CD85j+ and **(J)** TEMRA+ CMV-specific CD8 T cells in individuals with high (>1%) and low (<1%) tetramer+ cell frequencies. p-values were determined using Wilcoxon non-parametric paired **(B,D)** or Mann–Whitney unpaired **(I,J)** test.

derived from the same progenitor cells (**Figure 3A**). The total number of unique TRB sequences (TCR richness) was similar between the two populations, with median counts of 213 (range: 113–1,361) and 185 (range: 70–781) for CD85j+ and CD85j− populations, respectively (**Figure 3B**). *TRB* sequences unique for one subset were all highly infrequent and, therefore, likely reflected the low probability of reidentification rather than uniqueness for one particular subset. Sizes of T cell clonotypes in both subsets showed a similar logarithmic distribution with a few clones dominant and no depletion of infrequent clones in either subset. Accordingly, clonality indices, a measure of clonal expansion, were not different (**Figure 3C**). In addition, there was a strong correlation between individual TCR clone frequencies in CD85j+ and CD85j− populations (**Figure 3D**). Clonality indices also positively correlated with frequencies of CMV-specific CD8 T cells (**Figure 3E**). Thus, clonality reflects memory inflation but this clonal expansion occurs in the CD85j+ as well as CD85j− subsets and CD85j expression does not appear to increase with clonal size or to have major influence on selection of the antigen-specific repertoire.

Phenotypic and Functional Characterization of CD85j+ CMV-Responsive CD8 T Cells in Older Individuals

In initial studies using flow cytometry, stimulation with immediate early or late CMV antigens revealed that CD85j+ CD8 T cells produced significant levels of IFNγ (Figure S2 in Supplementary Material). To further interrogate the functional role of CD85j in

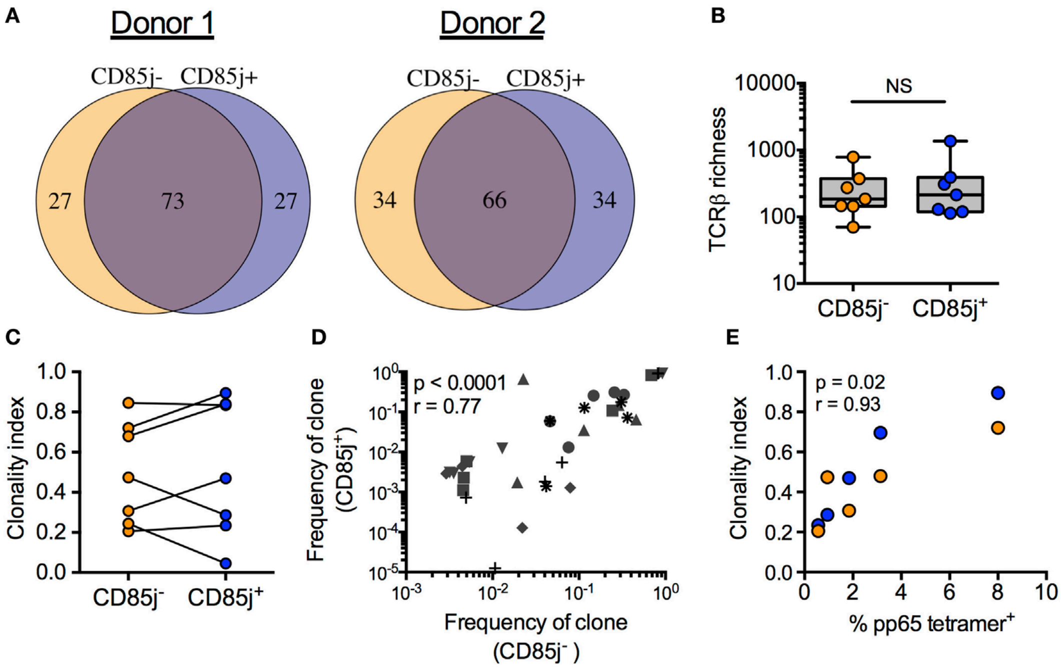

FIGURE 3 | *TRB* sequence diversity in CD85j⁺ and CD85j⁻ cytomegalovirus (CMV) pp65-specific CD8 T cells. **(A)** Overlap of the top 100 most frequent *TRB* sequences from pp65-specific CD85j⁺ and CD85j⁻ CD8 T cell populations in two representative donors. **(B)** TCR richness (the number of unique *TRB* sequences) and **(C)** clonality index (a modified Gini Simpson index to estimate contribution of clonally expanded sequences within a repertoire) in pp65-specific CD85j⁻ and CD85j⁺ CD8 T cells from seven older individuals. **(D)** Spearman correlation between the frequencies of the top five clones in CD85j⁻ population with corresponding frequency in the CD85j⁺ CD8 T cells from seven older individuals, each of them represented by a different symbol. **(E)** Spearman correlation between the percentage of pp65-tetramer⁺ CD8 cells and *TRB* clonality index from CD85j⁻ (orange) and CD85j⁺ (blue) populations (n = 5). NS = not significant.

antigen-specific responses, we utilized mass cytometry to determine simultaneous expression changes of 30 different parameters on CD8 T cells after CMV-specific peptide stimulation with immediate early or late antigen peptide super pools. These parameters included 5 T cell phenotyping markers, 7 cytokines, 2 cytotoxic factors, and 12 markers related to activation, exhaustion, and/or senescence. We termed all CD8 T cells expressing CD107a or one of the following cytokines, IFNγ, TNFα, IL-2, GM-CSF, and MIP1β, after stimulation as "CMV-responsive."

From our global clustering analysis, we found five distinct clusters of CMV-responsive cells (**Figure 4A**). The most prominent cluster, cluster 1, included 70.3% of all CMV-responsive cells (**Figure 4B**) and displayed no significant difference in cell frequencies between the two different peptide stimulations (**Figure 4C**). Cluster 4 was the only cluster that showed differences based on peptide stimulation, where most cells within this cluster were found with late, but not immediate early, peptide stimulation. CD85j expression differentiated into three groups; CD85j-negative, -intermediate, and -high (**Figure 4D**). At a single-cell expression level, clusters 3 and 5 were CD85j-neg/low, clusters 1 and 4 were CD85j-intermediate, and cluster 2 was CD85j-high (**Figure 4E**). Overall, CD85j^{intermediate} and CD85j^{-} clusters made up 81.0 and 14.7% of the CMV-responsive population, respectively. The smallest fraction of CMV-responsive cells was CD85j^{high} (cluster 2: 4.3% of all cells). The analysis of functional markers shows that CD85j^{high} cells expressed high levels of MIP1β and CD33. Further analysis revealed that this MIP1β^{+}CD33^{+}CD85j^{high} population was already present in unstimulated cells (Figures S3A,B in Supplementary Material). Therefore, it is undetermined whether these CD85j^{high} cells are truly CMV-specific T cells or a population of myeloid cells.

Median expression of (1) phenotypic markers, (2) response cytokines, and (3) other markers were individually compared between the five clusters using unbiased hierarchal clustering (**Figure 4F**). CD85j^{intermediate} clusters separated from CD85j^{-} and CD85j^{high} clusters in all comparisons. CD85j^{intermediate} clusters (clusters 1 and 4) strongly separated from CD85j^{-} clusters by phenotypic but not stimulation-induced cytokine expression. Similar to previous analysis of CD85j^{+} cell phenotypes, we found that CD85j^{intermediate} clusters contained primarily terminally differentiated (CD45RA^{+}CCR7^{-}CD28^{-}) CD8 T cells, whereas CD85j^{-} cells were CD27^{+} memory or naïve-like populations. Functionally, both CD85j^{intermediate} and CD85j^{-} cells expressed high levels of IFNγ, TNFα, and CD107a. However, the CD85j^{intermediate} population uniquely coexpressed high levels of CD57, granzyme B, and perforin, which are common markers of senescent cells or meditators of CTL responses (**Figure 4F**; Figure S4 in Supplementary Material). A subset of CD85j^{intermediate} cells from cluster 4 also coexpressed CD56 and NKG2C. Little to no expression of PD-1, an exhaustion marker, was detected on these cells. Median expression values and p-values for individual marker comparison between the different clusters are provided in Table S2 in Supplementary Material. Single-cell expression analysis of all CMV-responsive CD8 T cells demonstrated CD85j,

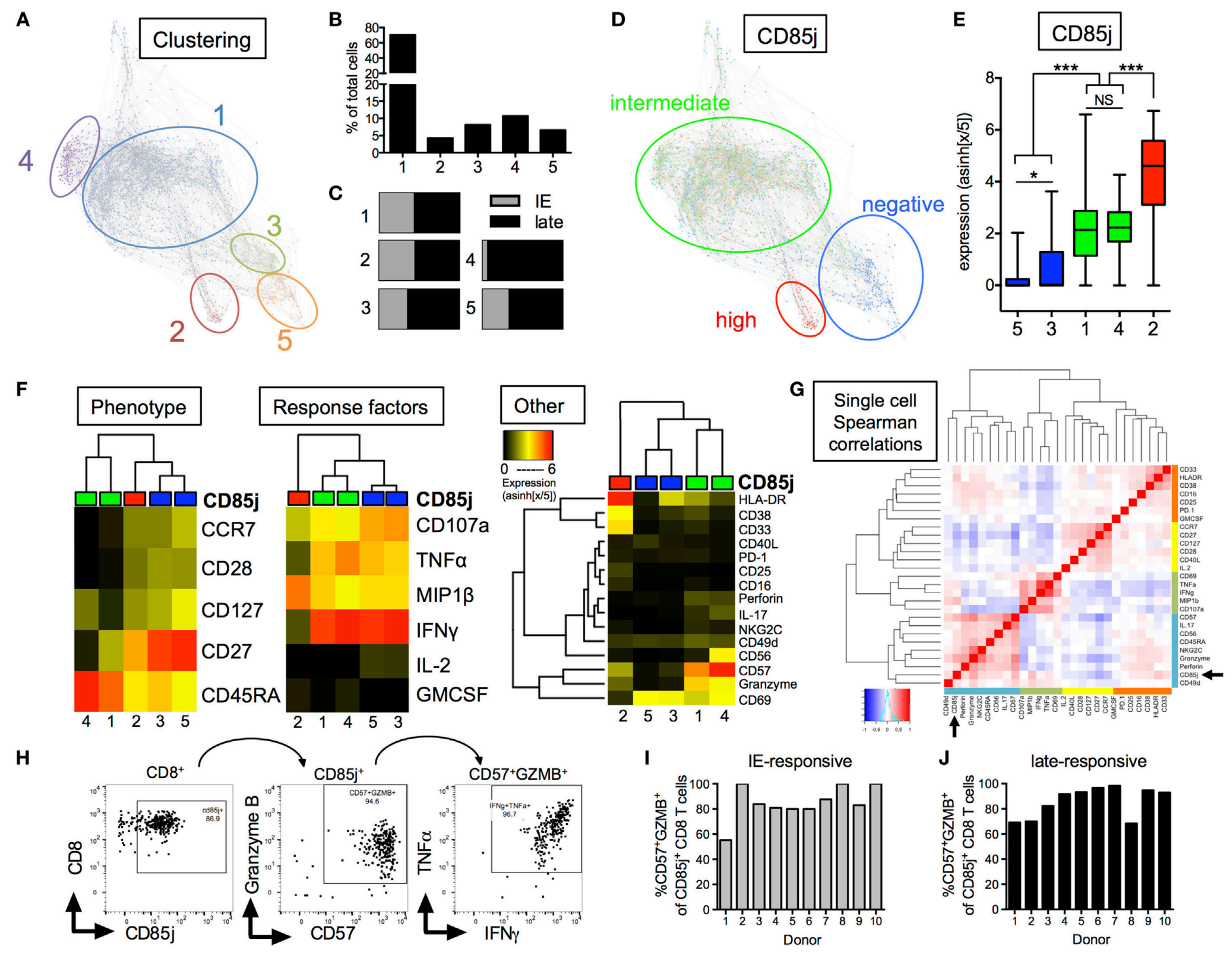

FIGURE 4 | CyTOF analysis of CD85j expression on cytomegalovirus (CMV)-stimulated CD8 T cells from older individuals. **(A)** Single-cell force-directed layout of X-shift clustering ($K = 55$) of all CMV-responsive CD8 T cells from CMV$^+$ elderly individual PBMCs ($n = 30$) stimulated for 18 h with peptide pools from immediate early (IE) and late CMV antigens, then stained for surface and intracellular markers. CMV-responsive CD8 T cells were defined as live/CD3$^+$CD19$^-$/CD8$^+$CD4$^-$ cells expressing IFNγ, TNFα, IL-2, GM-CSF, MIP1β, or CD107a after stimulation. Color code shows X-shift clusters. **(B)** Frequencies of cells with the individual clusters compared with all CMV-responsive cells. **(C)** Distribution of CMV-responsive CD8 T cells from IE or late peptide stimulation within each cluster. **(D)** Overlay of CD85j expression by individual cells within X-shift clusters. **(E)** CD85j expression on single cells from each of the X-shift clusters with *p*-values determined by one-way ANOVA. **(F)** Hierarchical clustering using phenotypic, CMV peptide stimulation-induced effector molecules, and other markers of activation, senescence, and exhaustion. Heat maps show median expression levels from each cluster, independent of CD85j expression. **(G)** Spearman correlation heat map of single-cell expression of all phenotypic, cytokine, and other markers from all CMV-responsive CD8 T cells. **(H)** Hand-gating of CD8$^+$/CD85j$^+$/CD57$^+$GZMB$^+$ cells in CMV-responsive cells from one representative donor after late peptide stimulation. Frequencies of CD57$^+$GZMB$^+$ cells in CD85j$^+$ CD8 T cells from 10 individual donors **(I)** after IE or **(J)** late peptide stimulation. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

CD57, and granzyme B expression positively correlated and clustered with NK or innate markers, CD56, perforin, and NKG2C (**Figure 4G**). In addition, hand-gating of CMV-responsive CD8 T cells confirmed that the majority of CD85j$^+$ cells coexpress CD57 and granzyme B (**Figure 4H**), and coexpression is maintained across multiple donors and CMV antigen responses (**Figures 4I,J**). CD85j$^+$CD57$^+$granzymeB$^+$ CD8 T cells were also found in the unstimulated population and single-cell analysis again revealed that CD57 and granzyme B positively correlated with CD85j in the resting CD8 T cell population (Figure S3 in Supplementary Material). Thus, CD85j$^+$ CMV-responsive CD8 T cells are phenotypically and functionally similar to that of terminally differentiated, not exhausted, CD8 T cells.

Inhibition of Proliferation but Not Cytokine Production by CD85j in CMV-Specific CD8 T Cells in Older Individuals

"Senescent" CD8 T cells are classically characterized by a reduction in proliferative capacity but maintained cytokine production, unlike exhausted cells, which also lost the ability to produce effector cytokines. Thus, we determined the potential role of

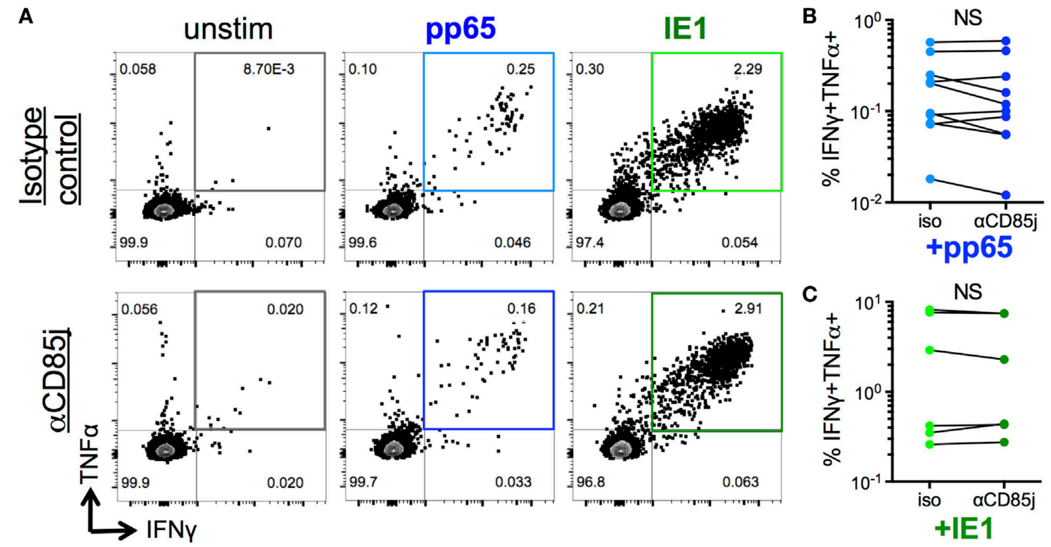

FIGURE 5 | Function of CD85j in cytomegalovirus (CMV) peptide-induced cytokine responses in CD8 T cells. **(A)** Representative flow plots of IFNγ and TNFα expression by CD8 T cells after stimulation with pp65- or IE-1-peptide pools in the presence of anti-CD85j or isotype control antibody for 13 h. **(B,C)** Frequencies of IFNγ^{+}TNFα^{+} CD8 T cells after stimulation with **(B)** pp65 (n = 10) and **(C)** IE-1 peptide pools (n = 7). p-values were determined using the Wilcoxon test. NS = not significant.

CD85j in antigen-specific cytokine responses and proliferation in CMV-infected, older individuals. Consistent with the functional data from CyTOF analysis (**Figure 4**), stimulation of PBMCs with immediate early (IE-1) and late (pp65) CMV antigen peptide pools both induced simultaneous production of IFNγ and TNFα by a subset of CD8 T cells (**Figure 5A**). However, blocking CD85j did not affect the frequencies of CD8 cells producing IFNγ and TNFα upon pp65 and IE-1 stimulation (**Figures 5B,C**). Thus, CD85j does not appear to play a role in the functional ability of CD8 T cells to produce cytokines in response to antigen.

Stimulation with pp65 peptide pool induced proliferation (CFSE^{low} cells) of a low-frequent T cell population (**Figures 6A,B**). Unlike cytokine production, CD85j blocking demonstrated a significant increase in the frequencies of proliferating CD8 T cells. CD85j blocking alone without stimulation was not sufficient to cause proliferation (**Figure 6A**; Figure S5A in Supplementary Material). To establish whether this inhibitory effect was specifically blocking CD85j engagement on CD8 T cells and not on CD85j-expressing antigen-presenting cells, we developed a cell culture system using immobilized pp65-loaded HLA-A*0201 tetramers to stimulate purified CD8 T cell *in vitro*. As observed with total PBMCs, CD85j blocking enhanced proliferation of CMV-specific CD8 T cells (**Figure 6C**; Figure S5B in Supplementary Material). Thus, engagement of CD85j on CD8 T cells reduced proliferation in response to antigen, indicating that CD85j may be a checkpoint regulator inhibiting clonal expansion of virus-specific T cells during aging.

DISCUSSION

Negative regulatory receptors on T cells, such as PD-1 and CTLA-4, are important checkpoint inhibitors that can be targeted to unleash T cell responses and improve the immunological control of tumors and, potentially, chronic viral infections. It is, therefore, of great interest whether this basic concept of checkpoint inhibition can also be applied to immune aging; where adaptive immunity and, in particular, CD8 T cell function falters and results in less efficient control of viral infections. Here, we focused on the inhibitory receptor CD85j and determined its cellular specificity and function in antigen-specific CD8 T cell responses during immune aging. We found that CD8 T cells acquire CD85j with effector cell differentiation, irrespective of the extent of clonal expansion, with maleness and age as predisposing factors. The association of CD85j expression with CMV infection is mainly due to terminal effector cell differentiation of CMV-specific CD8 T cells, not antigen-specificity. Moreover, the major function of CD85j is to constrain proliferation and clonal expansion, without impeding effector function upon antigenic stimulation. Together, these data indicate that CD85j plays an important function in checkpoint regulation during immune aging and that interfering with CD85j function may be useful to increase the frequencies of virus-specific T cells in older individuals.

Structurally, CD85j has high resemblance with the canonical checkpoint molecule PD-1, which has two ITIMs and one ITSM motif in its intracellular tail (29). Like PD-1, CD85j also demonstrates immune-suppressive functions in multiple cell types (30–32). These similarities suggest that CD85j may be a unique immune checkpoint factor and raises the possibility that CD85j plays an analogous role to that of PD-1 in anti-viral responses in immune aging. Indeed, previous studies using cross-linking of CD85j and CD3 have shown that CD85j can inhibit multiple cellular functions including cytokine production and cytotoxicity (33–35). The finding that a high frequency of CMV-specific T cells express CD85j permitted the phenotypic and functional

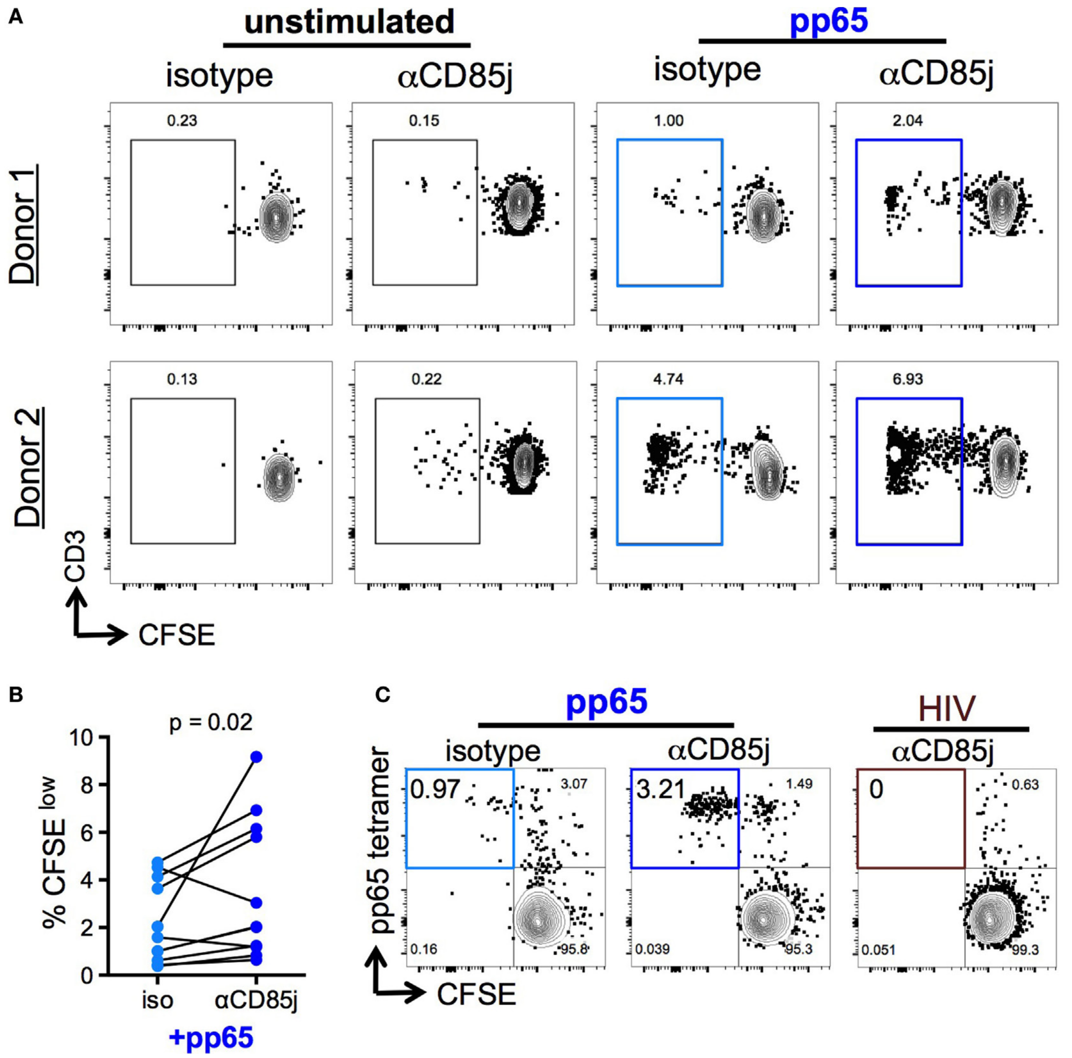

FIGURE 6 | Function of CD85j in cytomegalovirus (CMV) peptide-induced proliferation in CD8 T cells. **(A,B)** CFSE-labeled PBMCs stimulated with or without pp65 peptide pool in the presence of anti-CD85j or isotype control antibody for 7 days. **(A)** Representative flow plots of CFSE staining in CD8 T cells (gated on live/ $CD3^+$/$CD8^+CD4^-$) from two different donors. **(B)** The frequency of $CFSE^{low}$ (proliferated) CD8 T cells in stimulated PBMCs ($n = 12$) in the presence of anti-CD85j or isotype antibodies. **(C)** Flow plot of pp65-tetramer$^+$ cell proliferation in total CD8 T cell isolated from CMV^+ HLA-A2$^+$ PBMCs and stimulated with pp65-loaded tetramer-coated plates for 7 days, in the presence of anti-CD85j or isotype control antibody. HIV peptide-loaded tetramers were used as a negative control. These data are representative of two experiments. *p*-values were determined using the Wilcoxon test.

comparison of antigen-specific CD85j$^+$ and CD85j$^-$ T cells. CyTOF cluster analysis after CMV peptide stimulation clearly showed that CD85j expression does not differentiate antigen-specific effector function of CD8 subsets. Phenotypically, CD85j$^+$ cells most closely resemble TEMRAs, although a subset of EM can also express CD85j. However, these cells do not express PD-1, but more frequently the senescence marker CD57 and, therefore, are clearly different from exhausted cells. Indeed, these cells exhibit robust cytokine production and highest expression of granzyme B and perforin, demonstrating the retention of cytotoxic functionally.

It was surprising that we did not find any effect of CD85j on cytokine production after peptide stimulation, although the inhibitory function of CD85j is clearly directly related to MHC recognition; engagement of MHC-I by CD85j promotes intracellular inhibitory signaling and competitive binding of MHC-I with CD8 prevents positive costimulatory signaling (17). One possible explanation for our findings is that the inhibitory signals of CD85j are not of sufficient strength to block T cell activation. CD8 effector cells are also frequently of sufficient avidity to no longer require coreceptor engagement (36–38), so blocking CD8 engagement may not strongly influence antigen-induced cytokine production in TEMRAs. Moreover, the expression of CD85j on the surface of T cells is much lower than on other cell types on which it is expressed, due to lineage-specific differences in the transcriptional regulation (11). Additionally, although CD85j recognizes HLA-A and -B molecules, it has much higher affinity for HLA-G (17) and may therefore exhibit a lower inhibitory capacity in the context of our HLA-A *in vitro* stimulation system. However, we did see a distinct inhibitory activity of CD85j on the peptide-induced proliferative activity of CMV-specific cells that could be overcome by a blocking antibody. To confirm that the increased proliferative response was due to blocking CD85j on the responding T cells and not on the antigen-presenting cells, we used immobilized tetramers as peptide-presenting units in the absence of antigen-presenting cells and found essentially the same results. Therefore, it appears that proliferative responses by effector CD8 T cells are more sensitive to CD85j inhibitory signaling than cytokine production, consistent with our previous observation that sustained TCR signaling in TEMRAs, curtailed by engaging killer immunoglobulin-like receptors, leads to inhibition of proliferation but not cytotoxic activity (39). Mechanistically, it has been shown that partial phosphorylation of TCR-CD3 ITAMs reduces proliferation without affecting cytokine production (40), thus CD85j may functionally inhibit or reduce TCR-CD3 ITAM phosphorylation.

In aging humans, CD85j seems to dampen unopposed clonal expansion while leaving effector CD8 T cell functions intact. Such a mechanism could be very beneficial in the setting of chronic or latent viral infections such as by herpes viruses. Memory inflation is frequently seen with CMV infection to an extent that may compromise the overall T cell repertoire and therefore would have a negative impact on the ability to generate an immune response to unrelated antigens. Both memory inflation and frequencies of CD85j$^+$ effector T cells show high interindividual variability. We did not see any evidence for an inverse correlation between CD85j expression and memory inflation that would have indicated that failure to express CD85j is a risk factor for unopposed expansion.

It is of interest to note that CD8 TEMRAs employ several mechanisms in addition to CD85j expression to the same effect, namely curtailing proliferation while maintaining effector function. Akbar and colleagues have recently identified a disproportionate activation of the p38 MAPK pathway in TEMRAs that is directly involved in the loss of telomerase activity and proliferative capacity as well as the increased production of inflammatory cytokines (41). Activation of p38 is a consequence of DNA damage responses involving ATM and mitochondrial dysfunction causing ROS production (42). The failure to proliferate associated with upregulated production of inflammatory cytokines is reminiscent of the senescence-associated secretory phenotype in fibroblasts, and similarly involves the cyclin-dependent kinase inhibitors p16 and p21 (43). However, unlike cellular senescence in a strict sense, these functional deficits are reversed by inhibiting p38 (44, 45). Therefore, two pathways, p38 activation as well as CD85j expression, appear to be important checkpoints employed by effector T cells to maintain T cell homeostasis while keeping control of latent viral infection during immune aging.

If CD85j's function is to set a ceiling for clonal expansion in settings of chronic stimulation, one would expect that CD85j' expression correlates with clonal size. Surprisingly, this was not the case. Expression of CD85j was similar for CMV and EBV peptide responses although the frequencies of cells specific for these viral peptides differed by an order of magnitude. Large interindividual variations in CD85j expression on antigen-specific T cells correlated better with differentiation into TEMRAs than clonal sizes. Interestingly, maleness as well as age were demographic variables that were associated with CD85j expression and TEMRA frequencies. Most convincingly, the TCR repertoire of CD85j$^+$ and CD85j$^-$ CMV pp65-specific CD8 T cells was very similar; in particular, the CD85j$^+$ population was not enriched for large clones. These data suggest that CD85j expression occurs during effector cell differentiation irrespective of how many divisions a particular clone has undergone. They also show that CD85j does not induce a contraction of the antigen-specific repertoire, which is functionally important since it has been shown that not only the size of the CMV response but also the TCR diversity of CMV-specific cells determines how well CMV latency is retained (46).

Although CD85j may be an important checkpoint to maintain T cell homeostasis with age, it may also be harmful in certain contexts by excessively constraining clonal expansion. During aging, latent CMV infection is well-controlled without reactivation and there is no significant age-associated morbidity, while varicella zoster virus escapes latency causing shingles in up to 50% of individuals by the age of 80 years (47, 48). One possible reason for differential immune control is that the CMV response maintains the ability to undergo cellular expansion whereas VZV-specific T cells are unable to expand, causing a decline in the frequency of VZV-specific T cells with age (49, 50). Understanding the fine-tuning of signaling pathways involved in cellular proliferation, as well as effector functions, is a critical component to immune responsiveness. In the case of CMV infection, CD85j may provide a potential intervention to dampen excessive proliferation, while maintaining repertoire diversity and effector functions. Alternatively, inhibition of CD85j activity may be one way to improve anti-viral responses to other pathogens, such as VZV, with aging, similar to what has been proposed for the p38 pathway checkpoint. In addition to blocking CD85j's ability to interact with its ligand, one alternative intervention is to target CD85j transcription. Such an approach would be particularly attractive, since CD85j is widely expressed on hematopoietic cells but transcriptional and translational control is lineage-specific (11) and would, therefore, allow a more selective targeting of CD85j in TEMRAs.

ETHICS STATEMENT

This study was in accordance with the Declaration of Helsinki, approved by the Stanford Institutional Review Board, and all participants gave written informed consent.

AUTHOR CONTRIBUTIONS

CG: data collection, data analysis and interpretation, and manuscript writing. QQ: study design, data collection and analysis, and manuscript correction. JS and EN: study design and data collection. SG: data collection and analysis. RJ: data analysis and interpretation. HM: conception and design of study and financial support. CW: conception and design of study, data interpretation, and financial support. JG: conception and design of study, data analysis and interpretation, financial support, and manuscript writing. All the authors read, critiqued, and approved the final manuscript and also agreed to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00692/full#supplementary-material.

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Differential Relationships among Circulating Inflammatory and Immune Activation Biomediators and Impact of Aging and Human Immunodeficiency Virus Infection in a Cohort of Injection Drug Users

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As individuals with human immunodeficiency virus (HIV) infection live longer, aging and age-related chronic conditions have become major health concerns for this vulnerable population. Substantial evidence suggests that chronic inflammation and immune activation contribute significantly to chronic conditions in people aging with or without HIV infection. As a result, increasing numbers of inflammation and immune activation biomediators have been measured. While very few studies describe their *in vivo* relationships, such studies can serve as an important and necessary initial step toward delineating the complex network of chronic inflammation and immune activation. In this study, we evaluated *in vivo* relationships between serum levels of neopterin, a biomediator of immune activation, and four commonly described inflammatory biomediators: soluble tumor necrosis factor (TNF)-α receptor (sTNFR)-1, sTNFR-2, interleukin (IL)-6, and C-reactive protein (CRP), as well as the impact of HIV infection and aging in the *A*IDS *L*inked to the *I*ntravenous *E*xperience (ALIVE) study, a community-recruited observational study of former and current injection drug users (IDUs) with or at high risk for HIV infection in Baltimore, MD, USA. The study included 1,178 participants in total with 316 HIV-infected (HV+) and 862 HIV-uninfected (HIV−) IDUs. Multivariate regression analyses were employed, adjusting for age, sex, body mass index, smoking, hepatitis C virus co-infection, injection drug use, comorbidities, and HIV status (for all participants), and HIV viral load, $CD4^+$ T-cell counts, and antiretroviral therapy (for HIV+ participants). The results showed significant impact of aging on all five biomediators and that of HIV infection on all but sTNFR-1. In the adjusted model, neopterin had positive associations with sTNFR-1 and sTNFR-2 (partial correlation coefficients: 0.269 and 0.422, respectively, for all participants; 0.292 and 0.354 for HIV+; and 0.262 and 0.435 for HIV−, all $p < 0.0001$). No significant associations between neopterin and IL-6 or

CRP were identified. Such differential relationships between circulating neopterin and sTNFR-1, sTNFR-2, IL-6, and CRP may help inform their selection in future studies. These findings may also facilitate elucidation of underlying inflammatory and immune activation pathways that contribute to age-related chronic conditions, potentially leading to identification of key biomediators, particularly those upstream of CRP, as novel targets for intervention.

Keywords: neopterin, sTNFR-1, sTNFR-2, IL-6, CRP, human immunodeficiency virus infection and aging

INTRODUCTION

Human immunodeficiency virus (HIV) infection remains a major health problem worldwide. At present, approximately 34 million people are infected worldwide and there are about 50,000 new cases each year in the US alone. As effective treatment is available through combination antiretroviral therapy (cART) and new infections have increased among older adults, the number of older individuals living with HIV has risen dramatically over the past decade also. In fact, more than half of all HIV-infected persons in the US are now over the age of 50. Aging of the HIV-infected population is also evident in Asia and even in sub-Sahara Africa (1, 2). As HIV-infected (HIV+) individuals live longer, age-related chronic conditions commonly encountered in the geriatric population, some of which are also termed HIV-associated non-AIDS conditions, have become major health concerns for this vulnerable aging population despite suppression of HIV viral load to clinically undetectable levels by cART (3–6). One important example is frailty, a syndrome characterized by diminished physiologic reserve, increased vulnerability to stressors, and adverse health outcomes (3, 4). While much remains to be learned, it is believed that age-related senescent remodeling of the immune system, or immunosenescence, contributes significantly to the development of such chronic conditions and adverse health outcomes, and that HIV infection appears to accelerate immunosenescence (6–9).

Age-related chronic inflammation and dysregulated immune activation are considered cardinal features and likely mechanisms of immunosenescence. Biomediators commonly described in the literature include neopterin for immune activation and C-reactive protein (CRP), interleukin (IL)-6, soluble tumor necrosis factor (TNF)-α receptor (sTNFR)-1, and sTNFR-2 for chronic inflammation. In the geriatric population, we and others have shown that elevated serum levels of neopterin, CRP, IL-6, sTNFR-1, and sTNFR-2 are associated with frailty, disability, and mortality (10–13). In HIV infection, neopterin is shown to be elevated and its elevation is predictive of HIV disease progression (14–16). CRP, IL-6, and sTNFR-2 are also elevated with HIV infection and their elevated levels are associated with HIV disease progression and mortality in patients treated with cART (17–23). More recent studies in virologically suppressed HIV patients have shown persistent inflammation and immune activation, and that this persistent state of inflammation and immune activation is associated with aging, functional impairment, and AIDS defining events (24–29). In HIV+ men in the Multicenter AIDS Cohort Study (MACS), those with frailty had circulating CRP concentrations that were up to 50% higher than those in similar non-frail HIV+ men (30). However, *in vivo* relationships among these biomediators of chronic inflammation and immune activation have yet to be adequately investigated.

Neopterin, a GTP metabolite, is a well-established biomediator for immune activation primarily produced by monocytes and macrophages in response to stimulation with Th1-type cytokine interferon (IFN)-γ (31); its level increases with age (32) and in frailty (11). Both IL-6 and CRP are classic inflammatory biomediators and their elevated levels are considered the hallmark of age-related chronic inflammation, or "InflammAgeing" (33). TNF-α is a central player triggering inflammatory pathway or cascade as demonstrated in rheumatoid arthritis (34). TNF-α triggers inflammation through its two distinct but structurally homologous TNF receptors, the 55-kD receptor 1 (TNF-R1) and 75-kD receptor 2 (TNF-R2) (35, 36), and sTNFR-1 and sTNFR-2 are derived from TNF-R1 and TNF-R2, respectively, by proteolytic processing and have been shown to be reliable measurements for the *in vivo* activities of TNF-α. As such, sTNFR-1 and sTNFR-2 can be considered as more proximal inflammatory mediators than IL-6 and CRP. We have observed significant association between elevated levels of IL-6 and CRP among the *A*IDS *L*inked to the *I*ntra *V*enous *E*xperience (ALIVE) study participants, a large cohort of injection drug users (IDUs) with or at high risk for HIV infection (37). In the ALIVE study, we have also shown significant associations of serum levels of IL-6 and sTNFR-1 as well as an aggregate inflammatory index including IL-6 and sTNFR-1 levels with frailty and mortality (38). In addition, we have demonstrated significant *in vivo* associations between IL-6 and sTNFR-1 and sTNFR-2 levels (39). However, these are primarily biomediators of chronic inflammation. The objective of this study, therefore, was to further investigate the *in vivo* relationships between neopterin, a well-known biomediator of immune activation, and the above four inflammatory biomediators. This is built upon earlier work from Zangerle and colleagues who observed elevated neopterin and its association with sTNFR-1 in HIV-infected IDUs (40–42). We hypothesized that neopterin would have complex relationships with these inflammatory biomediators in which neopterin would be directly associated with sTNFR-1 and sTNFR-2 rather than IL-6 or CRP. As increasing numbers of immune activation and inflammatory biomediators have been evaluated in various settings, addressing this hypothesis is important in order to inform more accurate interpretation of existing data as well as their selection for evaluation in future studies. Delineating the *in vivo* relationships of these biomediators will advance our understanding of immune activation and inflammatory pathways as well as their role and interaction in contributing to chronic conditions and adverse health outcomes in the vulnerable aging population with HIV infection. To test this hypothesis, we conducted a cross-sectional analysis to evaluate the relationships between serum neopterin levels and levels of sTNFR-1, sTNFR-2, IL-6 and CRP in the ALIVE study, adjusting for age, sex, race, body mass index (BMI), cigarette smoking, comorbidities, hepatitis C infection, injection drug use, HIV status (for all participants), and HIV viral load, CD4 counts and cART (for HIV+ subgroup).

MATERIALS AND METHODS

Study Population

The *A*IDS *L*inked to the *I*ntra *V*enous *E*xperience (ALIVE) study is a prospective cohort consisted of IDUs based in Baltimore, MD, USA. Methodology has been previously described (43). During semi-annual visits, ALIVE participants completed standardized questionnaires and submitted biospecimens for testing. Smoking and illicit injection drug use per participant were self-report of behaviors over the past 6 months. Frequency of injection drug use during the past 30 days prior to the visit was also recorded. Multimorbidity including diabetes mellitus, hypertension, obstructive lung disease, anemia, chronic kidney disease, and liver fibrosis were confirmed clinical diagnoses by medical history and records. HIV serology was determined using enzyme-linked immunosorbent assay (ELISA) with Western blot confirmation (Dupont, Wilmington, DE, USA). Hepatitis C infection was determined by a positive antibody titer using standard laboratory assay. For those who were HIV positive, $CD4^+$ T cell counts and HIV viral load were measured routinely in a clinically certified laboratory. cART usage was self-reported by the participants and confirmed by medical or pharmacy record. Serum neopterin, sTNFR-1, sTNFR-2, IL-6, and CRP levels were measured as described below. A total of 1,190 participants had measurements of all 5 biomarkers at baseline. Residuals were examined and participants with high influence for one or more of the above biomediators were excluded ($n = 10$). Two participants were also removed due to incomplete data, leaving the sample size of 1,178 for this analysis. Johns Hopkins University Institutional Review Board approved the study and each participant provided written informed consent.

Measurements of Serum Neopterin, sTNFR-1, sTNFR-2, IL-6, and CRP

Serum samples were obtained from each participant according to the standard protocol, and stored in aliquots at −80°C until analysis. Serum neopterin was measured using a commercially available competitive ELISA (ALPCO Diagnostics; Salem, NH, USA). The immunoassay has a sensitivity of 0.8 nM and an inter-assay coefficient of variance of 5.29%. Serum sTNFR-1, sTNFR-2, IL-6, and CRP were measured using commercially available ELISA according to the procedures provided by manufacturers. Serum sTNFR-1 and sTNFR-2 were measured using DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA) with a sensitivity of 12.5 or 7.8 pg/ml and an inter-assay CV of 4.9 or 6.1%, respectively. Serum IL-6 and CRP were measured using High-Sensitivity Quantikine kits (R&D Systems) with detection ranges of 0.156–10.0 and 31.25–2,000 pg/mL and inter-assay coefficients of variance of 5.7 and 6.4%, respectively. Measurements were performed in duplicate and repeated if the measures differed by more than 15% or were out of the measureable range. The average of the two values in duplicate was used for analyses.

Statistical Analysis

Frequency distributions were determined for baseline population characteristics. Fisher's exact test and Student's *t*-test were used to determine differences between categorical and continuous data by HIV status, respectively. Medians and Interquartile Range (IQR) were calculated for all five biomediators and the non-parametric Wilcoxon–Mann–Whitney test was used to compare distributions between groups stratified by HIV status. Bivariate associations between neopterin and each of the inflammatory biomediators (sTNFR-1, sTNFR-2, IL-6, and CRP) were evaluated using cross-tabulations of means and SDs of sTNFR-1, sTNFR-2, IL-6, and CRP levels by quartiles of neopterin; analysis of variance was used to compare group differences. Multiple linear regression was used to examine the relationships between neopterin and each of the inflammatory biomediators (sTNFR-1, sTNFR-2, IL-6, and CRP). Separate analyses were performed for each pairs of biomediators adjusting for age, sex, race, BMI, cigarette smoking, number of comorbidities (0 or 1, 2, ≥3), hepatitis C infection, number of injection drug use within the past 30 days, HIV status (for all participants), and HIV viral load, CD4 counts and cART (for HIV+ group). To account for non-normal distributions, levels of neopterin, sTNFR-1, sTNFR-2, IL-6, and CRP were log transformed to approximate normality for linear regression analyses. Regression effects can be interpreted as a one percent change in the median covariate value for each unit change in the median outcome value. Multi-collinearity was examined using variance inflation factors with 2.5 as a cutoff. Assumptions were checked for all models by examining error properties and residual plots. All analyses were performed using SAS statistical software (Version 9.2, Cary, NC, USA).

RESULTS

Out of 1,178 participants (all participants) included in this analysis, 316 were HIV+ and 862 were HIV− participants, giving a 26.8% prevalence of HIV infection. The majority of the study participants were African-American (87.5%) and male (64.9%) with a mean age of 46.8 (range 21.2–78.1) years. Daily injection drug use was reported in 21.1% of the population. **Table 1** summarizes baseline demographic and clinical characteristics as well as medians (IQR) of neopterin, sTNFR-1, sTNFR-2, IL-6, and CRP levels of all participants as well as HIV+ and HIV− study groups. Compared to HIV− participants, those who were HIV+ were more likely to be African-American, never married, unemployed, use injection drugs less often, inject fewer times within the past 30 days, consume fewer alcoholic drinks/day, and have two or more comorbidities. The prevalence of hepatitis C infection was 86% among all participants included

TABLE 1 | Characteristics of ALIVE study participants: all participants and stratified by human immunodeficiency virus (HIV) status.

	All participants (n = 1,178)	HIV positive (n = 316)	HIV negative (n = 862)	p-Value
Age, mean in years (95% CI)	46.8 (46.3–47.2)	46.9 (46.2–47.6)	46.7 (46.1–47.3)	0.762
Sex				
Male	765 (64.9)	203 (64.2)	562 (65.2)	0.783
Female	413 (35.1)	113 (35.8)	300 (34.8)	
Race				
White/other	147 (12.5)	20 (6.3)	127 (14.7)	0.0001
African-American	1,031 (87.5)	296 (93.7)	735 (85.3)	
Marital status				
Never married	776 (65.9)	230 (73.3)	546 (63.4)	0.002
Ever married	399 (33.9)	84 (26.8)	315 (36.6)	
Employed				
No	870 (73.9)	253 (80.1)	617 (71.7)	0.004
Yes	306 (26.0)	63 (19.9)	243 (28.3)	
Cigarette smoker				
No	181 (15.4)	56 (17.8)	125 (14.5)	0.164
Yes	994 (84.4)	258 (82.2)	736 (85.5)	
IV drug use in past 6 months				
None	637 (54.1)	182 (57.6)	455 (52.8)	0.007
<Daily	293 (24.9)	87 (27.5)	206 (23.9)	
≥Daily	248 (21.1)	47 (14.9)	201 (23.3)	
Number of injections in past 30 days, median (IQR)	25 (4–60)	12 (4–35)	30 (4–60)	0.038
Number of alcoholic drinks/day				
0	549 (46.6)	173 (54.8)	376 (43.6)	0.009
1–2	339 (28.8)	78 (24.7)	261 (30.3)	
3–4	166 (14.1)	38 (12.0)	128 (14.9)	
≥5	124 (10.5)	27 (8.5)	97 (11.3)	
Comorbidities				
0	293 (24.9)	43 (13.6)	250 (29.0)	<0.0001
1	406 (34.5)	87 (27.5)	319 (37.0)	
2	294 (25.0)	105 (33.2)	189 (21.9)	
≥3	185 (15.7)	81 (25.6)	104 (12.1)	
Body mass index				
<30	931 (79.0)	259 (82.0)	672 (78.0)	0.135
≥30	247 (21.0)	57 (18.0)	190 (22.0)	
Hepatitis C infection				
No	166 (14.0)	0 (0.0)	166 (19.3)	<0.0001
Yes	1,012 (86.0)	316 (100.0)	696 (80.7)	
CD4$^+$ T-cells, cells/mm^3, median (IQR)		304 (180–437)		
HIV viral load, copies/ml, median (IQR)		961 (400–28,000)		
cART use in past 6 months				
No		148 (46.8)		
Yes		163 (51.6)		
Neopterin, nmol/ml, median (IQR)	16.52 (10.85–26.78)	25.98 (16.49–39.08)	14.36 (9.83–21.88)	<0.0001
Factor (TNF)-α receptor (sTNFR)-1, pg/ml, median (IQR)	1,491 (1,260–1,834)	1,484 (1,257–1,955)	1,492 (1,261–1,808)	0.416
sTNFR-2, pg/ml, median (IQR)	4,976 (3,833–6,900)	9,682 (5,113–9,213)	4,487 (3,592–5,964)	<0.0001
IL6, pg/ml, median (IQR)	1.61 (1.01–2.75)	1.81 (1.21–3.12)	1.50 (0.95–2.67)	0.0001
C-reactive protein, pg/ml, median (IQR)	1,566 (546–4,632)	1,307 (498–3,815)	1,723 (552–4,878)	0.069

Values are number (%) unless otherwise noted.
cART, combination antiretroviral therapy; IQR, interquartile range.

in this analysis with hepatitis C co-infection present in all (100%) HIV+ participants and 80.7% in HIV− participants ($p = 0.0001$). There was no statistical difference with regard to age, sex, smoking, or BMI between HIV+ and HIV− groups. For HIV+ participants, median (IQR) CD4$^+$ T-cell count and HIV viral load were 304 cells/mm^3 (180–437) and 961 copies/ml (400–28,000), respectively. Among them, 163 were treated with cART with majority being treated with protease inhibitors (57.7%) or non-nucleotide reverse transcriptase inhibitors (18.5) alone and the remaining 148 (46.8%) had no cART. Median (IQR) levels of immune activation and inflammatory biomediator for all participants were 16.52 nmol/ml (10.85–26.78) for neopterin, 1,491 pg/ml (1,260–1,834) for sTNFR-1, 4,976 pg/ml (3,833–6,900) for sTNFR-2, 1.61 pg/ml (1.01–2.75) for IL-6, and 1,566 pg/ml (546–4,632) for CRP (**Table 1**). HIV+ participants had significantly higher neopterin, IL-6, and sTNFR-2 levels than HIV− participants (median 25.98 vs 14.36, $p < 0.0001$, 1.81 vs 1.50 pg/mL, $p = 0.0001$ and 9,682 vs 4,487 pg/mL, $p < 0.0001$, respectively), while there was no significant difference in sTNFR-1 or CRP levels between the two study groups (1,484 vs 1,492 pg/mL, $p = 0.416$ and 1,307 vs 1,723, $p = 0.069$, respectively). The median neopterin levels in HIV− IDUs were higher than that typically seen in other HIV− populations, likely due to drug use or other infections.

Potential associations of age, sex, race, and HIV status in all participants were assessed by bivariate regression analyses (**Table 2**). Among all participants, neopterin and IL-6 levels were significantly associated with age, sex, and HIV status, but not race. Levels of sTNFR-1 were significantly associated with age and race but not sex or HIV status. Levels of sTNFR-2 were significantly associated with age and HIV status but not sex or race. CRP levels were significantly associated with age, sex, race, and HIV status. Median neopterin levels were increased by 68.2%, IL-6 levels were increased by 19.8%, and sTNFR-2 levels by 51.5% in HIV+ participants compared to HIV− participants (all $p < 0.05$). Females had median IL-6 and CRP levels that were higher compared to males by 17.7 and 33.0%, respectively, both $p < 0.05$. African-Americans had median sTNFR-1 and CRP levels that were lower compared to all other races by 10 and 34.4%, respectively, both $p < 0.05$.

The relationships between levels of neopterin and those of sTNFR-1, sTNFR-2, IL-6, CRP in all participants were evaluated next. First, the means and SDs of sTNFR-1, sTNFR-2, IL-6, and CRP levels were cross-tabulated across quartiles of neopterin levels. We found a stepwise increase in sTNFR-1 and sTNFR-2 levels across neopterin quartiles (**Table 3**). There were no significant differences across neopterin quartiles for IL-6 and CRP.

We then assessed the associations between log-transformed levels of neopterin and those of sTNFR-1, sTNFR-2, IL-6, and CRP in all participants as well as HIV+ and HIV− participants, adjusting for age, sex, race, BMI, cigarette smoking, number of comorbidities, hepatitis C infection, number of injections in the past 30 days, HIV status (for all participants), as well as $CD4^+$ T-cell counts, HIV viral load, and cART (for HIV+ group only). log(neopterin) and log(sTNFR-1) were associated with each other in all participants as well as in HIV+ and HIV− groups (partial correlation coefficient $r = 0.269$, 0.292, and $r = 0.262$, respectively, all $p < 0.0001$, **Figures 1A–C**). Similarly, log(neopterin) and log(sTNFR-2) were associated with each other for all participants, HIV+, and HIV− groups ($r = 0.422$, 0.354, and $r = 0.435$, respectively, all $p < 0.0001$, **Figures 1D–F**). log(neopterin) had minimal or insignificant associations with log(CRP) or log(IL-6) in all population, HIV+, and HIV groups (data not shown).

Multiple linear regression analyses were performed using log(neopterin) as predictor and log(sTNFR-1), log(sTNFR-2), log(IL-6), and log(CRP) as outcome measures for all participants, HIV+, and HIV− groups, adjusting for age, sex, race, BMI, cigarette smoking, number of comorbidities, hepatitis C infection, and number of injection drug use in the past 30 days. Analyses for all participants were also adjusted for HIV status and those for the HIV+ group were also adjusted for HIV viral load, $CD4^+$ T-cell counts and cART (**Table 4**). The results indicate that log(neopterin) had significantly positive associations with log(sTNFR-1) and log(sTNFR-2) for all participants [regression coefficients 0.467 (SE, 0.054) and 0.569 (0.039), respectively, both $p < 0.0001$] as well as for both HIV+ participants [0.542 (0.104) and 0.476 (0.073), both $p < 0.0001$, respectively] and HIV− participants [0.487 (0.061) and 0.622 (0.044), both $p < 0.0001$, respectively]. On the other hand, log(neopterin) had no significant associations or negligible regression coefficients (likely spuriously negative) with log(CRP) or log(IL-6) in all participants, HIV+ or HIV− participants.

TABLE 2 | Effects of age, sex, race, and human immunodeficiency virus (HIV) status on circulating levels of neopterin, sTNFR-1, Factor (TNF)-α receptor (sTNFR)-2, IL-6, and C-reactive protein (CRP) in ALIVE study participants as shown by regression coefficients (95% CI).[a]

	Age (years)		Female vs male		African-American vs White/other		HIV positive vs HIV negative	
	Coefficient	95% CI	Coefficient	95% CI	Coefficient	95% CI	Coefficient	95% CI
Neopterin (nmol/ml)	1.006*	(1.001–1.01)	0.893*	(0.828–0.963)	1.025	(0.919–1.144)	1.682*	(1.559–1.815)
sTNFR-1 (pg/ml)	1.006*	(1.004, 1.008)	1.006	(0.969, 1.045)	0.902*	(0.854, 0.953)	1.017	(0.977, 1.060)
sTNFR-2 (pg/ml)	1.005*	(1.002, 1.008)	1.018	(0.964, 1.076)	0.945	(0.872, 1.023)	1.515*	(1.435, 1.600)
IL-6 (pg/ml)	1.008*	(1.001–1.015)	1.177*	(1.053–1.315)	1.071	(0.912–1.257)	1.198*	(1.063–1.350)
CRP (pg/ml)	0.983*	(0.972–0.994)	1.330*	(1.104–1.601)	0.656*	(0.501–0.858)	0.804*	(0.658–0.983)

*$p < 0.05$.
[a]Since log-transformed scores were used in the regression analyses, values presented are exponentiated regression coefficients.

TABLE 3 | Mean (SD) of sTNFR-1, factor (TNF)-α receptor (sTNFR)-2, IL-6, and C-reactive protein (CRP) levels across neopterin quartiles.

Neopterin quartiles (range in nmol/ml)	0–25% (0–10.88)	26–50% (10.92–16.49)	51–75% (16.51–27.10)	76–100% (27.13–174.04)	*p*-Value*
sTNFR-1 (pg/ml)	1,500 (584)	1,522 (479)	1,641 (566)	1,899 (875)	<0.0001
sTNFR-2 (pg/ml)	4,406 (1,658)	4,867 (2,102)	6,067 (2,921)	8,522 (4,754)	<0.0001
IL-6 (pg/ml)	3.84 (8.92)	3.31 (6.40)	3.58 (9.17)	3.07 (5.89)	0.776
CRP (pg/ml)	3,491 (4,521)	3,539 (4,647)	3,999 (7,879)	3,246 (5,765)	0.444

*p-Value is from the trend test of the differences in sTNFR-1, sTNFR-2, IL-6, and CRP Levels across Neopterin quartiles.

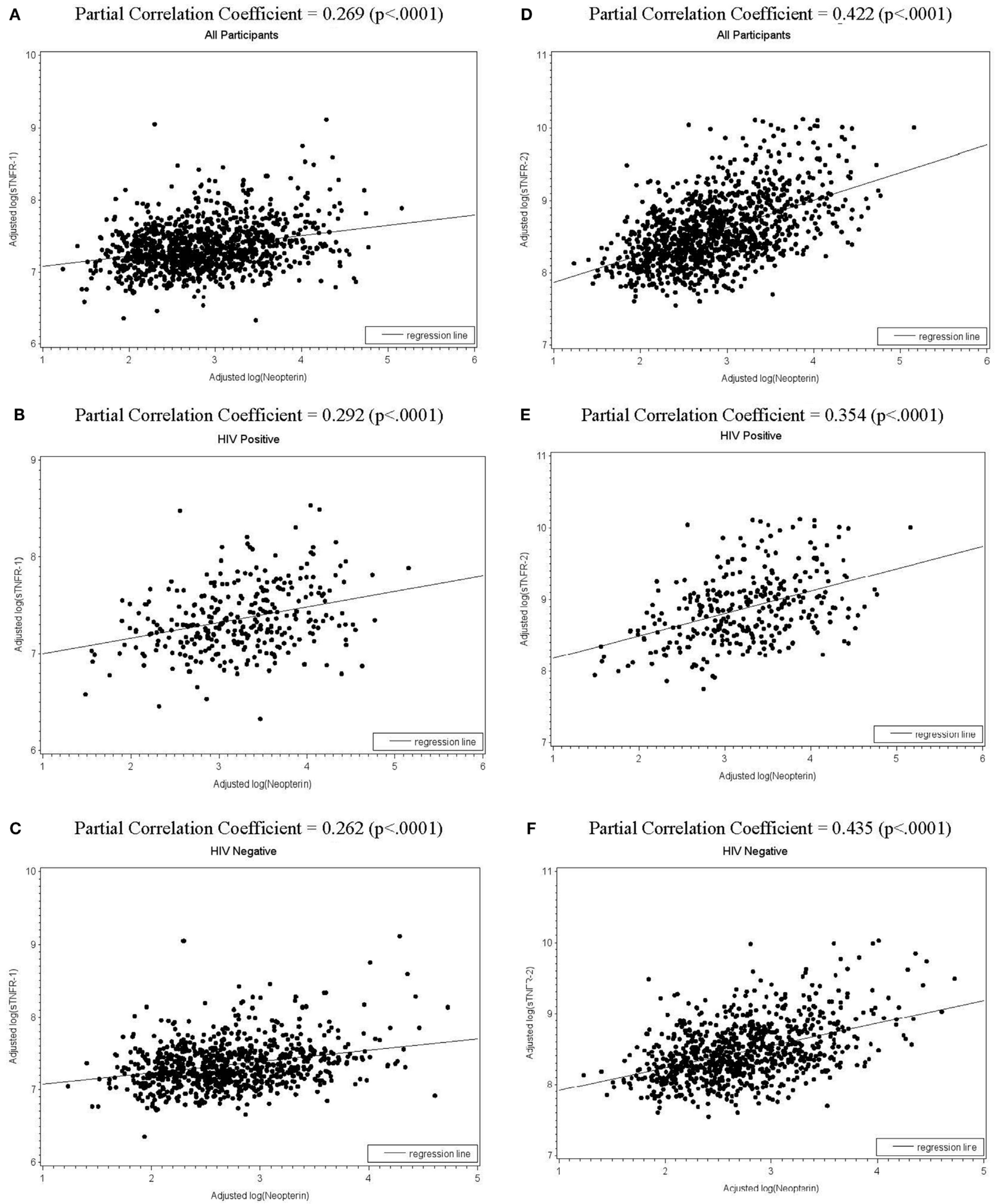

FIGURE 1 | Scatterplots of total (all participants), human immunodeficiency virus (HIV)-positive, and HIV-negative populations with regression line shows, respectively, the fitted relationships of log(neopterin) with log(sTNFR-1)**(A–C)** and with log(sTNFR-2) **(D–F)**, adjusting for age, sex, race, number of comorbidities (0 or 1, 2, 3 or more), cigarette smoking, hepatitis C infection, body mass index, and number of injections in the past 30 days. CD4+ T-cell counts, HIV viral load, and combination antiretroviral therapy (cART) use in past 6 months were also adjusted for HIV-positive subgroup.

TABLE 4 | Adjusted regression coefficients sTNFR-1, factor (TNF)-α receptor (sTNFR)-2, IL-6, or C-reactive protein (CRP) (SE).

		All participants	Human immunodeficiency virus (HIV) positive	HIV negative
log (Neopterin)	log (sTNFR-1)	0.467 (0.054)**	0.542 (0.104)**	0.487 (0.061)**
	log (sTNFR-2)	0.569 (0.039)**	0.476 (0.073)**	0.622 (0.044)**
	log (IL-6)	−0.042 (0.018)*	−0.034 (0.042)	−0.040 (0.020)*
	log(CRP)	−0.014 (0.011)	−0.030 (0.021)	−0.003 (0.013)

***p < 0.0001, *p < 0.05.*
All models adjusted for age, sex, race, number of comorbidities (0 or 1, 2, ≥3), cigarette smoking, Hepatitis C infection, body mass index, and number of injections in the past 30 days.
HIV status was adjusted for all participants.
CD4⁺ T-cell counts, HIV viral load, and cART use in past 6 months were adjusted for HIV-positive subgroup.

DISCUSSION

In this study, we have observed, for the first time, differential *in vivo* associations between circulating neopterin and four commonly tested inflammatory biomediators (sTNFR-1, sTNFR-2, IL-6, and CRP) with significant impact of HIV infection and aging in a cohort of IDUs with and at risk for HIV infection.

As the role of chronic inflammation and immune activation in HIV disease progression as well as in the manifestations of aging and age-related chronic conditions has become widely recognized, increasing number of biomediators (or biomarkers by some) of chronic inflammation and immune activation have been evaluated. The challenge is then to appropriately interpret the results and gain biologically informed insights from their evaluation. This study serves as an important initial step toward addressing this challenge through evaluating *in vivo* relationships between neopterin and four commonly tested inflammatory mediators in the ALIVE study. Theoretically, since immune activation leads to inflammation, neopterin would have significant associations with all four inflammatory biomediators. Our findings, however, demonstrate significant and positive associations of neopterin with sTNFR-1 and sTNFR-2 only, not with IL-6 or CRP, suggesting more complex *in vivo* relationships. One possible explanation for such preferential associations is that monocytes and macrophages are a main source of neopterin, sTNFR-1 and sTNFR-2 production. However, IL-6 is also considered as a monokine and no consistent association was observed between neopterin and IL-6 levels. Another biological plausible explanation is that neopterin, being a biomediator of immune activation is associated with more proximal inflammatory biomediators (sTNFR-1 and sTNFR-2) rather than distal biomediators (IL-6 and CRP). It is conceivable that the levels of IL-6 and CRP, the two classic and yet more distal inflammatory biomediators, are regulated by various factors and local milieu in addition to the underlying immune activation. This is illustrated by the result that CRP levels were lower in HIV+ participants compared to HIV− individuals with the difference reaching borderline statistical significance ($p = 0.069$, **Table 1**). This is because CRP is mainly produced by the liver (44) and hepatitis C infection, which was positive in all HIV+ study participants, could lead to significant liver damage. In fact, our previous study observed lower CRP levels in the ALIVE participants with HIV and hepatitis C virus co-infections (37). Alternatively, the observed associations of neopterin with sTNFR-1 and sTNFR-2 may be secondary to a Th1-type response as elevated neopterin and soluble TNF-α receptors are, at least under certain conditions, downstream products of IFN-γ activation (45), while IL-6 and CRP are typically considered as markers for Th2-type response.

The stronger association of neopterin with sTNFR-2 than with sTNFR-1 is not surprising but worthwhile emphasizing. sTNFR-2 is primarily produced by immune cells, particularly CD8⁺ T cells which could be activated by residual HIV infection and significantly impacted by immunosenescence (46, 47). In addition, sTNFR-2 rather than sTNFR-1 has shown consistent association with HIV infection and disease progression (48–50). Our findings confirmed the earlier work from from Zangerle et al. cited above (42) in a much larger sample and further expanded to include other inflammatory mediators. Stein and colleagues evaluated levels of sTNFR-2, neopterin, HIV RNA, and β_2-microglobulin levels in the MACS and reported that high baseline sTNFR-2 levels were predictive for HIV disease progression or death (23). However, that study was conducted only in men with early-stage of HIV infection and did not include sTNFR-1, IL-6, or CRP levels. Whether sTNFR-2 has important regulatory function to activate or accelerate inflammatory pathways in response to neopterin beyond TNF-α cascade in the setting of HIV infection and aging and, therefore, represents a potential interventional target upstream of CRP and IL-6, deserve further investigation. This therapeutic implication has been demonstrated in the field of cardiovascular disease as serum CRP measurement is now incorporated in routine clinical assessment of chronic inflammation and atherosclerosis and studies have started to move upstream to identify novel target for vascular protection (51).

A major strength of this study is the availability of data for all five biomediators measured in the same blood sample collected at the same visit from the same individual in a large cohort study. It makes this analysis feasible and the observed *in vivo* associations biologically meaningful. Additional strengths include that standard or high sensitive ELISA assays were employed as appropriate for measuring these mediators, avoiding pitfalls associated with multiplex assays [reviewed in Ref. (52)]. This study also has several limitations. First, this is a cross-sectional analysis. We could not determine causal directionality of the identified associations. We have adjusted for a number of potential covariates commonly known for IDUs (such as IV drug use, injection frequency, and hepatitis C infection) and HIV infection (viral load, CD4 counts, and cART therapy) as well as comorbidities. We have also vigorously excluded participants with outlier values. However, other potential confounding factors that were not in the dataset or unknown to the participants could not be completely eliminated. In addition, we only included neopterin, sTNFR-1, sTNFR-2, IL-6, and CRP in this study. Other immune and inflammatory mediators are also likely important in the setting of HIV infection and aging. Finally, the ALIVE study cohort is a rather unique population identified by the IDU behavior. Results from this study will need to be confirmed in other HIV+ and HIV− populations. Despite these limitations, findings from this study do support our original hypothesis and suggest significant and positive *in vivo* associations of immune activation biomediator neotperin with proximal inflammation biomediator, sTNFR-1 and sTNFR-2 rather than more distal ones, IL-6 and CRP. These findings, if confirmed and further expanded, may facilitate elucidation of underlying inflammatory and immune activation pathways that contribute to the development of age-related chronic conditions as well as the impact of HIV infection, aging, and immunosenescence. They may also help not only inform their selection of for evaluation in the future studies, but also promote further investigations into their role and regulation, particularly those upstream of CRP as novel interventional targets for frailty and other chronic conditions in older adults with or without HIV infection.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of human research subjects, the Johns Hopkins Institution Review Board (IRB)'s with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Johns Hopkins IRB.

AUTHOR CONTRIBUTIONS

GK, SM, DP, JM, and SL contributed to overall design and data interpretation of this study. SD contributed to data analyses. HL and YC contributed to biomediator measurements. GK and SM are lead investigators for the ALIVE study. All authors contributed to manuscript writing and editing.

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Adipose Tissue Inflammation Induces B Cell Inflammation and Decreases B Cell Function in Aging

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Aging is the greatest risk factor for developing chronic diseases. Inflamm-aging, the age-related increase in low-grade chronic inflammation, may be a common link in age-related diseases. This review summarizes recent published data on potential cellular and molecular mechanisms of the age-related increase in inflammation, and how these contribute to decreased humoral immune responses in aged mice and humans. Briefly, we cover how aging and related inflammation decrease antibody responses in mice and humans, and how obesity contributes to the mechanisms for aging through increased inflammation. We also report data in the literature showing adipose tissue infiltration with immune cells and how these cells are recruited and contribute to local and systemic inflammation. We show that several types of immune cells infiltrate the adipose tissue and these include macrophages, neutrophils, NK cells, innate lymphoid cells, eosinophils, T cells, B1, and B2 cells. Our main focus is how the adipose tissue affects immune responses, in particular B cell responses and antibody production. The role of leptin in generating inflammation and decreased B cell responses is also discussed. We report data published by us and by other groups showing that the adipose tissue generates pro-inflammatory B cell subsets which induce pro-inflammatory T cells, promote insulin resistance, and secrete pathogenic autoimmune antibodies.

Keywords: aging, obesity, inflammation, immunity, antibody responses

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AGING AND RELATED INFLAMMATION DECREASE ANTIBODY RESPONSES IN MICE AND HUMANS

Aged mice and humans have a poor immune response against infectious agents and vaccines (1). The antibody-mediated humoral immune response is qualitatively deficient with the production of antibodies of lower affinity (2–5) and with self-reactivity (6–8). Defects in T cells (9–11), B cells (12, 13), and antigen-presenting cells (14, 15) have been reported and all contribute to the age-related decrease in antibody production.

Our laboratory has characterized age-related autonomous B cell defects, which are responsible for sub-optimal antibody responses of elderly individuals to infections and vaccines (16–20). These include a reduction in activation-induced cytidine deaminase (AID), the enzyme necessary for class switch recombination, somatic hypermutation, and IgG production, as well as in E47 (13, 21), a key transcription factor regulating AID (22). Because AID correlates with optimal B cell function, it can be used as a predictive marker of optimal B cell response in humans (16, 17). The decrease in AID (4) leads to a reduced ability to generate higher affinity antibodies, e.g., to the influenza vaccine.

Aging is characterized by "inflamm-aging" (23), a low-grade chronic inflammation, which is a risk factor for morbidity and mortality of elderly individuals as it is implicated in the pathogenesis of several disabling diseases of the elderly such as type-2 diabetes mellitus (24), osteoporosis (25), Alzheimer's disease (26), rheumatoid arthritis (27), and coronary heart disease (28). Our results have shown B cell functional deficiencies with increased inflammation with age in both mice (29) and humans (19). In particular, we demonstrated that increased TNF-α either in serum or in B cells contributes to sub-optimal antibody responses and we consider this to be a condition where the B cells have been made "refractory" to further stimulation by chronic stimulation with inflammatory cytokines.

OBESITY AS A MECHANISM OF AGING

Among risk factors associated with disability and frailty, obesity seems to be a major contributor. Obesity is an inflammatory condition in which the innate immune system is chronically activated. Obesity contributes to pathologic conditions such as type-2 diabetes mellitus (30–32), cancer (33), psoriasis (34), atherosclerosis (35), and inflammatory bowel disease (36). Obesity is associated with sub-optimal immune responses in mice (37, 38) and humans (39).

The adipose tissue is generally separated into visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) (40). The SAT accounts for ~80% of human adipose tissue, but the VAT is more metabolically active (41), and VAT accumulation is a greater predictor of obesity-associated mortality.

Fat mass increases with age in mice (42–44) and humans (45) and this is associated with low-grade chronic inflammation which contributes to the development of insulin resistance (IR) which also increases with age. Aging induces a significant increase in fat mass, redistribution of body fat with increased VAT, and decreased SAT, as well as ectopic VAT deposition. All these are strongly associated with worse health conditions in healthy elderly individuals (46). Moreover, aging may significantly affect AT function by changing the profile of inflammatory mediators produced by the adipocytes, modifying pre-adipocyte number and function and AT infiltration of macrophages (46), and other lymphocytes (see below).

It has recently been proposed that increased cellular stress in the adipocytes with age induces cellular senescence, which in turn leads to impaired removal of lipotoxic fatty acids, and increased secretion of pro-inflammatory cytokines and chemokines, due to the activation of the innate, and adaptive immune systems (47). These pro-inflammatory processes not only amplify each other but also have systemic consequences. These results suggest that cellular senescence is a stress-induced adaptive response that develops through major metabolic and secretory readjustments. This can occur at any time during life.

Studies in humans have shown that individuals with higher total and abdominal adiposity have shorter telomeres (48), suggesting that obesity may accelerate the aging process. Telomere length is inversely associated with body mass index (BMI), waist to hip ratio, independently of sex, age, fasting glucose and insulin, lipid and lipoprotein concentrations, physical activity, smoking status, and other metabolic risk factors.

ADIPOSE TISSUE INFLAMMATION

The AT is a major immunologically active organ that contributes to systemic inflammation through the secretion of pro-inflammatory cytokines and chemokines, as well as adipokines (49). Immune cells represent two-thirds of the stromal vascular fraction, and therefore the expansion of the AT during high-fat diet increases its ability to act as an immunological organ able to control systemic inflammation and metabolism. Chronic inflammation and immune cell infiltration in the AT are hallmark of obesity-associated IR and glucose intolerance.

Increased inflammation in the AT is the result of increased intrinsic inflammation in the adipocytes, which operates in a positive feedback loop, whereby the accumulation of infiltrating immune cells secrete pro-inflammatory cytokines and chemokines following interaction with the adipocytes. This feedback loop explains not only local but also systemic inflammation *via* the circulating immune cells. Infiltrating immune cells are drawn to the AT and become more inflammatory and these cells would generate sub-optimal immune responses in obesity by circulating to the peripheral lymphoid organs.

Immune cells infiltrating the AT include macrophages, neutrophils, NK cells, innate lymphoid cells (ILCs), eosinophils, T cells, B1, and B2 cells. The cellular composition of AT is dynamic and is regulated by acute and chronic stimuli including diet, body weight, fasting. In general, neutrophils are the first cells that infiltrate the expanding AT during high-fat diet, followed by macrophages, B, T, and NK cells (43).

In response to energy increase, adipocytes undergo hypertrophy, hyperplasia, and die, releasing in the extracellular space their cytoplasmic content including the lipid droplets, which cause the release of danger-associated molecular patterns such as free fatty acids, excess glucose, ATP, ceramides, cholesterol. All these activate macrophages expressing TLRs and NLRs, activate the inflammasome and initiate the AT inflammatory response, leading to the recruitment of monocytes, and increased polarization of macrophages to an inflammatory M1-like phenotype. Macrophages represent the primary source of TNF-α in the AT (50).

Neutrophils promote IR through the release of elastase (51), myeloperoxidase, and extracellular traps (ETs) (52). Aberrant production and reduced clearance of ETs can lead to accumulation of immunogenic self-antigens and promotion of autoimmune diseases (53).

NK cells significantly increase in number in the AT of mice fed with a high-fat diet. NK cells regulate the number and the function of AT macrophages through production of pro-inflammatory cytokines, mainly TNF-α, and thereby contribute to the development of IR. Depletion of NK cells using neutralizing antibodies has been shown to protect from IR (54).

Innate lymphoid cells have also been shown to promote IR, in particular ILC1s, which trigger M1 macrophage activation and inhibit ILC2 function through IFN-γ, thereby contributing to chronic inflammation and possibly perpetuating obesity-associated IR (55, 56).

In obese individuals, pro-inflammatory Th1 cells infiltrate the AT (57) and activate M1 macrophages (58), whereas in lean individuals Th2 cells, T regulatory, and iNKT cells are predominant in the VAT and promote secretion of IL-10 and other anti-inflammatory cytokines from M2 macrophages which maintain insulin sensitivity. The abdominal SAT has been reported to be dangerous as well in promoting inflammation (59).

Studies elucidating B cell function in obesity are limited, although B cells have recently emerged as crucial players in regulating inflammation in murine AT, by presenting antigens to T cells, secreting pro-inflammatory cytokines, and pathogenic antibodies (43). In mice, B2 cells accumulate in the AT before T cells, shortly after the initiation of a high-fat diet (60). We have recently shown that the adipocytes in murine VAT make several pro-inflammatory chemokines (CXCL10/CCL2/CCL5), which may recruit B2 cells as they express the corresponding receptors (CXCR3/CCR2/CCR3) (61). We believe that B2 cells infiltrating the VAT become more inflammatory and these cells would generate sub-optimal immune responses once they circulate back to the peripheral lymphoid tissues (**Figure 1**). B2 cells may also be recruited to the AT through leukotriene LTB4/LTB4R1 signaling (62).

B1 cells can also be found in the AT, although at lower percentages when compared with B2 cells, with B1a (but not B1b) being increased in the AT mice fed high-fat diet (43). These cells secrete IgM antibodies which have no effect on metabolic parameters in contrast with IgG, but they clear self-antigens and therefore have a regulatory role by limiting B2 cell activation and by promoting B cell tolerance. B10 producing B1 cells in the AT have been shown to have protective effects against diet-induced obesity and IR (63).

The early recruitment of B cells promotes T cell activation and pro-inflammatory cytokine production (43). B cells are activated in the expanding AT by pro-inflammatory stimuli and release cytokines or chemokines, thus contributing to local and systemic inflammation (64, 65). Antibodies secreted by B cells can also regulate lipid absorption from the gut and B^{null} mice show reduction in lipid absorption (66). This role of B cells in lipid adsorption could also shape mucosal immunity and change the gut microbiota (43, 67). Moreover, murine (44) and human B cells (39) support T cell inflammation.

The ongoing apoptosis in the AT, due to increases in fat mass, and consequent hypoxia, induces the release of "self" antigens, including cell-free DNA, and the release of class switched IgG antibodies which form immune complexes with "self" antigens, which in turn activate complement (C1q/C1qR/C3/C3a) and Fc receptors on immune cells, leading to enhanced local inflammation, remodeling of the AT, impairment of adipocyte function and of nutrient metabolism, and exacerbation of obesity-associated conditions. This represents a novel mechanism by which DNA released from cells dying in the AT may attract immune cells expressing TLRs, which may propagate the inflammatory response, as recently shown in mouse macrophages (68).

Obesity is also associated with altered composition of the gut microbiota, increased intestinal permeability, and translocation of gut bacterial products into the blood. These include lipopolysaccharide and unmethylated CpG DNA, which may exert effects systemically or locally in the AT (69, 70). Aberrant production and recognition of nucleic acid antigens has been suggested to promote activation of immune cells in metabolic tissues, leading to the secretion of pro-inflammatory cytokines (71).

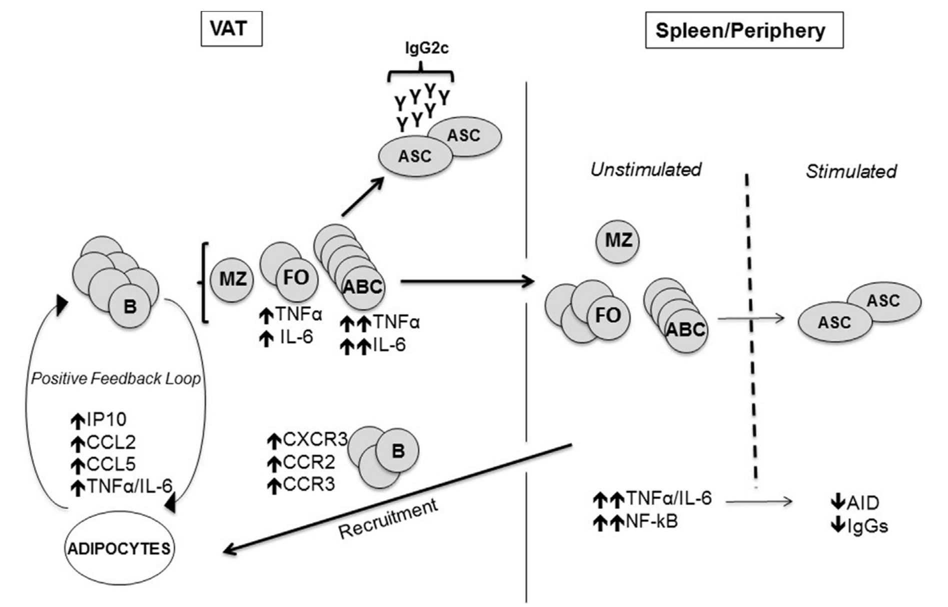

FIGURE 1 | Mechanisms by which the visceral adipose tissue (VAT) impairs antibody responses. The adipocytes in the VAT secrete more pro-inflammatory chemokines which attract B cells *via* chemokine receptors, as well as pro-inflammatory cytokines. Age-associated B cells (ABCs) are preferentially induced and we hypothesize that these cells make pro-inflammatory cytokines and pathogenic antibodies. Marginal zone B cells are not affected. Immune cells traffic to the spleen and periphery where there are more ABC in aged and obese mice. Before antigen stimulation the cells secrete increased amounts of inflammatory cytokines (TNF-α/IL-6), making them refractory to further stimulation. Fewer antibody-secreting cells producing less Ig are made after antigen stimulation of "refractory" B cells.

HOW THE ADIPOSE TISSUE AFFECTS ANTIBODY RESPONSES?

Obesity is associated with increased susceptibility to bacterial, viral, and fungal infections (72, 73) and obese individuals develop more post-surgical infections than lean individuals (74, 75). Moreover, overweight children have impaired antibody responses to tetanus toxin (76). Similarly, high-fat diet has been shown to increase mortality in mice infected with influenza (37). Contributing mechanisms seem to be defects in the generation and maintenance of memory CD8$^+$ T cells (77), as well as impaired lung wound healing (78). The response to the influenza vaccine (39, 79) and to the Hepatitis B vaccine (80) are also compromised in individuals with obesity.

B cell function has been shown to be affected by leptin, the adipocyte-derived cytokine, member of the IL-6 superfamily, linking nutritional status with neuroendocrine, and immune functions, whose plasma levels correlate with the amount of body fat and BMI. The role of leptin in inflammation is supported by the studies in *ob/ob* mice which are leptin-deficient and have reduced secretion of Th1 cytokines (IL-2/IFN-γ/TNF-α/IL-18), and increased production of Th2 cytokines (IL-4/IL-10) (81–84). Leptin suppresses appetite in mice and humans (85, 86). High-serum levels of leptin contribute to the inflammatory state of the adipose tissue associated with obesity (87, 88). In individuals with obesity, leptin levels are associated with leptin resistance (86). Leptin activates human peripheral blood B cells from both young and elderly individuals to secrete pro-inflammatory cytokines (IL-6/TNF-α) and this occurs through activation of JAK2/STAT3 and p38MAPK/ERK1/2 signaling pathways (89, 90). In our recently published article, we stimulated B cells from lean individuals *in vitro* with leptin. We found pro-inflammatory signaling pathways upregulated (phospho-STAT3, crucial for TNF-α production) and anti-inflammatory pathways down-regulated (phospho-AMPK, crucial for antibody production), similar to what we observed in B cells from individuals with obesity (39).

We have recently discovered further mechanisms through which AT inflammation contributes to decreased B cell responses in old mice (61). We found AID in stimulated splenic B cells negatively correlated with epididymal fat size, showing for the first time a role of AT in the down-regulation of B cell function in aged mice. When we measured the percentages of the major peripheral B cell subsets [follicular (FO), age-associated B cells (ABCs), and marginal zone] in the spleens and epididymal VAT of young and old mice, we found reduced percentages of the FO subset in the spleen of old versus young mice and concomitant increased percentages of the pro-inflammatory ABC subset as previously shown (29, 91–93). Importantly, percentages of FO were reduced (and percentages of ABC were increased) even more in VAT versus spleen.

We have recently shown that the VAT promotes the differentiation of FO into ABCs (61). We demonstrated this by co-culturing in transwells for 72 h adipocytes and splenic B cells, in the absence of any additional mitogenic stimulus. Results showed increased percentages of ABC, which was similar to what we have observed in the VAT. To clarify if this was the result of increased expansion and survival of ABC versus FO B cells, death of FO B cells, loss of cell markers, or a combination of these, we sorted splenic FO and ABC from old mice and measured by qPCR the expression of several markers described to be differentially expressed in these two subsets by a transcriptome analysis performed previously by the Marrack group (92). We selected five markers among those most differentially expressed in FO versus ABC: Prdm1 (Blimp-1), FcεRγ1, Tbx21 (T-bet), Kifc3, Stx3. All these markers were found expressed at higher levels in ABC versus FO, as expected. Then, we cultured sorted splenic FO B cells from old mice in the presence of adipocyte-conditioned medium (ACM) and we found that the ACM induced significant increased expression of ABC markers when compared with complete medium, suggesting that FO B cells differentiated into ABC. In order to evaluate if the ACM contains factors which may be responsible for FO differentiation into ABC, as suggested by an article recently published by Cancro's group (94), we measured by qPCR production of IL-21/IFN-γ by the adipocytes. Results showed that adipocytes express mRNA for both cytokines.

B cells have been shown to promote IR through activation of T cells and production of pro-inflammatory, pathogenic, autoimmune antibodies (43). We also found production of class switched IgG2c antibodies by B cells in the VAT, and these antibodies were detected by intracellular staining of VAT ABCs in the absence of stimulation, suggesting that ABC in the VAT are already highly preactivated, and are refractory to further stimulation (61). IgG2c have been noted to be more autoimmune (95). Our results have shown for the first time that IgG2c antibodies are made in the VAT by ABCs, and the expression of MHC class I and class II on B cells has been reported to be crucial (43), suggesting that B cell-mediated antigen presentation to T cells is required for their pathogenic effects.

It has been shown that B cell depletion with an anti-CD20 antibody ameliorates metabolic disease, and transfer of IgG from high-fat diet mice rapidly induces IR (43). T cells can also be necessary for a pathogenic effect, as adoptive transfer of CD4$^+$ T cells into high-fat diet RAG^{null} mice, lacking both B and T cells, was able to block weight gain and reverse IR for months, predominately through anti-inflammatory Th2 cells (96).

In conclusion, we have summarized emerging data on potential cellular and molecular mechanisms for the age-related increase in inflammation and how these lead to functional decline and decreased humoral responses in aged mice and humans. Overall, it appears that persistent inflammation is the driver of age-related diseases and that down regulation of inflammatory pathways may help to reduce onset and severity of age-related chronic diseases. Key challenges for the field will be to identify therapeutic strategies of intervention to lose weight will reduce body fat, systemic inflammation, and the pathogenic role of immune cells. Importantly, immune responses to fight infections will also be improved.

AUTHOR CONTRIBUTIONS

All authors were involved in writing the article and had final approval of the submitted and published versions.

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Chronic Inflammation in Immune Aging: Role of Pattern Recognition Receptor Crosstalk with the Telomere Complex?

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Age-related decline in immunity is characterized by stem cell exhaustion, telomere shortening, and disruption of cell-to-cell communication, leading to increased patient risk of disease. Recent data have demonstrated that chronic inflammation exerts a strong influence on immune aging and is closely correlated with telomere length in a range of major pathologies. The current review discusses the impact of inflammation on immune aging, the likely molecular mediators of this process, and the various disease states that have been linked with immunosenescence. Emerging findings implicate NF-κB, the major driver of inflammatory signaling, in several processes that regulate telomere maintenance and/or telomerase activity. While prolonged triggering of pattern recognition receptors is now known to promote immunosenescence, it remains unclear how this process is linked with the telomere complex or telomerase activity. Indeed, enzymatic control of telomere length has been studied for many decades, but alternative roles of telomerase and potential influences on inflammatory responses are only now beginning to emerge. Crosstalk between these pathways may prove to be a key molecular mechanism of immunosenescence. Understanding how components of immune aging interact and modify host protection against pathogens and tumors will be essential for the design of new vaccines and therapies for a wide range of clinical scenarios.

Keywords: pattern recognition receptor signaling, telomere shortening, inflammaging, myelopoiesis, NF-κB, toll-like receptor signaling

INTRODUCTION

Aging is a complex process that involves a gradual decline in critical cellular processes, signaling pathways, and regulatory mechanisms, leading to eventual disruption of tissue homeostasis (1). Accumulation of cell functional defects over time, commonly termed "senescence," is a driving force of human aging and confers increased risk of cardiovascular and neurodegenerative disorders, as well as autoimmune disease and infection (2). Cellular senescence-associated changes affect numerous processes including proliferation or changes in secretome. Recent studies have shown that chronic inflammation contributes to pathological aging by promoting stem cell exhaustion, impairment of cellular communication, and somatic cell loss of the repetitive nucleotide sequences known as telomeres that form protective "caps" at the ends of chromosomes (1).

To maintain telomere length and protect chromosomes against damage, cell types with high proliferative capacity such as hematopoietic progenitors (3, 4) and effector leukocytes (5, 6) employ the inducible enzyme telomerase to maintain telomere length. In addition, the multiprotein complex shelterin coordinates the formation of protective "loop" structures that prevent telomere ends from being recognized as DNA breaks (7). While a large number of studies have investigated telomere length and telomerase activity as prognostic biomarkers in human cancer, this review instead focuses on the potential interactions between inflammation and telomere biology in immunological aging. Indeed, telomerase activity is now known to be strongly influenced by leukocyte proliferative activity, ongoing inflammation, and production of reactive oxygen species (ROS), but the molecular basis of these effects is not yet fully understood. In particular, the transcription factor NF-κB, which has long been associated with pattern recognition receptor (PRR) signaling and inflammation, has recently been identified as an important regulator of the telomere complex. Better definition of potential immune crosstalk with telomerase activity may therefore yield a range of novel therapeutic targets for intervening in age-related and inflammatory pathologies.

IMMUNOSENESCENCE

Effective host immunity is essential for the maintenance of tissue homeostasis and health, but both innate and adaptive responses are subject to natural age-related functional decline termed "immunosenescence" (8). Key features of immunosenescence include a progressive loss of naïve T cells and accumulation of memory T cells in body tissues (9–11) as well as gradual deterioration of innate leukocyte defense mechanisms (8, 12). In this review, we focus mainly on senescence-associated changes in the innate immune compartment, which mediates first line of defense against infections. Senescence impacts on several major mechanisms of innate protection against pathogens, including phagocytosis and ROS production by neutrophils, as well as toll-like receptor (TLR) expression and cytokine release by macrophages and dendritic cells. Key defects in innate cell activity associated with senescence have been reviewed elsewhere (8, 12, 13). These include a range of deficits in myeloid cell functions, which are governed primarily *via* PRR signaling and have been identified as displaying significant impairment in various senescence-related disorders.

Myeloid cell-derived biomarkers of immunosenescence reportedly include increased production of the cytokines interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α), which correlate with elevated serum levels of C-reactive protein to predict increased patient frailty and higher overall rates of mortality (14). IL-6 and TNF-α are produced mainly by tissue macrophages and T cells and have already been implicated in multiple age-related disorders including osteoarthritis, cardiovascular disease, autoimmunity, and neurodegeneration (15). Both IL-6 and TNF-α are able to increase telomerase activity through NF-κB, STAT1, and STAT2 activation (16). However, the mechanism by which these mediators of inflammation impact on the aging process remains poorly defined. For example, serum IL-6 levels have previously been identified as a predictive biomarker of mortality risk in the elderly (17, 18), but this cytokine has also been shown to exert anti-inflammatory effects in certain age-related pathologies including rheumatoid arthritis (19). Therefore, additional studies will be required to identify the molecular mediators involved so that these can be targeted by future therapeutic strategies.

Although immunosenescence occurs naturally as the human body ages, early activation of senescence pathways has been observed in a wide range of human disorders (20, 21). Immunosenescence is also associated with hematopoietic dysfunction, leading to a decline in leukocyte numbers and function across both the innate and adaptive arms of the immune system (22–24). These detrimental effects are typically associated with prolonged, low-grade infection or inflammation (25, 26) and/or persistent infection by pathogens including cytomegalovirus (27, 28). Previous studies have indicated that low-grade inflammation induced by genetic deletion of NF-κB subunit can confer telomere dysfunction (29) and that bone marrow-derived macrophages from aged mice exhibit short telomeres and impaired inflammatory signaling (30). It seems likely therefore that mechanisms of telomere maintenance impact on immune function and *vice versa*, in particular, *via* interactions with the enzyme telomerase. Indeed, emerging data indicate that telomerase likely exerts a range of additional functions that could significantly impact on hematopoiesis and mitochondrial ROS production during age-related immune decline.

"INFLAMMAGING"

Immunosenescence is strongly driven by persistent infections and/or tissue inflammation (1, 31), leading some investigators to term this process "inflammaging" to better distinguish pathological events from natural age-related decline (20, 21, 32). In some settings, inflammaging is a consequence of unresolved "sterile" inflammation resulting from organelle/molecule damage, inappropriate immune signaling, and autoantigen (33). Although inflammation is primarily maintained by secreted cytokines, as already reviewed elsewhere (34, 35), another important factor is damaged cell/tissue release of stimulatory molecules that can activate myeloid cells by signaling through PRRs such as TLRs. PRRs recognize specific pathogen-associated molecular patterns (PAMPs) as well as host-derived damage-associated molecular patterns (DAMPs) that are produced by stressed, malfunctioning, and injured cells. Several DAMPs released by damaged mitochondria (36, 37) and nuclei (38, 39) or derived from the cytoplasm (40, 41) have already been linked with inflammaging. Failure to resolve low-grade inflammation can result in both innate and adaptive immune responses to self-antigens, progressive tissue damage, and pathological cellular aging. Accordingly, sustained activation of PRR pathways has already been identified in a number of chronic inflammatory disorders associated with aging (**Table 1**), and changes in PRR expression and signaling are now widely recognized as critical components of immunosenescence (12,

TABLE 1 | Chronic inflammatory diseases with reported telomere shortening, changes in telomerase activity, and a role for PRRs.

Disease category	Pathology/disease type	PRRs associated with disease and cell types affected	Cell-specific telomere shortening	Telomerase activity
Cardiovascular diseases	Atherosclerosis	TLRs (42–45); Mo, MF, DC, MC, aortic tissue	Leukocytes (46)	MF, aortic tissue, ↗ (47, 48)
	Chronic heart failure	TLRs, NLRs (49); MF, heart tissue	Leukocytes (50)	ND
Pulmonary diseases	Chronic obstructive pulmonary disease	TLRs (51); Mo, MF, lung tissue	Leukocytes (52, 53)	ND
	Sarcoidosis	TLR2 (54); BAL	Leukocytes (55, 56)	ND
Hepatic diseases	Non-pathogenic hepatitis	TLRs (57–60); hepatocytes, biliary epithelia, sinusoidal endothelia, MF, Mo	Liver tissue (61)	ND
	Primitive biliary cirrhosis	TLRs (62), Mo	Bile duct (63)	ND
Gastrointestinal diseases	Ulcerative colitis	TLR4, TLR5 (64, 65); mucosa	Leukocytes, mucosa (66–69)	Mucosa, ↗ (70)
	Celiac disease	TLR2, TLR4 (71)	Leukocytes (72)	ND
Joint and muscle diseases	Idiopathic inflammatory myopathies	TLRs, NLRs (73), skeletal muscle, MF, DC	No significant shortening (74)	Skeletal muscle, ↗ (74)
	Rheumatoid arthritis	TLRs (75, 76), synovial tissue	Leukocytes, T cells (77, 78)	Synovial ts., ↗ (79, 80)
	Juvenile idiopathic arthritis	TLRs (81), Mo	Naïve T cells (82, 83)	ND
	Systemic sclerosis	TLRs (84–86), synovial tissue	No significant shortening (87)	PBMCs, ↘ (88)
Other autoimmune conditions	Systemic lupus erythematosus	TLR7, TLR9 (89–91), mesangial cells	Leukocytes (92)	PBMCs, T cells, ↗ (88, 93)
Infectious diseases (chronic infections)	*Helicobacter pylori*	TLR2, TLR4 (94–96), gastric mucosa, gastric epithelial cells	Gastric mucosa (97)	Gastric mucosa, ↗ (98)
	Hepatitis B	TLRs (99, 100), PBMC	Hepatocytes (101)	PBMCs, ↘ (102)
Alcohol, smoking, and obesity-related diseases	Alcohol consumption	TLR4, TLR2 (103, 104), Kupffer cells, lung epithelia	Eosophageal epithelium (105)	ND
	Smoking	TLR4 (103, 106), Lung epithelia	Leukocytes (107, 108)	↗ (109), lung epithelia
	Obesity	TLRs (110, 111), adipose tissue	Leukocytes (108)	ND

Chronic inflammation plays a major role in progression of various disorders and autoimmune pathologies. This table lists diseases in which shortening of telomeres, changes in telomerase activity, and a role for TLR signaling have been reported. Although direct interaction between these processes has yet to be formally demonstrated, these events have been closely correlated in a range of different disorders and putative mechanisms are now beginning to emerge. While short telomeres have frequently been associated with human disease, telomere length is not always correlated with disease severity.
Mo, monocyte; MF, macrophage; DC, dendritic cell; MC, mast cell; BAL, bronchoalveolar lavage; PBMCs, peripheral blood mononuclear cells; PRR, pattern recognition receptor.

24). Inflammation-induced immune aging in host tissues is therefore a consequence of multiple detrimental pathways acting in concert over a prolonged period of time.

HEMATOPOIETIC STEM CELL EXHAUSTION IN CHRONIC INFLAMMATION

Natural age-associated changes in innate immune function have already been described in adults older than 40 years (112), whereas early-onset immunosenescence has been associated with various pathologies. Changes in TLR expression and function likely represent key components of both healthy and pathological immune aging (113). In particular, various types of hematopoietic progenitors have been shown to express TLRs (114, 115), which may play direct roles in senescence of the progenitor pool (113, 115). Steady-state differentiation of hematopoietic stem and progenitor cells (HSPCs) into myeloid lineage cells is controlled by growth factors including G-CSF, M-CSF, GM-CSF, and Flt3-L, but can be modified by pro-inflammatory cytokines such as IFN-γ during an immune response (116, 117). Chronic inflammation can also generate massive quantities of DAMPs including calgranulins (S100A8/9), high mobility group box-1 (HMGB1), and serum amyloid A, which can engage PRRs expressed by multiple cell types. Direct TLR stimulation of HSPCs in the bone marrow and circulation may therefore accelerate the immune aging process (113, 118).

Direct roles for HSPCs in inflammation have only recently been described by Griseri et al. who identified progenitor cell infiltration of the gut mucosa in experimental colitis (119). Most studies of PRR function in HSPCs have focused on the small number of cells that circulate in peripheral blood, where these progenitors can detect PRR ligands and enhance extramedullary hematopoiesis during inflammation (118, 119). HSPC stimulation with TLR ligands can potently modulate differentiation pathways and typically favors myeloid cell development (114, 115, 120), but prolonged TLR triggering eventually leads to progenitor exhaustion and loss of self-renewal capacity (121–123). Bone marrow HSPCs can also mediate "emergency hematopoiesis" in response to PRR ligation of DAMPs and PAMPs (114, 124), particularly in the context of bacterial infection (125, 126) or fungal invasion (127, 128). However, inflammatory modulation of hematopoietic activity is not restricted to the blood and bone marrow, since somatic cells and tissues also appear to influence this process (129, 130). It is also important to note that PRR signaling in HSPCs can play a role in cell reconstitution even under resting conditions, since TLR4/TRIF reportedly mediates the steady-state renewal of granulocytes (131). Taken together, these data indicate that inflammation can induce PRR signaling in HSPCs and accelerate/modify cellular differentiation to promote progenitor exhaustion and immune system dysfunction, both of which are important hallmarks of immunosenescence. To what extent stem cell telomeres and telomerase are involved in these events remains unclear, although HSPC skewing toward generation of myeloid-lineage cells has previously been linked with telomere dysfunction (132), and experimental mice lacking the telomerase subunits telomerase reverse transcriptase (TERT) or telomerase RNA component (TERC) exhibit increased myeloid progenitor cell numbers in bone marrow (133).

Formal demonstration of a direct influence of PRRs/inflammation on telomere length/telomerase activity in host leukocytes and stem cells is currently lacking, but experimental data consistent with this concept are continuing to accumulate. Indeed, age-related DNA damage and shortened telomeres have been observed in murine HSCs (134), and senescent progenitor cells with shortened telomeres exhibit increased activity of the pro-inflammatory transcription factor NF-κB (135). TERC-deficient mice also exhibit chromosome instability that enhances signaling through TLR4/NF-κB, leading to increased macrophage expression of pro-inflammatory cytokines and high susceptibility to endotoxin shock (136). These and other influences of PRR signaling on accumulation of DNA damage in host cells have been expertly reviewed elsewhere (137). It seems likely therefore that direct crosstalk between PRRs and telomerase activity will also prove critical to the immunosenescence process in humans. This could have major implications for the design of therapies to maintain effective host immunity in elderly patients and treat various inflammatory disorders. Indeed, immune aging has already been identified as a major determinant of bone marrow progenitor quality and functionality during transplantation (138). Inflammatory DAMP generation and PRR triggering of HSPCs have also been reported to increase pathology in disorders including atherosclerosis (42, 43, 139), colitis (119), and chronic dermatitis (140). Further detrimental effects of inflammation on HSPCs have been observed in models of chronic PRR triggering (117, 126, 141) as well as in human sepsis (142), while age-related change in hematopoietic function have also been shown to confer increased risk of anemic and malignant disorders (143). PRR-driven signaling has now been observed to correlate with altered telomere length or telomerase activity in numerous cell types and tissues from patients with chronic inflammatory disorders (**Table 1**), but the mechanistic basis of this link has not yet been defined. Despite their disparate origins and diverse pathological features, these diseases share common features of oxidative stress and inflammation together with telomere shortening, suggesting tight associations between inflammatory disorders and cellular senescence across a range of clinical settings.

MITOCHONDRIAL DAMAGE IN INFLAMMAGING

Mitochondrial ROS production is a key antimicrobial function of specialized immune cells including macrophages, dendritic cells, and neutrophils. Accordingly, age-related impairment of mitochondrial function can significantly impair host immune responses (144). Increasing age is typically accompanied by decreased mitochondrial output of antimicrobial ROS together with a parallel increase in oxidative stress. While a role for mitochondrial dysfunction in immunosenescence is now well established, the basis of this association may be more complex than initially thought. Recent reports have indicated that DNA release from damaged mitochondria is a major driver of ROS production and inflammation (145, 146) and may therefore promote host immunosenescence *via* a range of different mechanisms (147). ROS accumulation also promotes further mitochondrial dysfunction, oxidative stress, and release of DNA into the cytosol where this can activate the NLRP3 inflammasome (146). While neutrophils exhibit only a short half-life in blood and typically lack TERT expression or telomerase activity (148), during inflammation these cells are a major source of ROS and can reportedly acquire telomerase activity on infiltration of unstable coronary plaques (149). Further studies will now be required to resolve the exact role of cytoplasmic TERT expression in neutrophils that lack TERC (150) and to determine the contribution of these cells to immunosenescent pathology.

Reactive oxygen species have also been strongly implicated in pathological changes in blood vessel structure and function that characterize age-related vascular diseases such as atherosclerosis (151). In this context, Jurk et al. used a genetic model of chronic low-grade inflammation to demonstrate that ROS exacerbate telomere dysfunction (29). It now seems that oxidative stress, mitochondrial damage, and cellular aging are intimately linked in multiple species including yeast (152) and trypanosomes (153), although additional data from animal models and validation in human studies will be required to fully understand this.

INFLAMMAGING, TELOMERASE ACTIVITY, AND TELOMERE LENGTH

Telomere shortening during cell division is a critical process in progression to senescence (154), and telomerase may play an important role in immunological aging. Overexpression of telomerase subunit TERT can decrease oxidative stress in cancer cell lines (155), whereas TERT-deficient HSCs are characterized by ROS impairment and functional defects (156). Similarly, chromosome instability arising from TERC deficiency promotes TLR4 stimulation (136), while telomeric repeats (TTAGGG) can inhibit CpG binding to TLR9 to impair innate immune activation (157). Telomerase activity also appears to be subject to modulation by the activity of NF-κB (29) and/or exposure to pro-inflammatory cytokines (16, 158, 159) as summarized in **Figure 1**. However, it is important to note that telomerase expression level and enzymatic activity do not always directly correlate with senescent status or even telomere length; hence, further studies will be needed to better understand these complex interactions in human cells and tissues.

Even in the absence of NF-κB signaling, prolonged low-grade inflammation is sufficient to induce telomere dysfunction, likely involving accumulation of mitochondrial ROS (29). TERT can

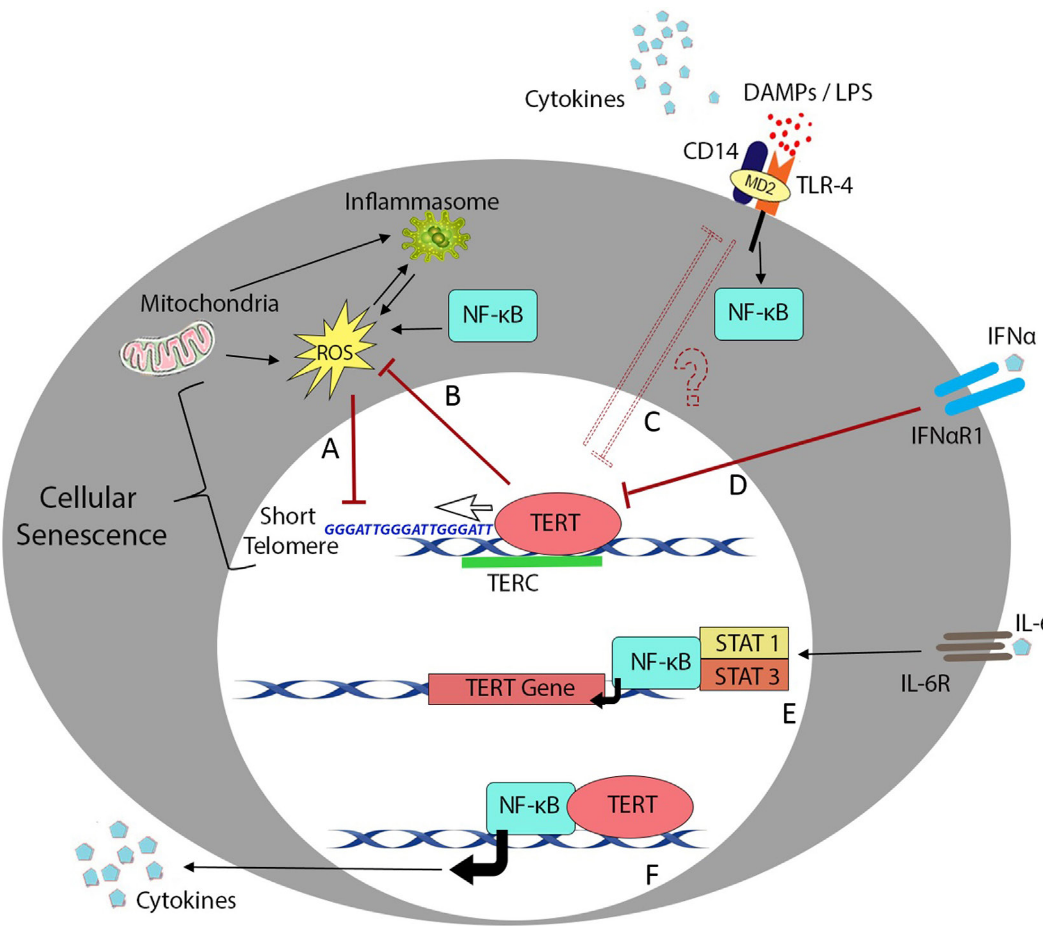

FIGURE 1 | Telomere length and telomerase activity during inflammation. Overview of the major cellular processes linking the telomere complex with inflammatory signaling and immunosenescence. Transcription factor NF-κB plays a crucial role in most inflammatory processes but also interacts with telomere control machinery and putative non-telomeric functions of the telomerase enzyme. **(A)** Low-grade inflammation in nfkb1$^{-/-}$ mice causes increased ROS production and results in telomere dysfunction in mouse hepatocytes and intestinal crypt stem cells (29). **(B)** One of the reported non-telomeric functions of human telomerase enzyme (TERT) is the ability to inhibit endogenous ROS production and regulate oxidative stress in cancer cell lines (155). **(C)** Mice lacking telomerase RNA component (TERC) succumb to LPS administration due to endotoxin shock arising from chromosome instability in splenocytes and macrophages (136). **(D)** Signaling downstream of inflammatory cytokines such as IFN-α plays an important role in downregulation of TERT activity in hematopoietic cells (159). **(E)** In contrast, interleukin (IL)-6 and tumor necrosis factor (TNF)-α reportedly upregulate TERT transcription and telomerase activity through activation and binding of NF-κB in macrophages (47) or NF-κB, STAT1, and STAT3 interactions with the TERT promoter in splenocytes and cancer cells (16, 158). **(F)** Ghosh et al. have also described the ability of TERT to directly regulate NF-κB-dependent gene expression in primary bone marrow blasts from leukemic patients (160).

integrate numerous upstream signals including Wnt/β-catenin developmental cues (161) and can regulate inflammatory signaling through binding to NF-κB promoters and subsequent transcription of NF-κB-regulated genes including IL-6 and TNF-α (160). This crosstalk is exemplified by an alcoholic liver disease model in which NF-κB was observed to regulate protein expression levels of the catalytic subunit TERT (158), which in turn modulated NF-κB signaling to promote macrophage polarization toward an inflammatory M1 phenotype with increased expression of IL-6 and TNF-α (162). Increased peripheral blood expression levels of IL-6 and TNF-α in patients with metabolic disorders have also been shown to correlate with elevated levels of telomerase activity (163).

The central role of NF-κB in regulating chronic, low-grade inflammation has long been established, but only recently have experimental data begun to indicate a possible role for NF-κB in control of telomerase expression or activity in the context of senescence-associated disorders. For example, Gizard et al. showed that inflammation-induced NF-κB activation regulates TERT expression in macrophages and that human atherosclerotic lesions are characterized by high expression of TERT (47). Disease-associated changes in PRR signaling and telomere biology have also been identified within individual cells or host tissues, including the inflamed gut mucosa in ulcerative colitis (64–70), synovial tissues in rheumatoid arthritis (75, 76, 79, 80), and smoke-exposed lung epithelia (103, 106, 109). However, these features have often been described across multiple separate reports; hence, definitive proof of functional links between these processes is still lacking. Indeed, while short telomeres in leukocytes have been identified as a key component of pathological immune aging (5, 6, 164), direct associations with human senescence have not yet been confirmed, and the majority of relevant mechanistic data have been generated exclusively in mouse models. This is a particular challenge given that mouse telomeres can be up to 10 times longer than their equivalent human sequences despite a much shorter animal lifespan (165). Nonetheless, substantial data have now been obtained using genetically engineered TERC/TERT-knockout mice, which replicate features of human telomere biology as observed in various inflammatory disorders. It will now be critical to perform additional studies of telomere biology/telomerase activity in human leukocytes during natural aging and inflammation before this axis can be exploited for therapeutic benefit in the clinic.

CONCLUSION

Immunosenescence is the culmination of a complex network of molecular processes. Despite intensive study over the last decade and improved understanding of the features of immunological aging, the molecular mediators of these events and the extent to which they interact remain poorly defined. Indeed, while the strong association of telomere length with cellular senescence has been known for decades, the direct/indirect relationship between telomerase activity and PRR signaling is only now coming to light. While the molecular basis of PRR interactions with telomerase activity has not yet been determined, better definition of this crosstalk will be essential to understanding the influence of PRRs and "inflammaging" on human hematopoiesis and tissue regeneration. The recently identified ability of stem cells to directly detect DAMPs and PAMPs *via* PRRs should lead to significant progress in developing methods of combating immunosenescence in a range of human pathologies. Together, these data underscore the importance of inflammaging as a major driver of senescence progression and reinforce the concept that an array of different pathways likely interact to determine the rate of this process (graphically represented in **Figure 1**). Recent analyses of complex data sets from large cohorts of elderly subjects and patients with various chronic disorders have already implicated key regulators of immunosenescence in determining clinical outcomes. However, a complete understanding of the molecular mechanisms at play will require more sophisticated animal models and validation in human studies before these can be effectively targeted for therapy in common diseases of aging and inflammation.

AUTHOR CONTRIBUTIONS

SSJ prepared the figure and wrote the manuscript, KB wrote the manuscript and prepared the table, TK and ZK advised clinical research interpretations, and JF conceptualized, wrote, and critically reviewed the manuscript.

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Differential Phenotypes of Myeloid-Derived Suppressor and T Regulatory Cells and Cytokine Levels in Amnestic Mild Cognitive Impairment Subjects Compared to Mild Alzheimer Diseased Patients

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Alzheimer disease (AD) is the most prevalent form of dementia although the underlying cause(s) remains unknown at this time. However, neuroinflammation is believed to play an important role and suspected contributing immune parameters can be revealed in studies comparing patients at the stage of amnestic mild cognitive impairment (aMCI) to healthy age-matched individuals. A network of immune regulatory cells including regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) maintains immune homeostasis but there are very few data on the role of these cells in AD. Here, we investigated the presence of these cells in the blood of subjects with aMCI and mild AD (mAD) in comparison with healthy age-matched controls. We also quantitated several pro- and anti-inflammatory cytokines in sera which can influence the development and activation of these cells. We found significantly higher levels of MDSCs and Tregs in aMCI but not in mAD patients, as well as higher serum IL-1β levels. Stratifying the subjects based on CMV serostatus that is known to influence multiple immune parameters showed an absence of differences between aMCI subjects compared to mAD patients and healthy controls. We suggest that the increase in MDSCs and Tregs number in aMCI subjects may have a beneficial role in modulating inflammatory processes. However, this protective mechanism may have failed in mAD patients, allowing progression of the disease. This working hypothesis obviously requires testing in future studies.

Keywords: Alzheimer's disease, myeloid-derived suppressor cells, regulatory T cell, amnestic mild cognitive impairment patients, cytokines, inflammation

INTRODUCTION

Alzheimer disease (AD) is the most frequent neurodegenerative disease for which aging is the most important risk factor (1, 2). The pathogenesis of AD remains unknown. The paradigmatic amyloid beta hypothesis (3) is being increasingly challenged (4). Although AD is a chronic inflammatory disease that mainly involves the brain in its clinical manifestations, it is also a systemic disease (5). The preclinical stages of AD may last for decades before overt manifestations of the first symptoms are recognized. For instance, this is the case of amnestic mild cognitive impairment (aMCI) that is now termed mild neurocognitive disorder according to The Diagnostic and Statistical Manual of Mental Disorders (DSM 5) (6, 7). Thus, the first clinical step is the condition of aMCI which progresses to full clinical manifestations of AD in a number but not all individuals. Noticeably, the progression to full-blown AD is also relatively slow, occurring over a number of years (8).

Several immune alterations have recently been reported in AD patients (9). It was shown that the number of naïve T cells was relatively decreased, whereas the number of effector memory T cells was increased (10). We have reported that cells of the innate immune system were differentially altered in patients with aMCI compared to mild AD (mAD) (11). These data suggested that there was an upregulated inflammatory activity associated with some type of innate cells such as NK cells and neutrophils in aMCI. We have previously postulated that in the case of aMCI subjects, these cells respond to some still unidentified challenge that may originate from chronic viral, bacterial, or fungal infections. The recent demonstration that beta amyloid peptides, the most important component of the characteristic amyloid plaques in AD, possess antimicrobial properties (12–14) is consistent with this notion. Overall, these observations have raised the level of interest in immune changes associated with the development and progression of AD (9).

The activity of the immune system needs to be tightly controlled to provide a fast and targeted response to challenges, followed by inhibition of the response during the resolution phase to prevent chronic inflammation and tissue damage. Several types of immune regulatory cells participate to this essential regulatory mechanism and these include myeloid-derived suppressor cells (MDSCs) (15–17) and regulatory T cells (Tregs) (18–21). These cells suppress the immune response to prevent chronic inflammation and autoimmune processes (22, 23) although, in some cases, the response may be diverted in favor of a pathological process such as cancer (23–27).

Myeloid-derived suppressor cells are the most important immune modulatory cells of the innate immune system (28). These are essentially immature myeloid cells which may be either neutrophilic ($CD15^+$) or monocytic ($CD14^+$) (29, 30) and they composed a very heterogeneous population of cells (15). In humans, MDSCs are defined by the phenotype $CD33^+HLA\text{-}DR^-$ and are lineage (CD3, CD19, CD56)-negative (31). MDSCs suppress innate and acquired immune responses in cancer (31) and are elevated in chronic inflammation and malignancies (25, 32). It is of note that MDSCs have been found to be increased in healthy elderly subjects (28). In aging as well as in the case of chronic inflammatory states such as cardiovascular disease, cognitive decline, and frailty, pro-inflammatory mediators (TNF, IL-6, and IL-1β) production is commonly increased and is related to the differentiation of suppressor cells (33, 34). The role of MDSCs in normal physiology is complex. It has been reported that these cells impair the functions of T cells, NK cells, and dendritic cells through several pathways that include expression of arginase I, inducible nitric oxide synthase and Gp91phox and, the release of reactive oxygen species and peroxynitrite during antitumor immunity (26, 35, 36). MDSCs mainly suppress T cell function and NK cell cytotoxicity and they may also modulate macrophage polarization and, chemotaxis and functions of neutrophils (15, 16). MDSCs have been implicated not only in cancer but also in psoriasis (36), inflammatory bowel disease, traumatic stress, rheumatoid arthritis, and infections (37–44). It has also been shown that MDSCs may induce Tregs possessing a $CD4^+CD25^+FoxP3^+$ phenotype in cancer settings such as hepatocellular carcinoma (45, 46).

Regulatory T cells (defined phenotypically in humans as $CD4^+FoxP3^+$) are essential to control immunity and self-tolerance. Accordingly, their dysregulation leads to unbalanced immune responsiveness, tissue damage and autoimmunity (23). Tregs are recognized as suppressors of host immune responses in antiviral immunity and promoters of tumor growth (18). Similarly to MDSCs, they consist of very heterogeneous cell populations in their origin and expression of different cell surface markers (47, 48). Their suppressive activity involves either or both cell-cell contact-dependent and cytokine-dependent (especially through secretion of IL-10 and TGFβ) action (21). Furthermore, they play a role in homeostasis and damage repair in non-lymphoid organs (47).

In addition to their essential role in lymphocyte homeostasis, Tregs play either detrimental or favorable roles in certain viral infections. Whereas Tregs have a detrimental role in chronic hepatitis C virus infection by contributing to viral pathogenicity (49), their role in HIV infection is equivocal, depending mostly on the clinical stage (50). Similarly, accumulation of Tregs is correlated with poor prognosis in many types of cancer including breast cancer and hepatocellular carcinoma (51). Tregs participation may be beneficial for reducing tumor progression and improving prognosis by suppressing the inflammatory activities of Th17 cells in colorectal cancers (52).

There are few reports on the role of Tregs in neurodegeneration and AD (53–55). In this connection, two studies in humans have reported an increase of Tregs in AD (54, 55). One of these studies reported that Tregs of aMCI patients were increased relative to AD patients (54). The authors concluded that the inflammatory process plays a major role in AD pathogenesis and that alterations of Tregs in AD patients may contribute to this pathology. However, the fundamental role of Tregs in AD has not been yet defined and it is not known whether they are beneficial by suppressing a specific immune-inflammatory response or whether they are harmful by suppressing a potentially beneficial immune response (56).

Accordingly, the role of MDSCs or Tregs in immune modulation may vary considering their development, phenotype, functions, and the pathological conditions under which they are activated (57). In the case of AD, it can be suggested that their activity may contribute to development and progression of a neuroinflammatory process by suppressing protective innate and adaptive immune functions mainly at the early stage of the disease. The fact that chronic inflammation is a progressive process in AD led us to investigate whether there were changes in the number of MDSCs and Tregs in aMCI or mAD patients. Modifications of the involvement of MDSCs and Tregs in AD may contribute to the suppression/modulation of the innate and adaptive immune responses. To answer this question, we quantitated the number of MDSCs and Tregs in the periphery of aMCI and mAD patients and compared the results to age-matched healthy elderly subjects. In addition, we quantitated the levels of various circulating cytokines, as potential drivers of the development of these cells. Results showed differential increases in the number of neutrophilic MDSCs and Tregs in aMCI subjects in contrast to healthy controls and mAD patients. IL-1β was the only proinflammatory cytokine that was increased in aMCI whereas IL-10 was decreased in both aMCI and mAD patients. Our data suggest that the increase in MDSCs and Tregs number in aMCI subjects may have a beneficial role in modulating inflammatory processes. However, this protective mechanism may have failed in mAD patients, allowing progression of the disease.

MATERIALS AND METHODS

Subjects

Amnestic mild cognitive impairment and mAD diagnosis was made according to NINCDS-ADRDA criteria (57, 58) and the guidelines of Grundman et al. (59). Healthy elderly individuals satisfied the SENIEUR standard protocol for immuno-gerontological studies (60). Subjects who had a history or physical signs of atherosclerosis or inflammation were excluded. Evaluation and classification of subjects followed the standard protocol of our memory clinic including clinical, neuropsychological, and imaging assessment. Mini-mental state evaluation and Montreal cognitive assessment were performed, as we have previously described in details (11). The study included 11 healthy controls, 11 aMCI subjects, and 15 mAD patients. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the Faculty of Medicine and Health Sciences of the University of Sherbrooke (protocol # 2010-21/Fülöp). *Cytomegalovirus* (CMV) seropositivity was determined at the clinical laboratories of the Centre Hospitalier Universitaire de l'Université de Sherbrooke (CHUS) hospital. Additional details of the patients' clinical data are summarized in **Table 1**.

TABLE 1 | Patients' clinical data.

Parameters		Healthy subjects (C) ($n = 13$)		Amnestic mild cognitive impairment (aMCI) patients ($n = 13$)		Mild AD (mAD) patients ($n = 15$)		*p* Values (Tukey's posttest)		
Type	Units	Mean	SD	Mean	SD	Mean	SD	aMCI versus C	mAD versus C	aMCI versus mAD
Age	Years	71.1	5.22	72.8	3.64	78.1	4.37	0.50	<0.001	<0.01
Sex	Women (%)	77	–	70	–	80	–	–	–	–
	Men (%)	23	–	30	–	20	–	–	–	–
MMSE	Score/30	29.31	0.75	26.75	1.50	25.00	2.00	<0.05	<0.0001	<0.05
MoCA	Score/30	27.54	2.19	24.00	2.95	17.69	2.59	0.08	<0.0001	<0.0001
ApoE4	Frequency	0.35	–	0.67	–	0.39	–	–	–	–
WBC	10^9/L	5.63	0.81	5.72	1.22	6.75	1.80	0.99	0.20	0.27
Lymphocytes (ab)	10^9/L	1.72	0.36	1.62	0.38	1.90	0.66	0.92	0.72	0.48
Monocytes (ab)	10^9/L	0.38	0.08	0.47	0.13	0.60	0.16	0.36	<0.01	0.11
PMN (ab)	10^9/L	3.37	0.72	3.33	0.85	3.96	1.08	0.99	0.36	0.32
NLR	–	2.12	0.77	2.29	0.68	2.22	0.53	0.87	0.94	0.98
Hemoglobin	g/L	133.8	7.53	135.4	8.49	133.5	9.43	0.93	0.99	0.89
Total cholesterol	mmol/L	4.53	0.96	5.21	0.95	4.44	0.74	0.35	0.98	0.26
Triglycerides	mmol/L	1.72	0.79	1.72	0.79	1.71	0.50	0.99	0.99	0.99
HDL	mmol/L	1.49	0.39	1.69	0.40	1.48	0.49	0.68	0.99	0.64
LDL	mmol/L	2.41	0.56	3.01	0.66	2.27	0.60	0.20	0.90	0.10
TC/HDL	Ratio	3.13	0.58	3.22	0.70	3.50	1.18	0.98	0.68	0.83
CMV	Frequency	0.38	–	0.54	–	0.53	–	–	–	–
CRP	mg/L	1.20	1.85	0.79	1.45	0.99	1.55	0.90	0.97	0.98

Serological parameters are from the clinical laboratories of the Centre Hospitalier Universitaire de l'Université de Sherbrooke (CHUS) and are analyzed by one-way analysis of variance and Tukey's post-test. Absolute number is defined as the percentage of cells counted multiplied by the total number of white blood cells.
ab, absolute number; ApoE, apolipoprotein E; C, healthy (control) subjects; CMV Cytomegalovirus seropositivity; CRP, C-reactive protein; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MMSE, mini-mental state evaluation; MoCA, Montreal cognitive assessment; NLR, neutrophil leukocyte ratio; PMN, polymorphonuclear neutrophils; WBC, white blood cells.

Cell Purification and Culture

Human peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood (80 ml) by density gradient centrifugation over Ficoll-Paque plus medium (GE Healthcare Life Sciences, Baie d'Urfé, QC) (10, 61). PBMCs were washed three times in phosphate-buffered saline (PBS) (Wisent, St. Bruno, QC) and resuspended in culture medium consisting of RPMI 1640, 10% fetal bovine serum and penicillin G (2.5 IU/ml) and streptomycin (50 μg/ml) (Wisent). PBMC viability was assessed by FACS analysis using near-IR LIVE/DEAD fixable kit (Life Technologies, Burlington, ON, Canada).

Tregs and MDSC Analysis by FACS

Peripheral blood mononuclear cells (1×10^6 cells) were washed twice with PBS (500 μl), suspended in PBS (1 ml) and stained with a LIVE/DEAD fixable Far Red IR Dead cells kit (1 μl) (Life Technologies Thermo Fisher Scientific, Waltham, MA) for 25 min at room temperature, in the dark for viability staining (data not shown). After washing twice (PBS 200 μl), cells were fixed using a fixation buffer (BioLegend, San Diego, CA, USA) containing 1% (w/v) paraformaldehyde (PFA) for 10 min at 4°C. Cells were treated for 10 min at 4°C with PBS containing 10% FBS to reduce non-specific binding. After washing twice with PBS, cells were incubated with the relevant antibody mix for 30 min at 4°C, in the dark. The antibody mix contained: CD3 brilliant violet 510 (BV510) (BD Biosciences), CD4 brilliant violet 421 (BV421) (BD Biosciences, San Jose, CA, USA), CD8 peridinin chlorophyll protein (PerCP) (BioLegend), CD25 allophycocyanin 7 (APC-Cy7) (BD Biosciences), CD28 alexa 700 (A700) (BioLegend), and CCR4 phycoerythrin-cyanin 7 (Pe-Cy7) (BD Bioscience). Permeabilization was performed according to the supplier's instructions (eBioscience, Thermo Fisher Scientific) for Foxp3 and transcription factor permeabilization buffer kit, starting with incubation for 30 min in the dark with 200 μl of fixation and permeabilization buffer. After one wash with the permeabilization buffer (200 μl), intracellular staining with Foxp3 phycoerythrin (PE) (eBioscience) was performed for 30 min in permeabilization buffer. The last step of the staining procedure consisted in two washings with 200 μl of permeabilization buffer.

Myeloid-derived suppressor cell staining was performed on total blood freed of erythrocytes by hypotonic lysis (NH_4Cl). Samples were fixed and saturated as described in the section above, then a staining step of 30 min with the antibody mix was done. The antibody mix contained the following antibodies: lineage allophycocyanin (APC) (BioLegend), CD33 fluorescein isothiocyanate (FITC) (BD Biosciences), CD11b phycoerythrin-CF594 (Pe-CF594) (BD Biosciences), HLA-DR brilliant violet 786 (BV786) (BD Biosciences), CD15 brilliant violet (BV510) (BD Biosciences). Then, two washings with 200 μl of PBS were performed. Cells were suspended in PBS, filtered through a nylon filter cloth (70 μm mesh size, Morgans Screening & Filters Ltd., Pickering, ON, Canada) to remove cell clumps and processed for analysis. Data were acquired on a FACSAria III (BD Biosciences) instrument using the FACSDiva v. 6.1 software. Analysis was performed using the FlowJo version 7.6.1 software (TreeStar, Ashland, OR, USA). Mean fluorescence intensity refers to the geometric mean of fluorescence intensity. An APC anti-human lineage cocktail (Lin 1) consisting of fluorescence-labeled monoclonal antibodies directed globally against CD3, CD19, CD20, and CD56 (BioLegend, product # 363601) was used to exclude these cells from MDSCs analysis. Examples of the strategy for cytofluorometric analysis are shown in Figure S1 in Supplementary Material.

Cytokine Quantification in Sera

Sera were collected following centrifugation of heparinized blood (1,300 × *g*, 10 min). Samples were frozen at -80°C until the day of analysis using the Luminex technology (62). Levels of TNFα, IL-6, IL-1β, IL-10, IP-10, and IFNα in sera were quantified using a human cytokine magnetic bead assay (Milliplex® MAP Multiplex Assays; EMD Millipore, Billerica, MA, USA). Quantification was performed according to the manufacturer's instructions with a sample incubation step overnight, at 4°C. Data were acquired on a Luminex® 200TM System using the Luminex xPonent® software and analyzed using the Milliplex® Analyst 5.1 software (EMD Millipore).

Statistical Analysis

One-way analysis of variance (ANOVA) was used to test for differences among the three experimental groups. The single-step multiple comparison Tukey's test was used in conjunction with ANOVA to assess differences in the means between the three experimental groups. Data were processed using the GraphPad Prism v 6.02 software (GraphPad Software, La Jolla, CA, USA).

RESULTS

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells are immune regulatory cells of the myeloid lineage. They express CD33 but do not express HLA-DR. They are further characterized as originating from the neutrophil lineage by the expression of CD11 and CD15. We had found a complex differential change in innate cell functions (NK cells and neutrophils) in earlier studies. As a follow-up, we were interested to determine the phenotype of the common MDSC lineages and, more specifically, those derived from neutrophils since there is no data in the literature concerning the number of these cells in aMCI or mAD. Results showed a significant increase of MDSCs in the peripheral blood of aMCI subjects in contrast to healthy controls and mAD patients (**Figure 1**). For example, the number of $CD33^+HLA\text{-}DR^-$ cells in whole blood was significantly ($p < 0.05$) higher in aMCI subjects than mAD and control individuals (**Figure 1A**). In addition, the percentage of $CD33^+HLA\text{-}DR^-$ cells in whole blood was significantly ($p < 0.05$) higher in the aMCI group than the mAD and control groups (**Figure 1B**). Similar observations were made in the number (**Figure 1C**) and percentage (**Figure 1D**) of $CD33^+HLA\text{-}DR^-CD11b^+CD15^+$ cells in whole blood. Furthermore, these cells were nearly all of the neutrophil lineage. These observations clearly showed that aMCI subjects are characterized by increased levels of MDSCs, suggesting that an inflammatory condition may be downregulated in these individuals.

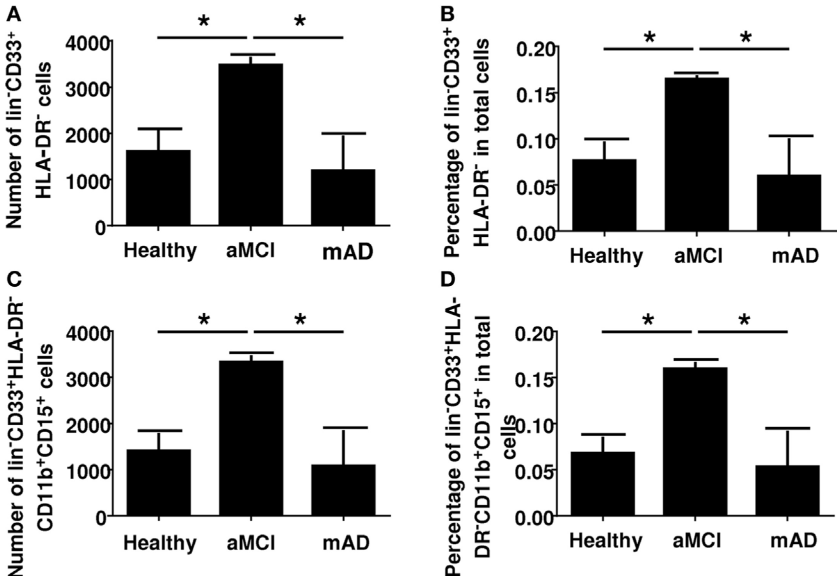

FIGURE 1 | Determination of myeloid-derived suppressor cells number and percentage in whole blood of healthy, amnestic mild cognitive impairment (aMCI), and mild AD (mAD) subjects. **(A)** Number of CD33$^+$HLA-DR$^-$ cells in whole blood. **(B)** Percentage of CD33$^+$HLA-DR$^-$ cells in whole blood. **(C)** Number of CD33$^+$HLA-DR$^-$CD11b$^+$CD15$^+$ cells in whole blood. **(D)** Percentage of CD33$^+$HLA-DR$^-$CD11b$^+$CD15$^+$ cells in whole blood. Each group was composed of five independent subjects with determinations made in triplicate. Data are shown as the mean ± SEM. The asterisks (*) correspond to $p < 0.05$.

Regulatory T Cells

Regulatory T cells are immunomodulatory cell derived from the CD4$^+$ Th1 lineage. They originate from thymus-derived CD4$^+$ T cells. They are characterized by expression of FoxP3 and CD25. Classically, Tregs express high levels of CD25, although the possibility cannot be excluded that this characteristic reflects a state of activation. Whereas Tregs suppress the hyperreactivity of the immune system by inhibiting autoimmune reactions, they also interfere with dysregulation of the effective immune response in aging. Here, results showed that the number of FoxP3$^+$ CD4$^+$ T cells was significantly increased in aMCI subjects compared to healthy controls and mAD patients (**Figures 2A,B**). However, double labeling using the additional CD25 marker showed an absence of differences between the three experimental groups (**Figures 2C,D**), suggesting that CD25 was only a marker of activation in these cells. In this connection, a newly discovered Treg subtype which is Foxp3$^+$ but CD25$^-$ has been reported to be increased in elderly subjects (48) and found here to be increased in aMCI subjects (**Figures 2E,F**).

Regulatory T cells also express other markers which may be useful in distinguishing their nature and activity. The chemokine receptor CCR4 has been reported be increased in mAD patients, potentially facilitating homing of these cells in the brain. Here, CCR4 (**Figures 3A,B**) and CD28 (**Figures 3C,D**) expression was similar in the three experimental groups. Analysis of potentially suppressive FoxP3$^+$CD8$^+$ T cells showed that expression did not differ between the three experimental groups (**Figures 4A,B**).

Circulating Cytokine Levels

Alzheimer disease may be viewed as a chronic inflammatory state with increased pro-inflammatory cytokines in circulation (63). To test the possibility that these cytokines influenced immunomodulatory activity of MDSCs and Tregs, we quantified the circulating levels of the pro-inflammatory cytokines TNFα, IL-6, IL-1β, IFNα, and IP-10. Results showed that the serum levels of TNFα and IL-6 were similar in the three experimental groups (**Figures 5A,B**). In marked contrast, the circulating levels of IL-1β were significantly higher ($p < 0.01$) in aMCI subjects than healthy controls and mAD patients (**Figure 5C**). With respect to the levels of the anti-inflammatory cytokine IL-10, data showed that these were higher in healthy subjects than in aMCI and mAD patients (**Figure 5D**). Whereas the concentration of IP-10 did not significantly differed between the three experimental groups (**Figure 5E**), it was significantly ($p < 0.01$) higher in healthy controls than in aMCI and mAD patients (**Figure 5F**). The bulk of these observations suggested that IL-1β, an important pro-inflammatory cytokine resulting from the inflammasome stimulation *via* pathogen recognition receptor stimulation, is specifically increased in aMCI. Furthermore, this observation suggested that IL-1β may play a beneficial role in aMCI but its increased production could also detrimental as it has been reported at the AD stage. In this connection, it has been shown that IL-1β specifically impairs microglial clearance of Aβ in AD (64, 65).

We stratified the subjects according to CMV-seropostivity. It has been reported that latent infection with this herpesvirus influences several peripheral immune parameters (66). There was nearly the same number of subjects CMV$^+$ or CMV$^-$ in each group. Data revealed significant ($p < 0.01$) increases in the levels of pro-inflammatory cytokines TNFα (**Figure 6A**) and IL-6 (**Figure 6B**) in CMV-positive aMCI and mAD subjects. However, levels of pro-inflammatory cytokine IL-1β were significantly

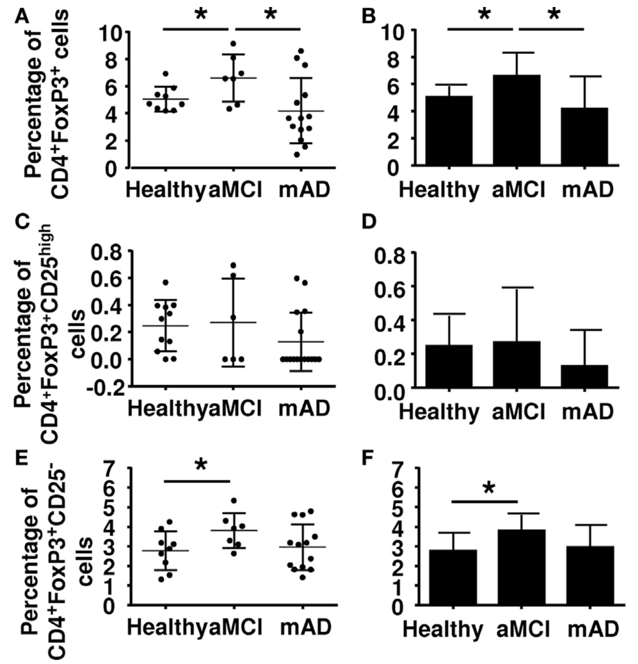

FIGURE 2 | Determination of the percentage of regulatory T cells (Tregs) in peripheral blood mononuclear cell of healthy, amnestic mild cognitive impairment (aMCI), and mild AD (mAD) subjects. **(A)** Tregs percentages defined by the $CD4^+FoxP3^+$ markers in individual participants. **(B)** Corresponding histograms. **(C)** Tregs percentages defined by the $CD4^+Foxp3^+CD25^{high}$ markers in individual participant. **(D)** Corresponding histograms. **(E)** T regs percentages defined by $CD4^+Foxp3^+CD25^-$ markers in individual participants. **(F)** Corresponding histograms. Each group was composed of at least five independent subjects with determinations made in triplicate. Data are shown as the mean ± SEM. The asterisks (*) correspond to $p < 0.05$.

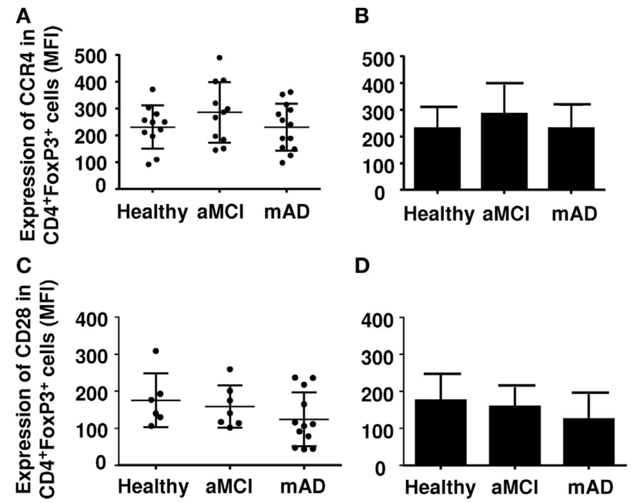

FIGURE 3 | Expression of CCR4 and CD28 on regulatory T cells (Tregs) of healthy, amnestic mild cognitive impairment (aMCI), and mild AD (mAD) subjects. **(A)** CCR4 number in Tregs defined by the $CD4^+FoxP3^+$ marker in individual participants. **(B)** Corresponding histograms. **(C)** CD28 number in Tregs defined by the $CD4^+Foxp3^+CD25^{high}$ marker in individual participants. **(D)** Corresponding histograms. Each group was composed of at least five independent subjects with determinations made in triplicate. Data are shown as the mean ± SEM.

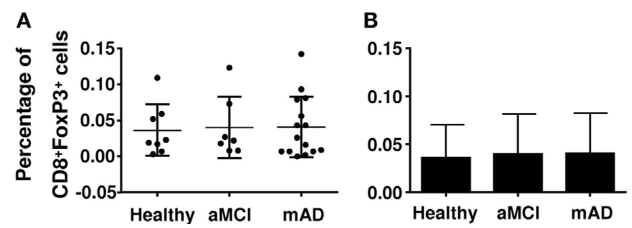

FIGURE 4 | Quantification of the percentage of $CD8^+$ regulatory T cells (Tregs) in healthy, amnestic mild cognitive impairment (aMCI), and mild AD (mAD) subjects. **(A)** Percentage of $CD8^+FoxP3^+$ Tregs in individual participants. **(B)** Corresponding histograms. Each group was composed of at least five independent subjects with determinations made in triplicate. Data are shown as the mean ± SEM.

higher ($p < 0.01$) only in the case of CMV-positive aMCI subjects (**Figure 6C**). In contrast, the serum concentration of IL-10 was significantly ($p < 0.01$) elevated only in the case of CMV-positive mAD patients (**Figure 6D**). The levels of IP-10 were not influenced by CMV serostatus (**Figure 6E**). Whereas the levels of IFNα were lower in CMV-positive healthy subjects, they were significantly ($p < 0.01$) higher in CMV-positive aMCI subjects but similar in mAD patients (**Figure 6F**). Overall, the bulk of the results suggested that CMV seropositivity influenced the production of pro- and anti-inflammatory cytokines in aMCI and mAD patients.

DISCUSSION

Although the cause(s) of the AD remains controversial, several immune-related alterations have been documented (9–11). The contribution of neuroinflammation to the pathogenesis of the disease has been acknowledged for some time, although the trigger(s) that sustains a state of inflammation is still uncertain (63). Recent observations have put forward convincing evidence that amyloid beta peptides possess antimicrobial activity (12–14, 67–69), suggesting that AD may have an infectious origin (69). In addition, there are data regarding the role of effector cell functions in AD (11, 70) but, to the best of our knowledge, the frequency of circulating MDSCs has not been reported. Here, we found an increase in total MDSCs, especially in the neutrophilic subset (PMN-MDSCs) in the aMCI group but not in the mAD and healthy groups (**Figure 1**). It is of note that the number of monocytic MDSCs were similar in the three experimental groups (data not shown). In these cases, percentages of monocytic MDSCs in whole blood or in PBMCs were similar between the three experimental groups, as reported by others in whole blood or PBMCs (16). It is of note that an increase in the number of MDSCs has been reported in peripheral blood of healthy aged and frail elderly patients (28). In many cancer patients, similar increases in MDSCs have been observed and their number correlated with the clinical

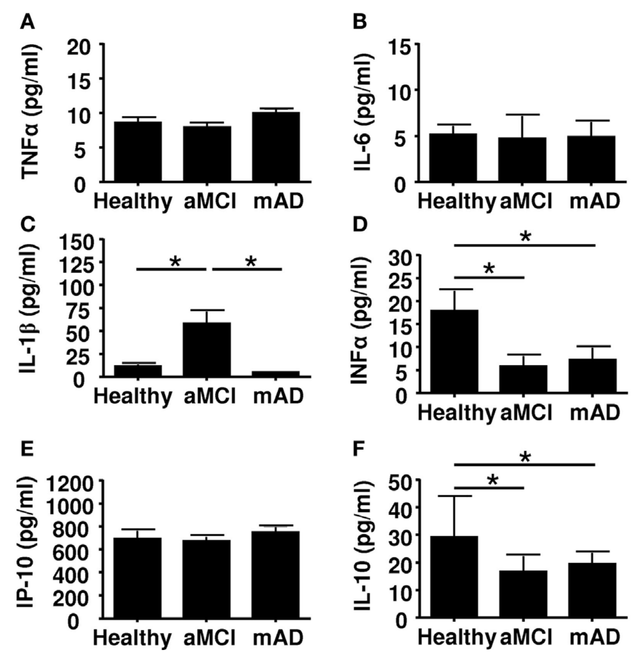

FIGURE 5 | Quantification of cytokine concentrations in sera of healthy, amnestic mild cognitive impairment (aMCI), and mild AD (mAD) subjects. **(A)** TNFα. **(B)** IL-6. **(C)** IL-1β. **(D)** Il-10. **(E)** IP-10. **(F)** IFNα. Each group was composed of at least 10 independent subjects with determinations made in triplicate. Cytokine quantification was done using the Luminex technology. Data are shown as the mean ± SEM. The asterisks (*) correspond to $p < 0.01$.

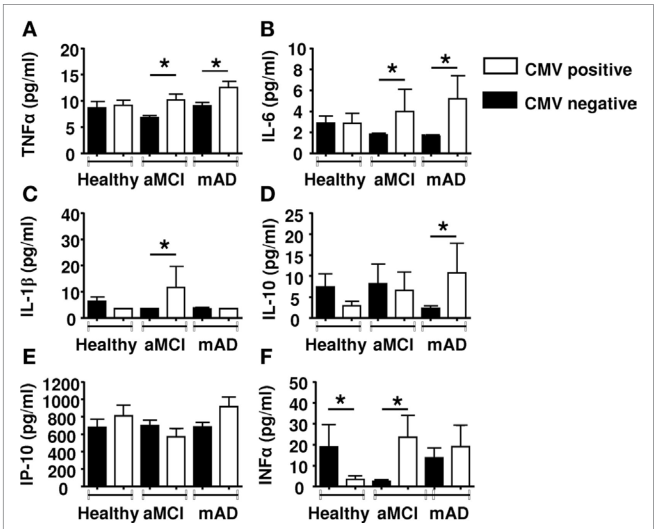

FIGURE 6 | Quantification of cytokine concentrations in sera according to CMV serostatus. **(A)** TNFα. **(B)** IL-6. **(C)** IL-1β. **(D)** Il-10. **(E)** IP-10. **(F)** IFNα. Each group (healthy controls, amnestic mild cognitive impairment [aMCI], and mild AD [mAD]) was composed of at least 10 independent subjects with determinations made in triplicate. Cytokine quantification was done using the Luminex technology. Data are shown as the mean ± SEM. The asterisks (*) corresponds to $p < 0.01$. Empty rectangle, CMV-positive cells, filled rectangle, CMV-negative cells.

outcomes as well as response to immunotherapy (68, 69, 71, 72). Similar increases have been reported in various bacterial and viral infections, mainly in the maintenance of chronicity (31). In these settings, the increased differentiation of these cells was correlated with increased levels of circulating pro-inflammatory cytokines (39). Here, we found only an increase in the levels of IL-1β in aMCI patients which may partly explain these observations, since IL-1 may mediate its effects by stimulating NO production as a result of increased iNOS expression (73). Our findings correlate with those that showed that IL-1β was increased in the AD brain as well as after Aβ stimulation and has a clinical stage dependent effect however mainly described as mediating neurotoxicity (74, 75). Furthermore, Il-1β has been shown to induce MDCSs accumulation and differentiation (16, 33, 76). It is also of interest, as infection was raised as a possible cause of AD, immunocompetent individuals rarely develop pro-inflammatory antifungal immune responses because as among many other pro-inflammatory IL1-β mediates MDSCs recruitment and modulate antifungal immune response (77). It is of note that no other pro-inflammatory cytokines were found to be increased in any of the experimental groups (**Figure 5**). The anti-inflammatory cytokine effect could not be established here, but may not be sufficient to compensate for the increase of IL-1β. We investigated the influence of CMV serostatus on cytokine production. We observed an upregulated production of pro-inflammatory cytokines (TNFα, IL-6, and IL-1β) in the aMCI and mAD groups. These observations suggested that CMV serostatus did not allow a distinction regarding production of pro-inflammatory cytokines between the two clinical stages of the disease studied here.

We observed an increase in Tregs in the aMCI group compared to healthy and mAD subjects (**Figure 2**). These observations are in agreement with the report of higher frequency of Tregs in elderly individuals and suppressive activity in neurodegeneration (55). However, when analysis of expression of the CD25 surface marker was done as a marker of $CD4^+FoxP3^+CD25^{high}$ in the antibody staining panel, results showed the absence of significant differences among the three experimental groups. This observation suggested that CD25 is an activation marker which cannot further distinguish between Tregs subpopulations. Interestingly, the newly characterized CD25-negative Treg subpopulation has been reported to be increased in aMCI subjects (48, 78). Of interest, this Treg subpopulation has also been found to be increased in SLE and in aging mice and shown to correlate with decreased T cell responses (78). Here, the expression of the surface markers CD28, CCR4 was found to be similar between the three groups of subjects. Together, the differences reported between aMCI and mAD patients concerning Tregs confirmed previous data with respect to the increased Treg specifically in aMCI (54). In this report, the authors further showed that in aMCI subjects, the increase in Tregs numbers was associated with upregulated suppressive activity toward T cell functions, which was decreased in AD patients. In the present study, we could not investigate the functions of Tregs because of an insufficient number of cells from each donor. This hurdle prevented us investigate, at this time, the influence of Tregs increase in aMCI subjects. However, it is to be noted that the increase of MDSCs was paralleled by the increase of Tregs as well.

There is no clear functional explanation for the increased number of MDSCs in aMCI subjects and the decrease in mAD patients. However, in the case of autoimmune diseases, their number has been reported to be increased, presumably as a mean to compensate for uncontrolled immune responses (36). This behavior can be a self-regulatory mechanism whereby a chronic inflammatory response induces the increase of immunoregulatory cells in an attempt to downregulate the hyper-response, even if an effector immunoparalysis appears concomitantly (11, 79). In cases of aMCI where there is still a strong inflammatory response, as demonstrated for NK cells and neutrophils, the increase in MDSCs may appear to be a compensatory mechanism. However, this situation may create a vicious cycle resulting in the increase of inflammation, along with progression of the disease toward AD where the immune response is already less inflammatory (9, 11). This possibility has been put forward in the case of Tregs by Saresella et al. (54). These authors have suggested that the increase in Tregs in aMCI subjects occurs as a mean to decrease uncontrolled inflammation, whereas the decrease in AD favors uncontrolled progression of the disease. Our recent studies on the immune response in aging and in various stress situations of elderly also support this hypothesis (79). However, an alternative explanation cannot be excluded, namely, that inflammation in aMCI subjects is still a necessary process to retard the deleterious effect of progression toward AD as was proposed in a murine model of AD (53). If this were the case, the involvement of MDSCs and Tregs may be harmful. More investigations are obviously needed to explore the mutual balanced effects of MDSCs and Tregs on the intrinsic and extrinsic disease environment. Until this question is resolved, all tentative efforts to modulate these cells in aMCI may be counterproductive or even dangerous.

AUTHOR CONTRIBUTIONS

TF participated in the recruitment of subjects, the conceptualization, the interpretation of the data, and writing of the paper. ALP has realized the experiments, analyzed the data and participated in the writing of the manuscript. HG has made the cytokine measurements by Luminex and participated in the analysis of the data. EF, AL, GD, JW, and GP have participated in the conceptualization, the interpretation of the data, and writing of the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00783/full#supplementary-material.

FIGURE S1 | Gating strategy used for analysis of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) by cytofluorometry. Far Red is a dye used to stain live cells (see Section "Materials and Methods" in the main text). Lin refers to an APC anti-human lineage cocktail (Lin 1) consisting of fluorescence-labeled monoclonal antibodies directed globally against CD3, CD19, CD20 and CD56 (see Section "Materials and Methods" in the main text). Abbreviations are: FSC, forward side scatter; SSC, size side scatter.

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REVIEW
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Role of Dendritic Cells in Inflammation and Loss of Tolerance in the Elderly

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Dendritic cells (DCs) play an important role in advancing age-associated progressive decline in adaptive immune responses, loss of tolerance, and development of chronic inflammation. In aged humans, DCs secrete increased levels of pro-inflammatory cytokines and decreased levels of anti-inflammatory and immune-regulatory cytokines. This may contribute to both chronic inflammation and loss of tolerance in aging. Aged DCs also display increased immune response against self-antigens contributing further to both inflammation and loss of tolerance. The secretion of innate protective cytokines such as type I and III interferons is decreased, and the function of DCs in airway remodeling and inflammation in aged is also compromised. Furthermore, the capacity of DCs to prime T cell responses also seems to be affected. Collectively, these changes in DC functions contribute to the immune dysfunction and inflammation in the elderly. This review only focuses on age-associated changes in DC function in humans.

Keywords: dendritic cells, inflammation, tolerance, aging, mucosa

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INTRODUCTION

Medical and technological advances have significantly enhanced the life expectancy of the human population (1–3). As a result, age expectancy has increased but is accompanied by a substantial increase in age-related diseases including cardiovascular, neurodegenerative disorders, infections, autoimmunity, diabetes, and cancers. Age-related immune dysfunction contributes to the increased incidence of these diseases due to impaired surveillance, repair, and regulation (4, 5). Advanced age affects the functions of both the innate and the adaptive arms of the immune system. Changes in adaptive immune T and B cells are more apparent as there is decline in cell numbers as well as functions (6). In contrast, functions of innate immune cells, such as dendritic cells (DCs), macrophages do not display major numerical or phenotypic changes; however, significant changes in regulation of the immune responses by these cells exist (7). DCs are well established as the most effective of the antigen-presenting cells (APCs) (8). They express high levels of the molecules that are required for antigen presentation such as the MHC II, CD80, and CD86 on activation (9, 10). DCs are thus highly effective in initiating an immune response (11). DCs are distributed throughout the body, including the mucosal tissues, where they are found below the epithelial cell barrier. DCs present at the mucosal sites, and in tissues, survey for external and internal danger signals using an array of pattern recognition receptors such as the toll-like receptor (TLRs), C-type lectin receptors, NOD-like receptors (NLRs), and others (12). These receptors can sense not only external infectious and environmental antigens but are also capable of responding to internal danger signals and molecules generated during tissue injury or malfunctioning of any of the other processes in the body (13).

Following uptake of antigens *via* the PRRs, DCs are activated and migrate to the lymph nodes to present antigens to the T cells, and initiate an adaptive immune response. In contrast, presentation of antigens, particularly self-antigens to T cells by unactivated DC prevents T cell activation and induces tolerance (14). DCs, thus play, a dual role that of generating immunity against danger signals and preventing immunity against self. Since increased susceptibility to infections as well as increased reactivity to self is a characteristic of aging, aberrant DC function can play a major role in age-related disorders. This review therefore focuses on the changes in human DC functions in the aged population.

DC NUMBERS AND PHENOTYPE

Dendritic cells can be divided into two major subclasses (1) plasmacytoid DCs (pDCs), which are of lymphoid origin and express B plasma cell markers; (2) myeloid DCs (mDCs) that are derived from myeloid progenitors (15). Hematopoiesis in aging is characterized by decrease in lymphoid cells with skewing toward the myeloid lineages (16, 17). In keeping with this, a decrease in pDC numbers in circulation has been observed in the aged population (18–20). Myeloid DC numbers have been reported to be largely unchanged in circulation (18, 20–22). Recent data from DC field have led to the further division of myeloid DC into several subsets (15). However, only two of these are present in the circulation, the $CD1c^+$ and the $CD141^+$ mDC subsets. We have observed a decrease in the $CD141^+$ mDC subset in the circulation of the aged subjects; however, the number of $CD1c^+$ mDCs was not affected with age (23). Monocyte-derived DCs (MoDCs) represent a third subset of DCs. MoDCs have similar functions as myeloid DCs in circulation though recent genomic studies suggest that these two populations are significantly different at the transcriptome level (24). The MoDCs numbers are also stable with age (21). The phenotype of the DCs is also reported to be largely unchanged though most of the data are from MoDCs (21, 25–27), and very few studies have examined the phenotype of pDCs and mDCs (18, 20, 22).

Information regarding the age-associated changes in tissue DCs is scarce due to the difficulty in obtaining human samples. Nevertheless, a very recent and comprehensive study by Granot et al. (28) has examined the $CD1c^+$ and $CD141^+$ mDC distribution, activation, and migration in various organs including the lung and the intestine and associated lymph nodes. The samples were from 78 organ donors of various ethnicities with ages ranging from infants to 93-year-old adults. The authors did not observe significant changes in DC subset frequencies throughout life in most tissues. They did observe a trend toward increased maturation of DCs particularly $CD1c^+$ DC subset in the lung, mesenteric, and inguinal lymph nodes indicating increased activation and migration of DCs with age.

PATHOGEN SENSING AND RESPONSE OF DCs

A hallmark of advancing age is an increased susceptibility to acute viral and bacterial infections. These infections are often more severe and prolonged, with a higher mortality rate among older adults (29). One of the primary functions of DCs as a part of the innate immune system is to sense and respond to external pathogenic stimuli *via* PRRs such as TLRs. The function of TLRs in aged subjects has been reported to be defective in both mDC and pDC subsets (18).

Among the DC subsets, pDCs play a major role in controlling viral infections *via* secretion of large amounts of type I interferons early in the immune response (30). A decrease in IFN secretion by pDCs from elderly in response to TLR7 and TLR9 ligands as well as to different viruses including influenza has been reported (18, 20, 31, 32). The data on the expression of TLR7 in pDC from aged subjects are rather conflicting; both decreased and normal expression has been reported (18); expression of TLR9 in pDC in elderly is comparable to young subjects. Impaired functions of pDCs are considered a major factor in increased susceptibility of elderly to viral infections.

In addition to pDCs, mDCs also play a major role in defense against microbes. mDCs in circulation express a wide array of PRRs including TLRs. Few studies have examined the expression and functions of TLRs in mDC in humans. Panda et al. (18) have performed an extensive analysis of the intracellular cytokine secretion by mDCs in response to multiple TLR ligands including TLR1/2, TLR2/6, TLR3, TLR4, TLR5, and TLR8. They observed a significant decrease in the production of TNF-α, IL-6, and IL-12p40 against almost all ligands tested. The decrease was consistent and was there even when the subjects were retested after an interval of 2 years. The decrease was attributed in part to reduced expression of TLR1, TLR3, and TLR8. TLR2 and TLR4 expression was reported to be unaltered. Della Bella et al. (33) have also reported a decreased IL-12 production by DCs from peripheral blood mononuclear cells from aged subjects in response to TLR4 ligand as compared to young individuals. There are no studies with purified mDCs probably due to very small number of circulating mDCs.

In contrast to mDCs in circulation, we have observed that MoDCs display increased secretion of pro-inflammatory cytokines TNF-α, CXCL-10, and IL-6 in response to TLR4 ligand, LPS (21). We also observed similar increase in these mediators in response to *Chlamydophila pneumoniae* (34). This increase was attributed to decrease in signaling *via* the PI3kinase/Akt pathway, which functions as a negative regulator of TLR signaling. Increased PTEN expression in DCs from aged subjects was found to be responsible for the deficient function of AKT (21). Most remarkably, we also observed a significant age-associated decrease in the secretion of the anti-inflammatory cytokine, IL-10, which is required to regulate inflammation. This defect in IL-10 secretion by DCs from aged subjects was a consequence of an inherent defect in DCs as addition of IL-10-inducing agents such as lithium chloride was unable to restore the production of IL-10 in DCs from aged subjects (35). This decrease in IL-10 production may also contribute to loss of tolerance in aging. In addition to impaired IL-10 production, DCs from aged subjects also displayed a defect in the production of type I and III interferons in response to both influenza and *Chlamydophila* (34, 36). The decrease in type I IFN production in older donors was also observed in MoDCs infected with West Nile virus (32). Interferons, both type I and III, are essential for blocking viral replication and preventing spread of infection (37, 38). In addition, the interferons also act on various cells to induce an antiviral state. Though the actions of both interferons are similar, type III interferons play a more important role in protection against infections of the mucosal surfaces. This is because the receptors for type I interferons are present on nearly all cells of the body, while type III display a more restricted pattern of expression with the receptors being present mainly on mucosal tissues (38). Type III IFNs thus contribute to the control of viral infections in epithelial cells of both respiratory and gastrointestinal tracts. Together, MoDCs from aged subjects display a selective deficiency in anti-inflammatory and protective cytokines accompanied with an increase in pro-inflammatory cytokines and chemokines (**Figure 1**).

DCs AND IMPAIRED VACCINE RESPONSES IN THE ELDERLY

Vaccine responses in the elderly are also compromised (39, 40). Reduced responses to influenza vaccines are well documented (40). Elderly display deficiency in mounting efficient immune response to new antigens or vaccines, but their capacity to generate recall responses to previously primed antigens is relatively better preserved (41). The underlying mechanisms are not well understood; in particular, the possible contribution of age-associated DC dysfunction has not been examined in humans. Successful response to vaccines requires presentation of the antigens by DCs as well as costimulation (42). DCs are important not only for the initiation of T cell responses but also for the generation of efficient effector and memory responses (43–47). Reduced phagocytosis as well as impaired migration of DCs from aged subjects may be one of the factors for the decreased vaccine responses (21). Furthermore, DCs also need to upregulate class I and II MHC molecules as well as costimulatory molecules, CD40, CD80 as well as CD86 for efficient antigen presentation. The response of human monocyte-derived DCs to TLR ligands indicates that the capacity of DCs from elderly to upregulate MHC and costimulatory molecules is not impaired (21, 25, 26). This is in contrast to murine DCs where reduced expression of CD80, CD86, and MHC II has been observed in DCs before and after infection (48–50). The discrepancy could be due to

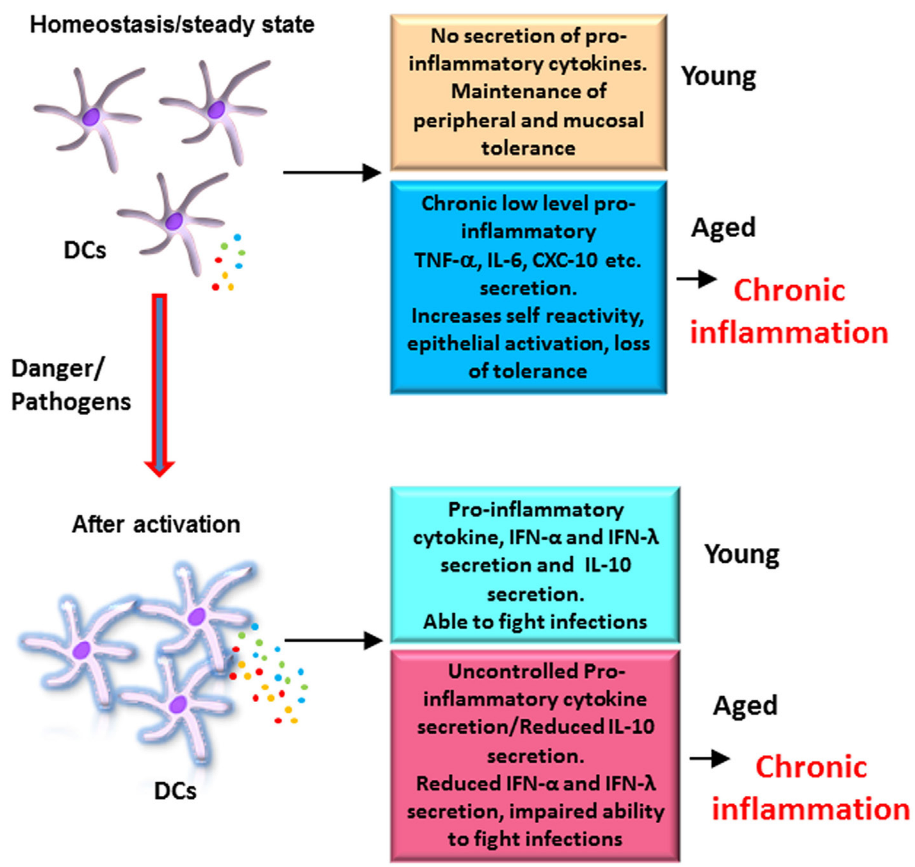

FIGURE 1 | Altered functions of dendritic cells (DCs) from elderly contribute to chronic inflammation: DCs from elderly display an enhanced basal level of activation, which increases their reactivity to self-antigens, affects the function of epithelial barrier, and results in erosion of peripheral and mucosal tolerance at homeostasis. After activation with pathogens, DCs from elderly secrete enhanced levels of pro-inflammatory cytokines, which are not regulated as the secretion of anti-inflammatory cytokine IL-10 is impaired. This also contributes to inflammation. In addition, secretion of protective cytokines such as the IFN-α and IFN-λ is also decreased resulting in a decrease in the ability of elderly to fight infections. Figure depicts the differences in the response of DCs from aged and young subjects at homeostasis and after activation.

difference in species. However, most of the information is derived from MoDCs, and the expression of costimulatory molecules on human DC subsets in circulation or in tissues after stimulation/infection has not been investigated. It is possible that these DCs may behave differently compared to MoDCs. MoDCs from young and aged subjects have also been reported to have similar stimulatory capacity to induce proliferation of T cell lines developed in long-term cultures (26). However, during respiratory syncytial virus infection, decreased IFN-γ-producing cells were observed in response to MoDCs from old individuals (51). We have observed increased proliferation as well as IFN-γ secretion by T cells primed with MoDC from older subjects in the absence of stimulation supporting the enhanced activated state of DCs in the elderly (34). However, MoDCs from aged subjects were not as efficient as MoDCs from young subjects in enhancing the T cell proliferation and IFN-γ secretion after stimulation (34). In another study also, elderly adults displayed a reduced ability to prime antigen-specific $CD8^+$ T cells (52). Furthermore, the expression of perforin and granzyme B was also reduced in the primed $CD8^+$ T cells. Reduced T cell proliferation has also been reported in another study where TLR agonist-stimulated PBMCs (minus CD3 T cells) from older subjects' induced lower proliferation of allogeneic adult T cells compared to stimulated PBMCs from adult subjects (22). In both these studies, reduced priming by DCs was believed to be one of the factors for the observed decreased activation of T cells, though direct priming of T cells by DC was not performed. In addition to MoDCs, we also observed a reduction in the capacity of pDCs from elderly to prime CD4 and CD8 T cell responses after stimulation with influenza (31). Altogether, these studies suggest that DCs from elderly display a deficiency in priming naïve T cell responses after stimulation. Preexisting inflammation in aged subjects could be one of the possible mechanisms, which modifies DC responses and reduces their capacity to prime T cells. This is supported by a recent clinical trial using an mTOR inhibitor, RAD001, which reduces inflammation. Treatment of older subjects with RAD001 prior to influenza vaccination was demonstrated to decrease the percentage of PD-1-positive CD4 and CD8 T cells compared to placebo and enhance the response to influenza vaccination (53). Enhanced basal level activation was also demonstrated to be responsible for the reduced response to yellow fever vaccine of African subjects compared to European subjects (54). Strategies to reduce inflammation prior to vaccination may therefore prove useful in enhancing vaccine responses in the elderly.

Emerging evidence indicates that different subsets of DCs may display differential ability to prime CD4 and CD8 T cell responses. For example, CD1c DC subset is believed to express high levels of molecules such as Ifi30 (GILT), HLA-DMA, and cathepsin H, which renders them more efficient in priming CD4 T lymphocyte responses (55). On the other hand, $CD141^+$ mDC subset possesses a superior capacity to cross-present antigens to CD8 T lymphocytes (56, 57). We have observed reduced percentages of $CD141^+$ mDC subset in the circulation of the elderly, but the alterations in DC subsets in tissues have not been studied (23). In this regard, a recent study by Yu et al. (58) has used a human $CD34^+$ hematopoietic progenitor cells reconstituted immunodeficient mice model to examine the $CD8^+$ T cell priming capacity of human respiratory $CD1c^+$ and $CD141^+$ DCs against intranasal live-attenuated influenza virus vaccination. Their results indicate that both DC subsets were efficient at activating antiviral $CD8^+$ T cell responses against influenza. Nevertheless, lung $CD1c^+$ DCs induced the expression of CD103 on the CD8 T cells, which allowed their retention in the lung epithelium and generate tissue-resident memory cells. Antigen-presenting capacity of different DC subsets in the elderly thus needs to be examined not only because of their capacity to prime different T lymphocyte subset but also since emerging evidence indicates different vaccines activate different DC subsets, and this differential activation is required for efficient adaptive immune responses (59). Fluzone was demonstrated to primarily activate MoDCs, while pneumovax activated monocytes. In contrast, Gardasil induced the activation of $CD1c^+$ blood DCs (59). The specialization of APCs in response to different vaccines will have to be kept in mind when designing vaccines for the elderly. The response of different DC subsets to vaccine antigens and adjuvants may be determined to obtain information about the induction of adaptive immunity. For example, to improve vaccine response to fluzone in the elderly, it may be beneficial to design adjuvants that activate monocytes. In addition to the above parameters, the strength and duration of antigen priming by DCs also affects memory generation. A recent murine study with vaccinia virus demonstrated that cross presentation of antigens by DCs to TCR-identical cells leads to generation of tissue-resident memory $CD8^+$ T cells versus the circulating memory cells (60). Studies examining the generation of tissue-resident memory in human subjects are not feasible due to ethical considerations. However, use of reconstituted humanized mice as described above (58) may be one of the approaches to examine tissue-specific human responses. Another approach is the emerging field of systems vaccinology (61), which may provide insight into the transcriptional and epigenetic signatures that could be predictive of reduced vaccine responsiveness in the elderly and may help design strategies to overcome the deficiencies.

DCs AND DAMAGE-ASSOCIATED MOLECULAR PATTERNS (DAMPs)

In contrast to exogenous danger threats, there is not much known about the response of DCs from aged subjects to endogenous, DAMPs. DAMPs are released upon cell or tissue damage, and enhanced tissue damage is a characteristic of aging. The inflammasome pathway plays a major role in recognizing a wide array of DAMPS including cholesterol crystals, uric acid crystals, extracellular ATP, amyloid-beta, and ceramides and lipids (62, 63), many of these molecules accumulate during aging (64). Studies in mice have linked impaired glucose tolerance and cognitive decline to enhanced expression of NLRP3 inflammasome pathway during aging (65). The increased TNF-α levels in aged mice were reported to enhance the expression of NLRP3 inflammasome in adipose tissue and liver, which results in impaired glucose tolerance. A recent study (66) in humans has demonstrated a direct correlation between specific inflammasome expression modules and age-related diseases such as hypertension, as well as with diminished longevity. The study identified two metabolites, adenine and N^4-acetylcytidine, which prime and/or activate the NLRC4 inflammasome and induce hypertension and inflammatory signatures. These studies indicate an enhanced activity of the inflammasome pathway during aging; however, these changes have not been determined in DCs. Aging also leads to changes/loss in protein homeostasis, proteostasis due to intracellular damage, which contributes to the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (67). It would be interesting to determine the response of DCs from healthy aged subjects to some of the pathological proteins such as amyloid-beta and compare it to response of DCs from AD patients.

DCs AND TOLERANCE

Maintenance of tolerance against self-antigens is another primary function of DCs (68). DCs are constantly being exposed to self-antigens generated during cell death, tissue injury, etc. Under steady-state conditions, DCs in the periphery uptake these antigens but do not get activated. Presentation of self-antigens to T cells in the absence of costimulatory molecules or activation signals leads to T cell tolerance *via* T cell anergy or induction of T regulatory cells. However, if the environment is inflamed and the DCs are activated, they may present self-antigens to T cells to generate an immune response against self-antigens. This can lead to autoimmunity and inflammation. Since our group and others (18, 69) have observed that DCs from aged subjects secrete low basal levels of pro-inflammatory cytokines, it is suggested that the DCs are activated. Indeed, we observed increased basal level of NF-κB activation in DCs from aged subjects (70). Furthermore, we also found increased reactivity of DCs from aged subjects to self-DNA. Self-DNA is released when apoptotic cells' clearance is defective and cells undergo secondary necrosis. In keeping with this, DCs from aged subjects exhibited reduced uptake of apoptotic cells (70). The increased immune response instead of tolerance to self-antigens contributes to the inflammation during aging (**Figure 1**).

In addition to peripheral tolerance, DCs also play a role in maintaining tolerance at mucosal surfaces (71). The lung and gut mucosa are constantly exposed to harmless and innocuous antigens in the form of particles from inhaled air and food. Furthermore, the commensal microbial communities present in the oral and gastrointestinal mucosa are essential for human health and thus an immune response against them would be detrimental. Significant progress has been made in the last few years identifying DCs as critical mediators of tolerance induction at these surfaces. The epithelial cells in the mucosa secrete factors such as retinoic acid and TGF-β, which act upon DCs to induce tolerance to prevent response against harmless antigens and commensal microbiome (71–73). Our studies suggest that DCs from aged subjects display impaired response to retinoic acid and are deficient in inducing T regulatory cells for tolerance (23). The infections of the respiratory mucosa such as the influenza and *Chlamydia pneumoniae* are more prevalent and severe in the elderly (74). The older population is also more susceptible to bronchitis, asthma, COPD, and emphysema (74–76). Reduced capacity of DCs from aged subjects to maintain tolerance in the airways may enhance inflammation and invasion by pathogens due to impaired remodeling of the airways. Furthermore, we have also demonstrated that the basal level of activation of DCs from aged subjects leads to low-level secretion of pro-inflammatory cytokines, which activates the epithelium even in the absence of infection (69). Exposure of airway epithelial cells to supernatants from unstimulated DCs from aged subjects, but not young subjects, led to an increase in permeability of the epithelial barrier, which was accompanied with secretion of chemokines and upregulation of activation molecules. Therefore, DCs from aged subjects are not only defective in their response to tolerogenic signals from epithelial cells but also act on the epithelium to compromise its barrier functions.

Although studies were performed with airway epithelial cells and DCs, a similar process may be occurring at the level of gut and skin. Infections of both these surfaces are also more common in the elderly (77). For example, *Helicobacter pylori* and *Clostridium difficile* infections of the gut are often more severe and result in hospitalization of the elderly (78). It is also well established that with advancing age there are significant changes in the composition of gut microbiota with increase in Gram-negative bacteria, like *Enterobacteriaceae* and other pathogens (79). These Gram-negative bacteria secrete lipopolysaccharides, and we have previously reported that inflammatory response to LPS is enhanced with age (21). In addition, the level of short-chain fatty acids (SCFAs), such as acetate, butyrate, and propionate, are also reduced in the intestine of aged subjects as compared to young subjects (80). SCFAs synthesized by gut microbiota can act on DCs to prevent their activation and enhance their capacity to induce T regulatory cells to maintain tolerance in the gut (81). Enhanced inflammation in the gut increases the susceptibility of the elderly to gastrointestinal infections. Similar to gut infections of the skin including viral infections like herpes zoster (shingles), pressure ulcers, bacterial, or fungal infections, methicillin-resistant *Staphylococcus aureus* are prevalent in the elderly. Aberrant functions of DCs at the mucosal surfaces may account for the increased mucosal infections observed in the elderly.

POTENTIAL MECHANISMS RESPONSIBLE FOR DC DYSFUNCTION IN THE ELDERLY

The above studies highlight the age-associated alterations in DC functions; however, the mechanisms responsible for the changes are not well understood. Changes in signaling mechanisms such as enhanced basal level activation of NF-κB are thought to be responsible for the increased inflammatory responses of the DCs from the elderly. This has been observed both in MoDCs and circulatory DCs (18, 21, 70). Nevertheless, it is still not defined whether the changes are due to an intrinsic defect in DCs or external senescent microenvironment drives the changes. Evidence regarding both mechanisms is present. The age-associated increased circulatory pro-inflammatory mediators such as TNF-α, prostaglandins can cause DCs to mature and secrete pro-inflammatory cytokines at homeostasis. This process can start during DC differentiation in the bone marrow since the fat content in bone marrow has been shown to increase with age (82), and adipocytes are major producers of pro-inflammatory cytokines (83). DC intrinsic mechanisms such as epigenetic changes including chromatin and methylation alterations or changes in micro RNA may also account for observed changes in DC function. We have observed increased binding of type I and type III IFN promoters to inhibitory histone, H3K9 (36), but studies comparing the chromatin accessible elements at a global level have not been performed in DCs from aged and young subjects. The inability to obtain sufficient number of DCs from the blood of the elderly has been a major hindrance. The advent of novel next generation techniques such as ATAC-seq (84) as well as single-cell sequencing (85) may enhance the feasibility of performing such studies. Similarly, changes in DNA methylation in T lymphocytes have been well documented in aging (86). However, methylation changes in DCs have not been studied in aged subjects. This is an area of importance for future studies as methylation has a significant impact on gene function (87). Studies focused on age-associated alterations in the expression of long non-coding RNAs (88) are also of potential importance to understand the mechanisms underlying DC dysfunction in the elderly.

CONCLUSION

In summary, aging impacts DC functions in multiple ways. The enhanced activated state of DCs from elderly leads to the erosion of peripheral and mucosal tolerance and induction of inflammation. The secretion of inflammatory cytokines by activated DCs is also uncontrolled due to a defect in IL-10 secretion. In addition, the secretion of type I and III interferons is compromised, which enhances the susceptibility of the elderly to viral and bacterial infections. More studies are required to understand the effect of age on various DC subsets particularly in the tissues.

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Functional and Homeostatic Impact of Age-Related Changes in Lymph Node Stroma

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Adults over 65 years of age are more vulnerable to infectious disease and show poor responses to vaccination relative to those under 50. A complex set of age-related changes in the immune system is believed to be largely responsible for these defects. These changes, collectively termed immune senescence, encompass alterations in both the innate and adaptive immune systems, in the microenvironments where immune cells develop or reside, and in soluble factors that guide immune homeostasis and function. While age-related changes in primary lymphoid organs (bone marrow, and, in particular, the thymus, which involutes in the first third of life) have been long appreciated, changes affecting aging secondary lymphoid organs, and, in particular, aging lymph nodes (LNs) have been less well characterized. Over the last 20 years, LN stromal cells have emerged as key players in maintaining LN morphology and immune homeostasis, as well as in coordinating immune responses to pathogens. Here, we review recent progress in understanding the contributions of LN stromal cells to immune senescence. We discuss approaches to understand the mechanisms behind the decline in LN stromal cells and conclude by considering potential strategies to rejuvenate aging LN stroma to improve immune homeostasis, immune responses, and vaccine efficacy in the elderly.

Keywords: aging, immunity, lymph nodes, fibroblastic reticular cells, lymphatic endothelial cells, naïve T cells

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INTRODUCTORY REMARKS

Older adults exhibit a greater susceptibility to infection and reduced responses to vaccination relative to young adults, and infectious diseases remain among the leading causes of morbidity and mortality in the elderly (>65 years of age) (1). While multiple changes occur in the organism with aging, immune senescence is believed to be the key culprit for this susceptibility. Immune senescence affects both the innate and adaptive branches of the immune system, as well as the stromal microenvironments that affect T cell development and homeostasis (2–4). It has been well established that the thymus begins involution relatively early in life, becoming progressively smaller, more disorganized, and functionally inferior, with reduced naïve T cell output (5). The

Abbreviations: BEC, blood endothelial cells; DC, dendritic cell; ECM, extracellular matrix; FDC, follicular dendritic cell; FRC, fibroblastic reticular cell; HEV, high endothelial venule; ILC, innate lymphoid cells; LEC, lymphatic endothelial cell; LN, lymph node; LTi, lymphoid tissue inducer; LTo, lymphoid tissue organizer; SCS, subcapsulary sinus.

changes in output of naïve T cells from the aging thymus have long been associated with the numerical decline in naïve T cells in the periphery of aged animals, while memory T cells accumulate proportionally (4). However, memory cells do not increase in absolute numbers with aging unless persistent infection with cytomegalovirus is also present (6). Substantial research has dissected the changes that occur to both T cell development with age (5) and to peripheral T cell homeostasis and function (2). However, less attention has been paid to the aging stromal environment that is expected to maintain these lymphocytes throughout the lifespan. Here, we discuss the series of changes that affect the aging lymph node (LN) architecture and function as a critical factor contributing to poor age-associated immune responses and propose new therapeutic targets to rejuvenate the aging immune system.

FUNCTION AND ORGANIZATION OF LN STROMA

The primary function of the LN is to coordinate immune responses to antigens trafficking from peripheral tissues. The non-hematopoietic stromal cell subsets provide the architecture and scaffolding necessary to guide cellular trafficking and compartmentalization, facilitate antigen presentation to circulating naïve T and B cells and thereby promote immune surveillance against infection. In addition, LN stromal cells are responsible for the production and presentation of chemokines that coordinate this trafficking of lymphocytes into and throughout the LN (7, 8). LN stromal cells also provide a crucial microenvironment for immune homeostasis and lymphocyte maintenance *via* presentation of pro-survival cytokines such as IL-7 and IL-15 to T cells (7, 8), and CXCL13 and B-cell activating factor of the TNF family (BAFF) to B cells (9).

Phenotypic Characteristics of LN Stromal Cells

The stromal cells of the LN are a numerically small, $CD45^{-}TER119^{-}$ population derived from endothelial and mesenchymal progenitors, relative to hematopoietic-derived $CD45^{+}$ or $TER119^{+}$ cells, which make up the vast majority (>98%) of LN cells (10) (Ter119 marks red blood cells). Within the stromal fraction, cell surface expression of podoplanin (PDPN, also known as gp38), CD31 (PECAM-1), and CD35/CD21 (complement receptor 1 and 2) distinguish five major, functionally important subsets: fibroblastic reticular cells (FRCs; $gp38^{+}CD31^{-}CD35/CD21^{-}$), lymphatic endothelial cells (LECs; $gp38^{+}CD31^{+}CD35/CD21^{-}$), blood endothelial cells (BECs; $gp38^{-}CD31^{+}CD35/CD21^{-}$), follicular dendritic cells (FDCs; $gp38^{\pm}CD31^{-}CD35/CD21^{+}$), and double/triple negative (DN) cells ($gp38^{-}CD31^{-}CD35/CD21^{-}$) (11, 12) (**Figure 1**; **Table 1**).

Functional Characteristics of LN Stromal Subsets

The endothelial derived LECs and BECs help mediate transport of both circulating cells and tissue-derived antigens into and out of the LNs. Entry into the lymphatics from the tissues occurs through lymphatic collectors and vessels lined with LECs (17). LECs also line sinuses in the LNs delivering antigen from the tissues and providing a route for cells to travel to the next LN (18). In general, BECs line blood vessels. A specialized BEC subset, called HEVs facilitates entry of circulating lymphocytes into the LN *via* a multistep adhesion and extravasation process utilizing chemokines, selectins, addressin and integrins (18).

Mesenchymal cells create the reticular network within the LN and are critical for the maintenance of its architecture; FRCs, FDCs, and DN stromal cells partake in this task. FRCs are a specialized type of reticular fibroblast that create a large proportion of the stromal network within the LN (19). FRCs ensheath bundles of collagen fibers to create conduits for the transport of small molecules, including antigens/antigen complexes and provide a transport system that guides DC and T cell movement (20). FDCs are also specialized reticular fibroblasts (9) that secrete CXCL13, guiding B cells, and follicular helper T cells into the germinal center (GC) to facilitate high-affinity antibody production (21). While the function of DN/TN cells is largely unknown, gene profiling studies suggest that some of these cells may be mesenchymal progenitors, consistent with their positioning as pericytes (20, 22). Pericytes within the double negative fraction may also help regulate blood vessel integrity, as well as permeability within the LN (22).

Hematopoietic Cells Facilitate LN Stroma Maintenance

Lymph node stromal cells have close bidirectional relationships with hematopoietic cells, each contributing to the homeostasis of the other (23). Innate lymphoid cells (ILC) are a broad category of cells that develop from common lymphocyte progenitors but do not have rearranged antigen receptors (24). ILC include lymphoid tissue inducers (LTi), which are a sub-group of ILC group 3 cells (25). During LN development, LTi are an important source of lymphotoxin beta (LTβ), which combines with lymphotoxin alpha to make the heterotrimer $LT\alpha_1\beta_2$ (25, 26). This heterotrimer can signal mesenchymal stem cells through the LTβ receptor (LTβR) to differentiate into lymphoid tissue organizers, which are critical in inducing proper development and architecture formation of other stromal cells, particularly FRC. Although LTi were originally recognized for their role in LN developmental, they are present in the adult LN and appear to also mediate adult tissue regeneration (24). LTi help induce regeneration of FRC networks in the spleen and LN following lymphocytic choriomeningitis virus infection (27). It should be noted that while LTi are an important source of LT, other lymphocytes including T, B, and NK cells also secrete LT and contribute to LT availability in the LN (28). Therefore, it is possible, and indeed likely, that naïve T and/or B cells contribute to the health and maintenance of FRC and other stromal cells, which, in turn, provide trophic factors for naïve lymphocyte survival and maintenance.

Other signals from hematopoietic populations in the LN influence the structure, function, repair, and regeneration of LN stroma. C-type lectin receptor 2 (CLEC-2) is expressed by megakaryocytes, platelets, neutrophils, DCs, and NK cells (12, 29). CLEC-2 serves as a ligand for PDPN expressed on

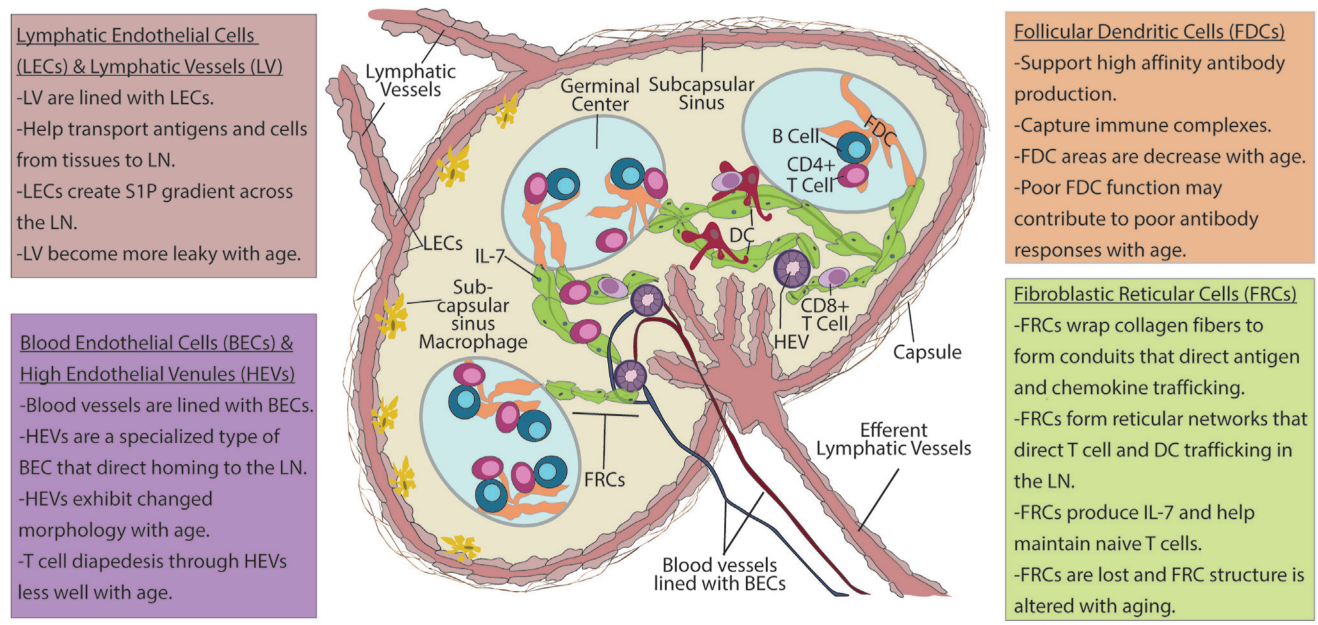

FIGURE 1 | Lymph node (LN) stroma elements and their changes with aging. *Upper left box*: lymphatic vessels (LVs) are lined with lymphatic endothelial cells (LECs). These vessels transport antigens and cells from peripheral tissues to draining LNs. LECs also produce sphingosine-1-phosphate (S1P) that forms a chemotactic gradient for migration of T cells into efferent lymphatics. Migratory dendritic cells (DCs) enter LN *via* LVs and into subcapsulary sinuses (SCSs) before entering the LN parenchyma. As antigens drain into the SCS, which are also lined with LECs, SCS macrophages pick up antigen and transfer it to follicular dendritic cells (FDCs). *Upper right box*: FDCs present immune complexes to B cells to enhance high-affinity antibody formation. *Lower left box*: blood endothelial cells (BECs) line blood vessels that transport blood borne cells into LN. High endothelial venules (HEVs) are specialized BECs with cuboidal morphology, T cell diapedesis across HEVs to enter the LN parenchyma. *Lower right box*: after entering the LNs naïve T cells from the blood stream crawl along fibroblastic reticular cells (FRCs) that form the reticular network in search of DCs bearing cognate peptide–MHC and costimulation to become activated. FRCs also have critical roles in the maintenance of naïve T cells through the production chemokines and IL-7. *Age-related changes*: with age, LVs become leaky and less capable of facilitating movement between of cells and antigens between the peripheral tissues and the LN to coordinate immune response. HEVs have altered morphology with age, and T cells have difficulty moving across HEVs with increased age. FRCs exhibit numerical reduction as well as disorganization of reticular networks with aging. This is likely to impair naïve T cell homeostasis, as well as movement of T cells within the LN and may impact the ability of aged LN to generate productive T cell responses. FDC areas are also reduced with age. Changes to FDCs may contribute to poor affinity of antibody responses that are observed with increased age.

stromal cells and triggers the relaxation of FRC networks (30), which in turn impacts how many antigen specific T cells can be recruited into the LN to respond (31). FRC lines isolated from LN are dependent on lymphocytes for production of ER-TR7 [which identifies the extracellular matrix (ECM) produced by FRC, but the antigen has not been identified]; reticular networks fail to form in the absence of this interaction *in vitro* (7, 23). Therefore, a picture is emerging of intense cross talk between hematopoietic and stromal cells, critical to the homeostasis and function of both compartments in the LN, although many mechanistic details still remain to be defined.

FUNCTIONAL CONSEQUENCES OF AGE-RELATED CHANGES TO LN STROMAL CELLS

Stromal Cells in Aged LN

While the contribution of LN stromal cells to both immune homeostasis and function is evident, age-related changes affecting stromal cells have been under investigated (32). Therefore, age-related dysfunction and/or disorganization of LN stromal cells may be an underappreciated contributor to immune senescence. Several groups have described chronic and progressive changes that occur in LN with age (33–35). In general, with aging, LN in both mouse and man become smaller and less cellular (33). Similar to thymic involution, histological studies of LN highlight that the organization is less distinct (especially between T and B cell areas) (13, 14), with an accumulation of adipocytes (33) and signs of fibrosis (34). Similar disorganization between T and B cell areas occurs in the aging spleen (36). It should be noted that not all LNs undergo the same age-related changes; skin-draining LNs are more affected than mucosal LN (33). Below, we discuss key defects in aging LN stroma that have been identified to date.

TRANSPORT IN AND OUT OF LN

LVs and LECs

Afferent LV function as conduits for trafficking of both antigens and immune cells. DCs that have captured antigen in tissues move *via* LVs from peripheral tissues into draining LN (16). Imaging studies demonstrated that aged mice show a diminished capacity to transport bacteria (*Cryptococcus neoformans*,

TABLE 1 | Age-related changes to lymph node (LN) stromal cell populations.

Cell type	Markers	Known functions	Changes with age
Fibroblastic reticular cells (FRCs)	gp38⁺, CD31⁻, CD35/CD21, CD45⁻, Ter119⁻ ER-TR7⁺ in histology	• Help form conduits and reticular network • Regulate naïve T homeostasis • Regulate naïve T cell movement • Secrete CCL19, CCL21, and CXCL12 • IL-7 presentation	• Becklund et al. found that FRCs are decreased in aging LN in homeostasis (13), while Turner and Mabbott found that FRC numbers are unchanged (14) • FRC structure altered (13)
Follicular dendritic cells (FDCs)	CD35/CD21⁺, gp38⁺/⁻, CD31⁻, CD45⁻, Ter119⁻	• Make reticular network for B cells • FDC secrete CXCL13 • Support production of high-affinity antibodies • Capture immune complex	• FDC area decreased in aged mice (14) • Less CXCL13 produced in aged mice (protein) (14) • More CXCL13 expressed in aged mice by qPCR (13) • Less CXCL13 produced in response to infection in aged mice (15)
Double negative stromal cells (DN)	gp38⁻, CD31⁻, CD35⁻, CD45⁻, Ter119⁻	• Thought to be FRC like pericytes • Function of these cells is mostly unknown	• Decreased in number in aged mice (14)
Blood endothelial cells (BECs)	gp38⁻, CD31⁺, CD35⁻, CD45⁻, Ter119⁻	• BECs construct cortical blood vessels and capillaries, including high endothelial venules (HEVs)	• Unchanged between old and adult mice (14)
HEVs	These are a type of BEC PNAd⁺ in histology	• Main route of entry for lymphocytes • HEVs have cuboidal morphology	• Impaired T cell diapedesis at aged HEV (13, 15) • HEVs reported as more dense and compressed in aged LN (13)
Lymphatic endothelial cells (LECs)	gp38⁺, CD31⁺, CD35⁻, CD45⁻, Ter119⁻ LYVE-1⁺ in histology	• Transport antigens and lymph from peripheral tissues to LN. • Connection between LN • Help create sphingosine-1-phosphate gradient across LN	• No change in LECs (14).
Lymphatic vessels (LVs)	LYVE-1⁺	• Transport antigens, immune cells, and lymph from peripheral tissues to LN	• LV showed a 20% decrease in contraction amplitude and a 70% decrease in contraction frequency (16) • LV leakiness and impaired pathogen clearance in aged mice between footpad and popliteal LN (16)

Mycobacterium smegmatis, and *Staphylococcus aureus*) from peripheral tissues into the draining LN, as seen by bacteria leaking out of lymphatics and into the surrounding tissue (16). This was due to both increased LV permeability (an LEC defect) and reduced contractility of the musculature that surrounds the LVs (16). Using paraquat to induce oxidative stress to LECs in a transwell system, the same study found increased LEC permeability to FITC–dextran (16). The authors proposed that the impaired *in vivo* bacterial transport was caused by increased oxidative stress to LECs (16).

Within the LN, the lymph enters through afferent lymphatics into subcapsulary sinus (SCS) lined with LECs and SCS macrophages (SCSM) (18). LECs provide routes in and out of the LN while the SCSM trap pathogens, antigen, and immune complex as they come into the LN (14, 37). Thus, the SCSM network, positioned at the entry of afferent lymphatics, acts to reduce pathogen dissemination and to increase the chance that antigen-presenting cells will come into contact with the rare T cell that might recognize them (37). SCSM additionally transfer incoming immune complexes to non-cognate B cells, which then transfer the complexes to FDCs (38). FDCs shuttle these antigens into non-degradative endosomal compartments, allowing long-term retention and presentation of the antigens (38). Turner and Mabbott showed that aged mice exhibit a significant increase in SCSM as a fraction of hematopoietic cells (14). Despite this increase in SCSM, the FDCs in aged mice fail to retain immune complexes (14). Further research is needed to address whether in old mice this increased SCSM population fails to efficiently capture/handoff antigen to FDCs, or whether these antigens are being shuttled into degradative endosomes, rather than the usual non-degradative endosomes that allows immune complexes to be retained by FDCs.

BECs and HEVs

Circulating naïve B and T cells enter the LN through HEVs, which are a specialized subtype of BEC (18). HEVs have a cuboidal shape and a polarized expression of adhesion molecules so that circulating cells in the blood can anchor to the HEVs and extravasate into the LN (39). BECs (including HEVs) appear to be unchanged numerically in aged mice (14); however, aged HEVs appear to have a more dense and compressed morphology (13). There is some evidence that aged BECs show changes similar to that of the aging vascular system, including increased permeability, inflammation, and number of senescent endothelial cells (32, 40). Moreover, aged HEVs may poorly facilitate lymphocyte entry into the aged LN, based on experiments showing pronounced defects in recruiting adoptively transferred adult naïve T cells into old LN (13, 15).

CELLS OF THE RETICULAR NETWORK

The reticular network provides the structure and architecture of the LN (23). The reticular network is composed of reticular fibers, ECM, and mesenchymal lineage cells such as FRCs and FDCs. Collectively, the reticular network creates specific microanatomical sites within the LNs that support and coordinate immune cells through the production of cytokines and chemokines (41).

Fibroblastic Reticular Cells

Fibroblastic reticular cells are a category of cells representing at least five different populations (10). FRCs are uniquely important in both organizing the T cell zone within the LN and in maintaining naïve T cell viability and function. The conduits formed by FRCs extend across the T cell zone from the SCS to the HEVs and construct the reticular network of the LN (37). FRCs are specialized myofibroblast (10) that, like other myofibroblasts, express α-smooth muscle actin (7). Unlike other myofibroblasts, FRCs ensheath ECM-like collagen bundles, whereas fibroblasts in connective tissues are embedded within the ECM (23). Also, FRCs have higher expression of genes involved in cytokine signaling, as well as genes involved in antigen presentation pathways (42). FRCs can directly present antigens to promote either T cell activation or T cell peripheral tolerance (37). FRC expression of chemokines CCL21 and CCL19 controls T cell motility. It has been proposed that CCL21 interacts in a unique manner with glycosaminoglycans on FRCs to facilitate T cell movements (37). Specifically, CCL21 has a 32 amino acid long C-terminal tail containing 12 basic amino acid residues. This allows it to bind to glycosaminoglycans and other molecules like PDPN, a proteoglycan expressed by LECs and FRCs (43).

Naïve T cells decline in number with age (2). This has been primarily attributed to age-related thymic involution and the consequent decline in new naïve T cells produced. However, naïve T cells can have a long lifespan if provided the appropriate survival signals (7, 44). Link et al. demonstrated that FRCs play a key role in naïve T cell survival *via* production and presentation of IL-7 and CCL19 (7). Genetic knockout or antibody-mediated depletion of IL-7 results in a gradual loss of peripheral naïve T cells, whereas IL-7 transgenic mice exhibit a larger naïve T cell pool (45). Bajénoff et al. used intravital microscopy to show that T cells enter the LN *via* HEVs, then use FRCs to crawl to the LN parenchyma (19). When FRCs are depleted [e.g., in CCL19-diphteria-toxin (CCL19-DTR) mice], the total cellularity of the LN declines, with a significant loss of T cells beginning 24 h after FRC depletion (46).

Becklund et al. extended these findings testing whether LN in old mice can support adult T cell homeostasis. Both naïve TCR-transgenic and polyclonal populations from adult donors failed to survive, and proliferated less in old LN compared to adult LN after transfer (13). Further, old LN exhibited reduced numbers of FRCs, and their reticular network appeared less reticular and more condensed than in adults (13). Despite possessing normal levels of IL-7 mRNA in LN and IL-7 protein in circulation in old individuals, naïve T cells parked in old hosts exhibited lower levels of phosphorylated signal transducer and activator of transcription 5, a signaling molecule downstream of IL-7R (13). The discrepancy between the levels of IL-7 and the homeostatic signals received by naïve T cells suggested that IL-7 presentation by FRCs is altered in aged microenvironments. Mechanistic understanding of the naïve T cell maintenance programs across aging and across species (47) will be exceptionally important to immune rejuvenation strategies.

In addition to these problems of homeostatic maintenance, aged FRCs likely also contribute to the compromised immune responses to infection. An adult LN can expand up to 10-fold during an infection (48). Old LNs expand modestly, but never reach the cellularity seen during an adult immune response (15, 49). During infection, chemokines and cytokines are transported through FRC networks to HEVs where they are transcytosed and displayed on the luminal side of HEVs to recruit naïve T cells (50). Antigen-bearing DCs crawl first through LV into LN through afferent lymphatics, then along FRCs in search of their cognate T cell. Loss of LN organization and boundaries between B and T cell zones make the host more susceptible to infection (50). Depletion of FRC in adult CCR19-DTR mice resulted in reduced responses to replication incompetent influenza A or to a coronavirus-based vector, likely due to the reduction in chemokines reaching HEVs to recruit lymphocytes into the LN, and/or a reduction in coordination of immune cells within the LN (46). Somewhat unexpectedly, FRC depletion also had a profound negative impact on both B cell homeostasis (reduced B cell follicle size and disrupted T/B boundary), and decreased T-dependent and T-independent antibody responses.

Fibroblastic reticular cells are also a key source of collagen in the LN. Appropriate thickness and abundance of collagen, which is essential as part of the ECM, is an important physiological parameter of organ architecture and function (51). Increased thickness and abundance of collagen fibers, termed fibrosis, is a frequent change to many organs during aging and is associated with impaired function (52). Wound-healing cytokines, dominantly TGFβ and type 2 cytokines like IL-13 (53), induce fibrosis in LN and other organs and are also known to be increased with aging (52, 54). This process is believed to operate *via* FRC in some pathogenic conditions (55, 56) and is likely to also occur in the same manner during aging.

Follicular Dendritic Cells

Follicular dendritic cells are specialized cells of mesenchymal origin (10, 57), named for their long cytoplasmic "dendritic" processes and are unrelated to classical hematopoietic DC (58, 59). The role of FDCs during homeostasis is less clear, but during infection FDCs support B cell movement and proper localization to GCs by producing CXCL13, as well as B-cell activating factor of the TNF family (BAFF) and a proliferation-inducing ligand (50). FDCs additionally help generate high-affinity antibody responses (10) by allowing prolonged antigen presentation to B cells undergoing somatic hypermutation (38).

Defects in FRCs and FDCs are a potential factor underlying poor humoral immunity in the elderly. Antibody responses are of lower affinity and impaired function compared to young adults (60). For example, during chikungunya virus infection, high antibody titers are found in old mice, but they show poor neutralizing function compared to adults (49). Further, aging results in fewer B cells within the LN (49), and B cell localization is less defined (13, 14). GC formation is reduced in the LN of aged mice infected with West Nile virus relative to adult controls (15). GC size has also been reported to decline with age in humans (33). FDCs are responsible for coordinating these events but have a decreased area in LN of aged mice compared to their adult counterparts (14), and less CXCL13 protein is produced in response to infection and in homeostasis (14, 15). Turner and Mabbott also found that immune complexes were retained less by aged FDCs (14). This loss of antigen may suggest that a degradative endocytic pathway is being used by aged FDCs, although this has not been directly demonstrated. These observations are consistent with the idea that age-associated impairments in FDCs (14) are a contributing factor to poor antigen retention, impaired GC formation, and decline in the production of high-affinity, functionally neutralizing antibodies.

MECHANISMS BEHIND LN INVOLUTION AND REJUVENATION

Understanding the Mechanisms behind LN Involution

As mentioned above, the mechanisms driving LN changes with aging remain incompletely understood. Heterochronic parabiosis, the surgical joining of two organisms of different ages (adult and old) (3, 61), can be a powerful tool to discern cell-intrinsic vs. to cell-extrinsic (environmental) defects that occur with age (3). Both pro-geronic (62) and anti-geronic (63) factors have been identified using this technique. Using heterochronic parabiosis, we found a surprisingly marked loss of naïve T cell maintenance in the LN of the adult parabiont, with numbers reduced to that in the old parabiont (64). After surgical separation of the parabiosed adult and old mice, the adult LN returned to normal, while the old remained hypocellular. While joined, the frequencies and numbers of stromal subsets in the adult parabiont were similar to the old parabiont than to those in isochronic parabiosis (adult–adult pairs). Together, these results suggest that a circulating soluble or cellular factor, present in the old parabiont, can influence the structure and cellularity of the adult LN *in trans*. Research is in progress to test this hypothesis.

Possible Molecular Targets to Rejuvenate Aging LN

Based on the observed defects, discussed above, one can hypothesize about candidate molecular pathways responsible for LN defects. One attractive target is LT signaling. The receptor for LTβ (LTβR) is expressed on LECs, HEVs, FRCs, and FDCs (28, 39). The bidirectional relationship between T cells and FRCs in homeostasis is critical for both populations of cells (23). Known producers of LT, such as DCs, B cells, and T cells (37) are less abundant in the aged LN (15, 49). When LTβ is conditionally depleted in young mice, there is a decline in LN organization and impaired induction of antiviral immune responses (37), similar to that described in aged LN (14, 15).

Approaches that limit fibrosis should also be considered. Regardless of tissue type, aging is the biggest risk factor for fibrosis (52), including in skin-draining LN in humans (34, 35). Serum levels of transforming growth factor-beta (TGF-β) are increased with aging in both mice and humans (65) and may be related to the age-associated increases in T-regulatory cells (10, 53, 66). Within the LN, fibrosis has been most studied in the context of simian immunodeficiency virus (SIV) in non-human primates (55). Increased levels of TGF-β, pSMAD2,3 signaling, and increased levels of fibrosis were found in LN of SIV-infected animals, where immune reconstitution is limited after antiretroviral therapy (55). As SIV infection proceeds, naïve T cells in fibrotic regions undergo apoptosis (10, 67). Administration of the anti-fibrotic drug pirfenidone to reverse fibrosis restored naïve $CD4^+$ T cell populations in SIV-infected monkeys in combination with antiretroviral therapy (55). Along these lines, we have observed increased fibrosis in aged mouse LN compared to adults. Understanding the interactions between FRCs and lymphocytes, and how fibrosis may impact these communications could have important therapeutic potential (10).

CONCLUSION

Age-related changes to LN stroma are emerging as an important area of research. Full understanding of these changes will likely be critical to understand, and, perhaps, correct age-related disorganization of T cell homeostasis and immune function. LN stroma is critical for naïve T cell homeostasis, providing both chemokine gradients for effective trafficking into the LN, and survival signals to the naïve T cell upon arrival (7, 13). Further, stromal cells control influx of antigen, and there is initial evidence that this process may be adversely affected by aging LN (15, 16). Within the LN, FRCs (13) and FDCs (14), both decline numerically and exhibit disorganized network formation, with a potential to impair interactions with T and B cells. A key challenge in front of us is to (1) understand how aging alters the structure and function of each of the stromal cellular components and their interaction and (2) dissect the functional consequences of such changes for protective immunity. The ultimate goal should be to manipulate and restore stromal cell function in response to vaccination or infection and thus provide new targets to improve immunity in the elderly.

AUTHOR CONTRIBUTIONS

HT, MS, and JN-Z wrote the paper. CS contributed to critically revising the paper. All the authors extensively discussed the topic, read and approved the final version of the manuscript.

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REVIEW
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Immunobiography and the Heterogeneity of Immune Responses in the Elderly: A Focus on Inflammaging and Trained Immunity

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Owing to its memory and plasticity, the immune system (IS) is capable of recording all the immunological experiences and stimuli it was exposed to. The combination of type, dose, intensity, and temporal sequence of antigenic stimuli that each individual is exposed to has been named "immunobiography." This immunological history induces a lifelong continuous adaptation of the IS, which is responsible for the capability to mount strong, weak or no response to specific antigens, thus determining the large heterogeneity of immunological responses. In the last years, it is becoming clear that memory is not solely a feature of adaptive immunity, as it has been observed that also innate immune cells are provided with a sort of memory, dubbed "trained immunity." In this review, we discuss the main characteristics of trained immunity as a possible contributor to inflammaging within the perspective of immunobiography, with particular attention to the phenotypic changes of the cell populations known to be involved in trained immunity. In conclusion, immunobiography emerges as a pervasive and comprehensive concept that could help in understanding and interpret the individual heterogeneity of immune responses (to infections and vaccinations) that becomes particularly evident at old age and could affect immunosenescence and inflammaging.

Keywords: inflammaging, trained immunity, human aging, macrophages, NK cells, immunobiography

INTRODUCTION: THE IMMUNE SYSTEM (IS) AS A COMPLEX SYSTEM

Life is a continuous exposure to a large variety of threatening and potentially damaging agents collectively indicated as stressors, which can be divided into two basic categories: external and internal stressors. The first category includes not only all sorts of bacteria, viruses, fungi, and parasites but also nutrients that are basically foreign material that are ingested as a source of energy. The second category includes all types of material produced by living organisms as a consequence of cell turnover and metabolism, i.e., cell components or debris, metabolites, and molecular aggregates resulting from incomplete degradation or non-enzymatic reactions, considered as "molecular garbage" (1). All along the evolution, animals from invertebrates to vertebrates have developed adaptive strategies to recognize and neutralize such complex and dynamic combination of stressors that all together represent the "ecospace" where each animal lives (2). On the basis of studies on the evolution of stress response, from invertebrates to mammals (3), we argued that an integrated set of immune–neuro-endocrine responses co-evolved to cope with internal and external stressors (4, 5). It is important to note that, according to this conceptualization, "antigens" can be considered as a particular type of stressors (6). The IS is composed of cells and receptors devoted to the recognition of, and response to antigenic stressors, and is considered a paradigmatic example of complex system. As such, it is characterized by specific features, such as *degeneracy* (the capability of a single receptor to recognize a variety of molecular patterns); *networking* (the capability of IS cells to interact and cross-talk with each other); *plasticity* (the capability to adapt to different situations); and finally, the so-called *bow tie* architecture has been conceptualized to integrate all these characteristics of the IS. This latter is an organizational module that foresees a core of elements that can integrate different input signals and produce a range of output signals (7) (**Figure 1**). The way the IS ages and what are the changes that accompany and characterize this aging process have been the subject of intense studies in the last decades. In year 2000, our group proposed to call *inflammaging* the chronic, low-grade, sterile, inflammation that is almost universally present in old age and seems to be a hallmark of immunosenescence (6). The origins and sources of inflammaging are still matter of debate. In this review, we will discuss the possible involvement for the development and maintenance of inflammaging of a relatively newly described immunological phenomenon, i.e., innate immune memory or trained immunity. Trained immunity entails a cross-protection from different pathogens, and the first antigenic contact appears to be important in determining what kind of protection will be evoked. Therefore, it appears evident that type, intensity, and temporal sequence of antigens we are exposed to during the whole life are of extreme importance in determining the type of trained immunity that will rise up. More in general, the same concept is valid also for all the responses of the IS as a whole. The combination of these elements (type, intensity, and temporal sequence of antigens) is called "immunological biography" or *immunobiography*, and it can be considered unique for each individual. This uniqueness can explain how the same antigenic molecule, depending on the immunobiography of the host, can become either a strong or weak antigen or can induce tolerance. We will use the concept of immunobiography as a *fil rouge* of this review.

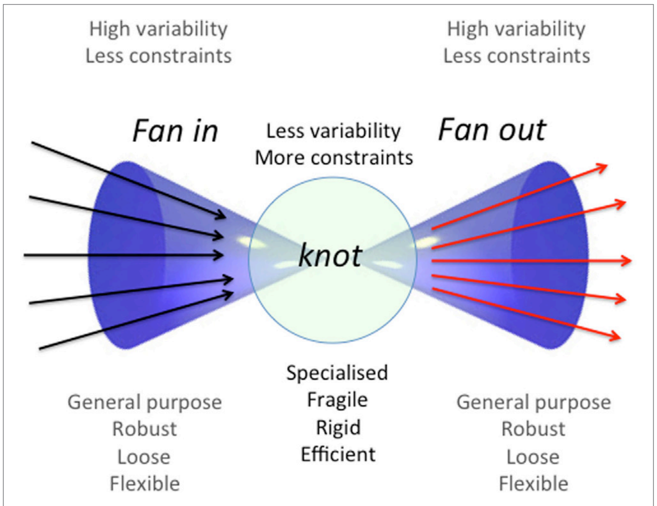

FIGURE 1 | General scheme of a bow-tie architectural module. This operational module is schematized as a bow tie due to the fact that it is composed by a conserved and relatively rigid core ("knot") of elements and by two wings of inputs and outputs (fan in and fan out, respectively). The core can accept a wide range of inputs that are integrated according to rules and protocols specific for every bow-tie, yielding a wide variety of outputs. These features confer flexibility, robustness, and evolvability to the system. Adapted from Ref. (7).

IMMUNOBIOGRAPHY AND THE PLASTICITY OF THE IS

As mentioned above, a basic characteristic of the IS as a whole is *plasticity* (8), which means that the cells of the IS are not only able to recognize external and internal stressors but also to adapt and modify according to the variety of stimuli they are exposed to. To this regard, a large body of literature [reviewed in Ref. (8)] suggests that not only the type of molecular stimuli and their doses are critical but also their temporal sequence. The combination of these factors is integrated into a bow tie-shaped core (i.e., IS cells) to produce a variety of outputs (strong response, weak response, anergy, tolerance, memory, etc.). This integration occurs at every contact with an antigen/stressor. The whole history of antigenic encounters (and consequent integrations into *bow tie* architectural modules) or *immunobiography* can be represented as a Waddington Landscape (8) (**Figure 2**). Immunobiography starts *in utero* and continues lifelong since the very first day of life and is thus strongly influenced by early life events, as illustrated in **Figure 2**. In the event, the immune responses of each individual will be unique, owing to his or her immunological "history," i.e., the summation ("immuneΣ") and interaction of all the immunological experiences/stimuli. We argue that temporal and geographical dimensions, as well socio-economic and psychological status, nutrition (oral tolerance and gut microbiota), and new potential source of unexpected epitopes produced by proteasome splicing (9) are integral component of immunobiography and could impinge upon the IS, thus inducing its continuous reshaping.

In conclusion, a variety of testable predictions derives from this conceptual framework, the most straightforward, suggesting that the immune responses to potentials antigens, including pathogens, food, and vaccines, will be quantitatively and qualitatively different according to the overall immune-biographical background of the host, including age, sex, lifestyle, socioeconomic and psychological status, and geography/genetics.

IMMUNOBIOGRAPHY AND INFLAMMAGING

We surmise that immunobiography is the best conceptual framework to understand the immune heterogeneity among

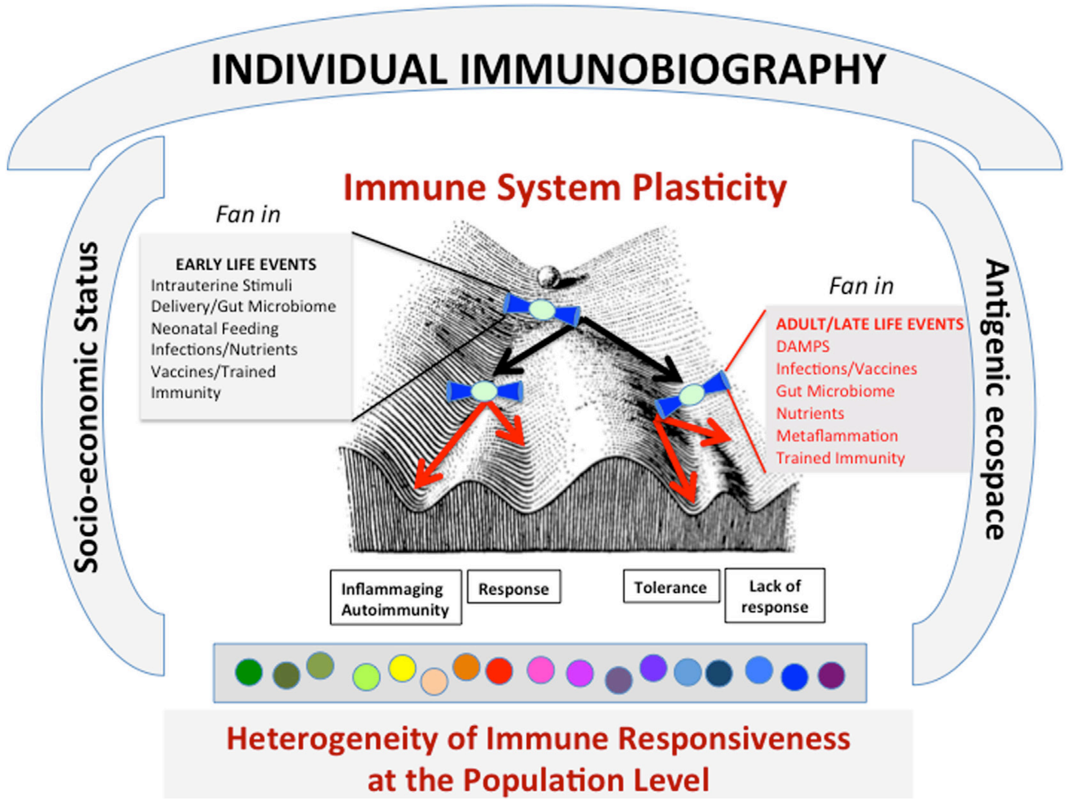

FIGURE 2 | The lifelong personal history of antigenic exposure (Immunobiography), represented as a Waddington Landscape, modulates the immune response to specific antigens. The response to every single antigenic molecule depends on the conditions of immune system (IS) when it meets the antigen. A variety of conditions, including socioeconomical status and antigenic ecospace, impinge upon the IS. The antigens can be met during life under different environmental conditions that can shape the immune response (i.e., what slope the ball will follow in its path). It is surmised that these environmental conditions act and are integrated as "fan in" signal by a bow tie-like module of conserved elements. These conditions include both early life and adult-late life events. As a whole, this process can lead to the creation at population level of a large heterogeneity of immune responsiveness to specific antigens.

individuals, including the difference in immune responses between men and women, and among different populations, whose genetics and IS have been molded by their evolutionary ecosystems and cultural habits (2). Moreover, the concept of immunobiography could explain the increased immune heterogeneity of old individuals and the age-related changes of the IS (i.e., immunosenescence and inflammaging). In fact, the IS undergoes a profound remodeling with age, contributing to the increased risk of infections, cancer, and autoimmune diseases (10). This remodeling affects both the innate and adaptive arms of the IS (11), and in general, it is thought to be a phenomenon associated with loss of functions and activities. However, this is not always true, as some features of the innate immunity seem to be preserved or even increased in immunosenescence (12, 13). In particular, inflammation is not dampened with age, and a low-grade, chronic, sterile inflammation (*inflammaging*) seems to be an almost universal phenomenon associated with advanced age (6, 14, 15).

The hyperproduction of innate immunity cytokines in elderly donors, including IL-6, TNF-α, and IL-1β, was first demonstrated in *in vitro* stimulated peripheral blood mononuclear cells (PBMCs) from aged people (16). The age-related activation of innate immunity was further confirmed in terms of blood levels of cytokines (17, 18) and chemokines (19–21). Accordingly, the age-related increase of pro- and anti-inflammatory mediators in peripheral blood was recently demonstrated on a large longitudinal cohort of Italians aged 20–102 years (22), underlying both the activation of innate immunity with age and the simultaneous activation of anti-inflammatory molecules, such as IL-10. Importantly, the presence of anti-inflammatory compensatory mechanisms was previously shown to be present also in centenarians (14), thus highlighting possible pathways of adaptation that likely favor longevity.

Since its very beginning, inflammaging was pigeonholed within an evolutionary framework where a central role of the macrophage was foreseen. This cell is indeed able to perform not only phagocytosis of foreign pathogens but also to produce a variety of soluble mediators, mainly but not exclusively pro-inflammatory (6, 23). The activation of this versatile cell (that is, now recognized to possess also a form of memory, see next paragraphs) likely accounts for the so-called physiological inflammation postulated since the beginning of the twentieth century by the great immunologist Il'ja Metchnikoff (24). Now, the available data indicate that other cell types (not necessarily belonging to the IS) can contribute to the setting up of the inflammaging, such as adipose and skeletal muscle cells. Moreover, an important contribution to inflammaging can arrive from senescent cells (1, 15), which are provided with a specific senescence-associated secretory phenotype characterized by the production of pro-inflammatory cytokines such as IL-6, IL-1β, IL-8 and chemokines such as CXCL1, CXCL2, matrix metalloproteinases, serine proteases, and regulators of plasminogen activators (PAI-1, PAI-2), etc. (25, 26). If the innate immune cells do not efficiently clear out these senescent cells, they can accumulate and contribute to the creation of a pro-inflammatory environment.

We have recently proposed that an age-related increase in the production of danger-associated molecular patterns (DAMPs) can impinge upon the level of inflammaging more importantly than pathogen-associated molecular patterns (PAMPs), through innate immune cells receptors, leading to innate inflammatory response (1). These DAMPs include, among others, high-mobility group B1 (HMGB1) protein, sodium monourate and uric acid crystals, oxidized fatty acids, and proteins. In particular, evidence exists for some specific molecules such as oxidized LDL, HMGB1, and uric acid. Oxidized LDL can train monocytes to secrete more pro-inflammatory cytokines (27, 28) (IL-6, IL-8, TNF, and MCP-1) and to express more pattern recognition receptors (PRRs) and LDL receptors (28). Mouse splenocytes that had been pretreated with HMGB1 responded with significantly higher TNF production when restimulated with PAMPs such as Pam3Cys, lipopolysaccharide (LPS), CpG, or other DAMPs like S100A12 (29). Finally, also uric acid appears to be able to prime the production of Il-1β and other pro-inflammatory cytokines in PBMC or monocytes (30).

The lifelong interaction between the gut microbiota and the IS could contribute to inflammaging. As recently summarized, host genetics, prenatal environment, and delivery mode can shape the newborn microbiome at birth (31). Moreover, a variety of other postnatal events such as antibiotic treatment, diet, exposure to infectious agents, among others can impinge upon and modify the development of the infant's microbiome and IS, with long-term effects (risk for several diseases) in adult life. The age-related trajectory of the gut microbiota composition, from young adults to centenarians, and its possible contribution to inflammaging has been recently described (32, 33). A complex, lifelong remodeling of such a complex ecosystem emerged, where the decrease of potentially beneficial species and the increase of potential pathobionts related to systemic inflammation (32) is continuously counteracted by the increase of sub-dominant species, some of which likely exert a protective effects (33).

Inflammaging appears to be associated with decreasing health, but is also compatible with longevity, being present in centenarians. This apparent paradox can be understood in the light of immunobiography. According to this concept, a clinical history or an environmental circumstance could shape the IS to counteract inflammaging by setting up effective anti-inflammatory responses.

IMMUNOBIOGRAPHY, TRAINED IMMUNITY, AND THE MEMORIES OF THE IS

A paramount feature of the IS (and of immunobiography too) is *memory*, i.e., the capacity to give rise to a more rapid and efficient response at the second contact with a previously met antigen. Until few years ago, a tenet in immunology was that memory was an exclusive feature of the adaptive IS of vertebrates. A classic example reported since many years is the phenomenon of the "original antigenic sin" (34), which influences the type of response to a second challenge with a pathogen. Upon a primary response toward a pathogen (e.g., a virus), a subsequent exposure to the same pathogen elicits a secondary amplified and quicker response. However, if the second pathogen is very similar but not identical to the first, the IS can mistakenly identify the second pathogen as the first one encountered and progress to a classical memory response, which may be ineffective toward the second pathogen.

Actually, several observations have challenged this tenet, as examples of memory involving the innate branch of the IS were already reported since many decades (35). To this regard, it is known since long time that in plants and invertebrates, which only display innate immunity mechanisms, memory characteristics are present in the response to pathogens. In plants, a phenomenon called systemic acquired resistance (SAR) is well documented (36–38). This sort of primitive immunization protects plants for long periods of time against infections different from the one that elicited SAR, including viruses, bacteria, fungi, and oomycetes. In invertebrates, the existence of a form of memory where the information on a first encounter with a pathogen is stored and rapidly used on demand has now been demonstrated in a wide range of species (39, 40). Intriguingly, this type of responses can vary in degree and specificity in relation to different priming. Moreover, a phenomenon similar to allograft rejection after tissue transplantation has been demonstrated in some invertebrates (41, 42). For example, in second grafting experiments, leech responses to the second transplant were always faster and stronger than those occurring in first set grafting experiments. In second set experiments, two cell populations are evidenced, and some of them expressed CD56 and CD8-α and some others CD8-β and TNF-β allowing to postulate the existence of a sort of positive immune memory. In addition, the presence of CD8β- and TNF-β-positive cells in the graft area could suggest the existence of leukocyte-like cells that had previously responded to antigenic stimulation and have thus become able to respond rapidly to subsequent antigenic challenges. As a whole, these data support the idea that in invertebrates a sort of immunological memory exists even if with different features compared to the classical memory of the adaptive immunity present in vertebrates. In recent years, an ancestral network of cells with a thin, elongated morphology called "telocytes" (TCs) has been described in both invertebrates and vertebrates, including humans (see **Box 1**). As detailed in the **Box 1**, the TC ancestral network is able to integrate many different functions shared with players involved in trained immunity, such as complex innate

BOX 1 | Telocytes (TCs) as possible players in trained immunity.

Recently a new type of cellular system is described as ubiquitous in both vertebrates and invertebrates (40, 43–47). These cells named TCs are stromal cells strategically spread in various types of tissues from invertebrates up to humans. TCs are characterized by a very small spindle-shaped cell body, essentially occupied by a large nucleus, from which very long convoluted cytoplasmic processes, the telopods, originate. Thanks to these thread-like telopods, TCs communicate among themselves, with any other type of cells and interact with collagenic bundles, forming a key extensive intercellular network. The interaction among these different players take place directly by cell–cell contacts and indirectly *via* the release (in autocrine, paracrine, endocrine manner) of microvesicles and exosomes, which can transport a variety of soluble factors involved in the regulation of different physiological processes (47–51). TCs immunophenotype is quite complex. Apart the specific markers (co-expressed CD34/vimentin and Oct-4/c-kit) (44), these cells express markers of the immune-surveillance such as Toll-like receptors (TLRs) 4 and 5, allograft inflammatory factor-1 (Aif-1 also known as IBA-1) involved in inflammatory responses, adrenocorticotropic hormone implicated in the immune and neuroendocrine responses, and endogenous pro-inflammatory cytokines such as IL-18 (47). TCs respond to chemical or physical stimuli changing their morphology and behavior. These cells, by acquiring migratory phenotype, numerically increasing and overexpressing the previously mentioned factors, are able to rapidly move toward the injured area where they also participate in repair and regenerative processes (47, 52). Moreover, it has been observed in the leech *Hirudo medicinalis* that TCs originate from precursor circulating cells during the angiogenesis that ensues the graft rejection inflammatory phase. These invertebrate/vertebrate cells organized in a 3D network are equipped to function as an immune-neuroendocrine system. This evolutionarily conserved system is formed by resident cells working as outposts to signal the presence of non-self/damaged-self molecules and to alert the internal defenses of the organism. Owing to the fact that they are tissue resident, TC networks are able to respond promptly and faster than migrating immunocytes that need time to reach the stimulated (injected with LPS or injured) area.

immune responses, regenerative processes in wound healing, and secretion, so it is tempting to speculate that it might also play a role in trained immunity.

Trained immunity appears to be based on innate immune cells that are also present in vertebrates. It was therefore conceivable that also in vertebrates similar phenomena were present. Consistently, studies performed in the past indicated the existence of an innate memory also in mice. In fact, vaccination with BCG was reported to protect mice against secondary infections with *Candida albicans* or *Schistosoma mansoni* through T cell-independent mechanisms (53), involving activated tissue macrophages (54). Moreover, infection with attenuated strains of *Candida* was observed to induce protection not only from reinfection with *Candida* itself but also from other pathogens such as *Staphylococcus aureus*, and this phenomenon was present also in athymic animals (55). More recently, it has been demonstrated that challenge of mice with CpG confers protection against *Listeria monocytogenes* infection (56). As a whole, it appears that in both invertebrates and vertebrates, innate immunity cells are provided with a capacity to respond more promptly to a second challenge, a feature that resemble the memory reactions typical of the adaptive immunity, with the crucial difference that such memory seems to be not limited to the specific antigen that triggered the first response. To describe this kind of innate memory, the group of Mihai Netea proposed the term "trained immunity" (57, 58). Trained immunity is evoked not only by microbial, viral, or fungal challenges (e.g., β-glucans, LPS) but also by molecules that are contained in vaccine adjuvants. Actually, adjuvants include TLR agonists such as monophosphoryl lipid A, CpG oligonucleotides, aluminum phosphate, or hydroxide salts. These adjuvants act mainly by inducing mild local inflammatory reactions that can boost the adaptive immune response toward the challenging antigen(s) (59). It has been shown that trained immunity is responsible for non-specific effects of vaccines such as BCG, OPV, and MMR (60, 61). It is known actually that these vaccines offer a protection from overall mortality that is not explained simply by the protection against the targeted pathogens (62). It is possible that this non-specific protection could be accounted for by the capability of adjuvants of inducing trained immunity responses (63).

At present, there is evidence that macrophages and NK cells are the main innate immune cells provided with this memory (64–66); however, also other cell types of both myeloid and lymphoid lineages (such as γ/δ T cells) seem to display similar features (67, 68), including NK-like $CD8^+$ T cells, invariant NKT cells, and innate lymphoid cells (ILCs), even if more data are needed to clarify the underpinning mechanisms (see also next paragraph).

The basic molecular mechanisms involved in and responsible for the trained immunity memory appear to be of epigenetic nature. In fact, one of the mechanisms responsible of macrophages and dendritic cells (DCs) trained immunity is the capability to undergo epigenetic modifications following exposure to PAMPs or DAMPs (69). As it will be described in detail in **Box 2**, these epigenetic modifications induce high concentrations of inflammatory cytokines, including IL-1, IL-12, IL-18, and IL-23, which promote IL-17 and IFN-γ production by innate lymphocytes, including γδT cells, innate lymphoid cells (ILCs), and NKT cells, that exert protective effector functions against the second pathogen (58, 69, 70).

The clear-cut distinction of innate and adaptive immunity based of the presence of memory is now much more blurred, and memory appears to be a shared property of the two branches of the IS, even if the memory of innate immunity (trained immunity) has different features. Therefore, the IS has at least two ways to remember previously encountered antigens. If and how these two "memories" do interact with each other is still unclear. They could act synergistically, or, on the contrary, trained immunity could dampen the adaptive one. This interaction could explain at least in part the heterogeneity of immune responses observed in the elderly. Urgent studies are needed to clarify this point. However, it is not known how trained immunity can change during aging and what contribution these possible changes can give to immunosenescence and inflammaging.

CELLS AND RECEPTORS INVOLVED IN TRAINED IMMUNITY DURING AGING

In this paragraph, we will briefly discuss the present knowledge on the changes that occur with age in cells and their receptors presently known to be involved in trained immunity such as monocytes/macrophages, NK, and γδT cells.

BOX 2 | Trained immunity and epigenetics.

There is evidence that trained immunity, at variance with adaptive immunity, does not imply genetic recombination, but relies upon epigenetic remodeling that influences gene expression profile without changing the DNA sequence of the cells. The first evidence that trained immunity is largely dependent from epigenetic mechanism derives from studies on plants (71).

Even though epigenetic changes tend to be maintained over time, they are less stable than the genetic rearrangement that occurs in adaptive immunity, and for this reason, trained immunity duration is shorter than adaptive immunity (that relies on clonal expansion of memory lymphocytes with specific receptors originated by genetic recombination). In general, the mechanism behind trained immunity can be recapitulated as follows: innate immunity cells, such as monocytes, macrophages, and NK cells, respond to antigenic stimuli by undergoing a shift in energy metabolism; this in turn causes an epigenetic rewriting that remains stable over time and have the potential to be inherited during cell differentiation. In particular, a shift of glucose metabolism from oxidative phosphorylation to aerobic glycolysis, increased glutamine metabolism, and cholesterol synthesis have been observed to play a crucial role in determining the establishment of the epigenetic modifications typical of the trained immunity phenomenon (72). Such epigenetic modifications lead to transcriptional programs that rewire the intracellular signaling of innate immune cells and induce an increase in the capacity to respond to the stimuli. A shift from phosphorylation to glycolysis has been observed in β-glucan-trained monocytes (70). There are different mechanisms by which a change in energy metabolism can impinge upon epigenetic setting. As an example, glycolysis results in higher ratios of NAD^+/NADH, and this has been shown to activate Sirtuin 1 and 6 (73). Furthermore, it has been demonstrated that end products of glycolysis can inhibit histone deacetylases, thus causing genes to be more accessible (74).

Depending on the nature of the stimuli and the type of epigenetic modifications, cells maintain a hyperactivated phenotype for weeks or months. Accordingly, the specificity of the hyperactivation in response to the activating signal/agent is correlated with the epigenetic modification involved in the first response (58). Data obtained on monocytes indicate that upon vaccination with BCG, trained immunity was induced through the NOD2 receptor and mediated by increased histone 3 lysine 4 trimethylation (75). Epigenetic modifications can be triggered even in bone marrow precursors of immune cells. To this regard, a study on mice showed that bone marrow epigenetic remodeling of DC progenitors can be also stimulated by the gut microbiota (76). These data are of the utmost interest as they open a new perspective on the relationship between trained immunity, chronic inflammation, and a wide range of physiological and pathological conditions such as aging, obesity, and type 2 diabetes, where consistent changes in GM composition have been reported (32, 33, 77).

Beside histone modification, that is the prominent epigenetic mechanism involved in the trained immunity acquisition, other mechanisms are involved, such as DNA methylation and miRNA expression. DNA methylation was correlated with trained immunity after CMV infection (78, 79). In these studies, NK cells underwent large changes in the overall methylation profile, which altered profoundly their secretory capacity (78, 79). This result is particularly interesting when considering trained immunity under the perspective of aging, since it is known that the DNA methylation structure undergoes profound changes with age in all the tissues and organs (80). Accordingly, it will be of great interest to investigate the effect of such age-related modifications on trained immunity efficacy and plasticity.

A specific contribution is also played by microRNAs. They are short RNAs that play a critical role in influencing gene expression by silencing genes hierarchically high in the expression cascade of specific pathways. A critical characteristic of microRNAs is their long life in cells, thus providing a concrete contribution to the trained immunity setup (81). Among all the microRNAs, miR-155 is of particular interest, since its upregulation in response to external agents has been correlated with the activation of myeloid cells (82). Moreover, miR-155 constitutes a direct link between trained immunity and inflammaging since is one of the microRNAs involved in the regulation of inflammation (the so-called inflamma-mir) active in the aging process (83, 84).

Monocytes/macrophages are perhaps the most characterized cells involved in trained immunity, and it is well established that a great heterogeneity within this cell type does exist. Three different populations based on the differential expression of the LPS (CD14) and the FcIII (CD16) receptors (85) have been identified. This circulating monocyte pool dynamically changes during aging. In particular, $CD14^+$ $D16^+$ non-classical monocyte subset increases with age in healthy adults (21) but, importantly, displays reduced HLA-DR surface expression in elderly donors, suggesting a decline of antigen presentation function. Further, many data suggest that TLR expression and signaling efficiency in monocytes and DCs is modified during aging. A highly significant increase in TLR5-induced production of IL-8 from monocytes of older individuals has been reported along with an incomplete activation of NF-κB in response to TLR5 signaling (86). Moreover, in a large cohort of healthy human donors, peripheral blood monocytes from elderly donors showed a decreased expression and function of TLR1 (87). Similarly, reduced TLR levels and signaling responses in DCs were found (88). Interestingly, dysregulation of TLR3 in macrophages and lower production of IFN by DCs from elderly donors after infection with West Nile virus was reported (89). In addition, Metcalf et al. (90) have recently showed in a small cohort of donors that PBMCs from old subjects exhibited a slower immune response to TLR4, TLR7/8, and RIG-I agonists compared to cells from adult individuals. This was evident by the rapid induction of the IFN-signaling pathway in PBMCs from adults treated with different PRR agonists, including LPS among others. However, old subjects did produce higher levels of CCL1 in response to LPS and analogs.

Of note, TLR4, the receptor for LPS, is downregulated in macrophages that have been challenged with repeated exposures to low doses of LPS, a process known as endotoxin tolerance (91). Recently, it has been reported that the expression and activation of TLR4 induced by exposure to *Mycobacterium leprae* was downregulated upon the previous exposure to BCG (92). This suggests that trained immunity could involve TLR4 and that this involvement does not always entail activation, but also possible phenomena of tolerance. In particular, TLR4 and TLR2 can be responsible for tolerance, while other receptors like NOD2 and Dectin-1 can be responsible for trained immunity (30).

As far as NK cells, age-associated changes in phenotype and function have been described (93–95). First, NK cells express different functional TLRs (96, 97) recognizing bacterial PAMPs and activating their response (98–100). Other molecules, such as natural cytotoxicity receptors (NCRs), including NKp30 and NKp44, are key receptors in the recognition and the killing of virally infected or tumor cells. The recent identification of the cellular ligands for NKp44 and NKp30 such as exosomal proliferating cell nuclear antigen implicates that NCRs may also function as receptors for DAMPs (101). Therefore, the activation of NK cells could be amplified during aging due to the increased availability of DAMPs, according to the Garb-aging hypothesis (1). Further, immunosenescence is associated with the increase of $CD56^{dim}$ NK cell subset, which expresses a mature phenotype, characterized by the augmented expression of markers such as CD57 (102) and KLRG1 (103, 104). The CD57 antigen (HNK-1, LEU-7) is also used to identify terminally differentiated "senescent" T cells with reduced proliferative capacity and altered functional properties as recently reviewed (105), but it seems to have a different expression pattern in NK cells. In fact, CD57 characterizes two typical NK subsets, i.e., the CD16$^+$CD56^{dim} cytotoxic NK cells and the CD16$^+$CD56$^-$ inflammatory NK cells, whereas the CD16$^-$CD56^{bright} regulatory NK cells do not express this marker even during chronic infections (102, 106). To this regard, infection with viruses including HIV and CMV could drive the expansion of CD57$^+$NKG2C^{high} NK cells (107, 108). It has been proposed that CD57$^+$NKG2C^{high} NK cells might represent human CMV-specific "memory" NK cells, thus highlighting the "adaptive characteristics" of NK cells (109). Remarkably, CD57$^+$NKG2C$^+$ NK cells expansion was observed in patients positive for both CMV and HIV, reaching levels >70% of all circulating NK cells, in comparison with patients who were positive only for either CMV or HIV (110). These data suggest that this NK subset may be trained by CMV and likely undergoes a great expansion when CMV reactivation occurs, a condition more frequently found in HIV-infected individuals. Hypothetically, a reactivation of latent virus can occur many times during aging and could stimulate CD57$^+$NKG2C^{high} NK cells, therefore triggering expansion of this cell subset.

At variance, the subset of CD56^{dim} KLRG1^{high} NK cells is expanded in the elderly, displaying impaired cytotoxicity and proliferation as well as other features of senescence (103). Interestingly, KLRG1 or the killer cell lectin-like receptor G1 is also considered a marker for T cell senescence (111, 112) like CD57 molecule (113).

As a whole, these data suggest a convergence of adaptive and innate immunity during immunosenescence (114). A progression toward terminal differentiation (or senescence) of CD8$^+$ T cells appears in fact to be associated with the acquisition of the hallmarks of innate-like T cells and the use of recently acquired NK cell receptors. These phenotypic, functional, and transcriptional changes would be a sort of compensation for functional deficits of conventional NK cells and T cells (115). Different health and environmental conditions, such as autoimmunity, inflammation, viral antigen re-exposure, or the presence of persistent tumor antigens, have been shown to allow the differentiation or "adaptation" of NK-like CD8$^+$ T cells, as recently reviewed (116).

As far as γ/δ T cells, these cells can be activated independently from TCR and APCs. Receptors used by γ/δ T cells include NOTCH (117), NKG2D, and TLRs. To this regard, almost all TLRs were found in human γ/δ T cells (118). No data are currently available on age-related changes in expression or function of these receptors, even if it is known that a decline of total peripheral blood γ/δ T cell frequency occurs with age, along with changes in phenotype and TCR repertoire (95, 119), phenomena accentuated by CMV infection (120, 121). Interestingly, some data show that peripheral blood Vδ2(neg) γ/δ T cells are significantly increased in CMV-seropositive healthy individuals compared to CMV-seronegative controls in all age groups (122), thus reinforcing the idea that persistent antigenic load may modulate T cell repertoire with important effects also on innate immunity and inflammation (123).

Finally, it has been observed that also other innate immune cells such as group 2 innate lymphoid cells (ILC2s) display memory features (124). In the lung, ILC2s are stimulated by inhaled allergens and produce Th2-type cytokines inducing T cell-independent allergic lung inflammation. After the resolution of the inflammation, some ILC2s persist as allergen-experienced cells, can respond to unrelated allergens more potently than naive ILC2s, and exhibit a gene expression profile similar to that of memory T cells (124). Nothing is known at present on the possible modifications of the activity (and memory) of such cells during aging. Moreover, it is possible that also other innate immune cell types such as neutrophils or TCs (as proposed here) can be provided with memory features. Further studies are needed to test this hypothesis.

Overall, a complex scenario emerges for cells and receptors of innate immunity: some of them undergo consistent age-related impairment, while others are preserved or even hyper-regulated. Thus, trained immunity could dramatically change at advanced age due to the fact that some cell types (and their receptors) but not others undergo complex reshaping, possibly driven by the persistence of specific antigens (such as viral ones) or increased availability of DAMPs.

IMMUNOBIOGRAPHY INTEGRATES IMMUNOSENESCENCE, INFLAMMAGING, AND TRAINED IMMUNITY

The heterogeneity inherently present in any population is at the basis of a variety of important immunological, largely unclear aspects, such as the different responsiveness of individuals to various antigenic stimuli, i.e., bacteria, viruses, parasites, and vaccines. This heterogeneity also increases with age, thus becoming particularly important not only in immunology but also in gerontology and geriatrics, as it affects the risk of developing age-related diseases. The basic assumption and suggestion proposed here is that we have to pay particular attention to immunological anamnesis of each individual to reconstruct as accurately as possible the own immunobiography. Immunobiography goes beyond the simple, erratic measurement of immunological parameters (e.g., immunoglobulin level and lymphocyte subsets, or antibody titer in the blood at a certain time point). We think that an effort is required to put all the immunological information regarding a single person altogether, in a standardized and easily accessible way (chip?). This integrated perspective is at present largely neglected, likely because of a lack of standardized tools to collect the information necessary to describe the immunobiography of each individual. Information regarding the type of delivery (natural *vs* caesarian), of early nutrition (breast *vs* bottle feeding) and diet, the use of antibiotics, the composition of microbiota, the different types, sequence and number of infectious diseases and vaccinations, to mention only a few, is extremely informative in order to predict individual's immune responses. Not less important are the data regarding ethnicity, socioeconomic, and psychological status that are an integral part of immunobiography.

As discussed all along this review, the knowledge on trained immunity in aging is still very scanty; accordingly, new experimental data are necessary to clarify the possible role of trained immunity in immunosenescence and inflammaging. At present, the available data, summarized in the previous paragraphs, are compatible with different possible scenarios. Trained immunity could undergo a functional impairment/decline with age, thus contributing to immunosenescence. However, it is also possible that trained immunity is hyperactivated with age, thus contributing to inflammaging and exerting deleterious effects on the onset of age-related chronic diseases (125, 126). Indeed, the main feature of cells of trained immunity is an enhanced production of pro-inflammatory cytokines, such as TNF-α, IFN-γ, and IL-1β in response to a subsequent challenge (127), and the receptors of innate immune cells can bind not only pathogen components but also "self" components (DAMPs) (1). LPS and other PAMPs can train monocytes/macrophages to become more pro-inflammatory when exposed to a second stimulus, but can also be rendered less responsive to pathogen or PAMPs through induction of tolerance or immunosuppression. The factors that determine whether a pathogen or a PAMP induces a state of trained immunity or tolerance/immunosuppression is unclear but may be influenced by the dose, timing, and nature of the exposure to the pathogen or PAMP. Moreover, factors related to immunological history and the life experiences could influence the trained immunity favoring one response or the other. This observation is particularly interesting for old persons characterized by a high heterogeneity that could be at least in part explained by different responses of monocytes/macrophages and other cells of the innate immunity to stimuli as a consequence of the different conditions they have experienced throughout life.

An urgent public health problem is to understand the immunological basis of the unresponsiveness to vaccines observed in a consistent percentage of elderly. On the basis of what we have discussed in this review, we surmise that the response to vaccines, in terms of both trained immunity and adaptive memory, is depending not only on the type/dose of immunological stimuli/vaccines that are encountered but also on the host immunobiography that shapes the responses of the IS. We surmise that this component, if adequately considered, will contribute to understand the poor responsiveness to vaccines and to newly encountered pathogens observed in a consistent number of elderly.

AUTHOR CONTRIBUTIONS

All authors checked literature data articles and reviews. MC, SS, PG, ME, and DM wrote the paper and critically discussed literature data; CF wrote the paper and coordinated the research.

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