THE AGING IMMUNE SYSTEM AND HEALTH

EDITED BY: Valquiria Bueno, Rafael Solana and Annemieke Boots
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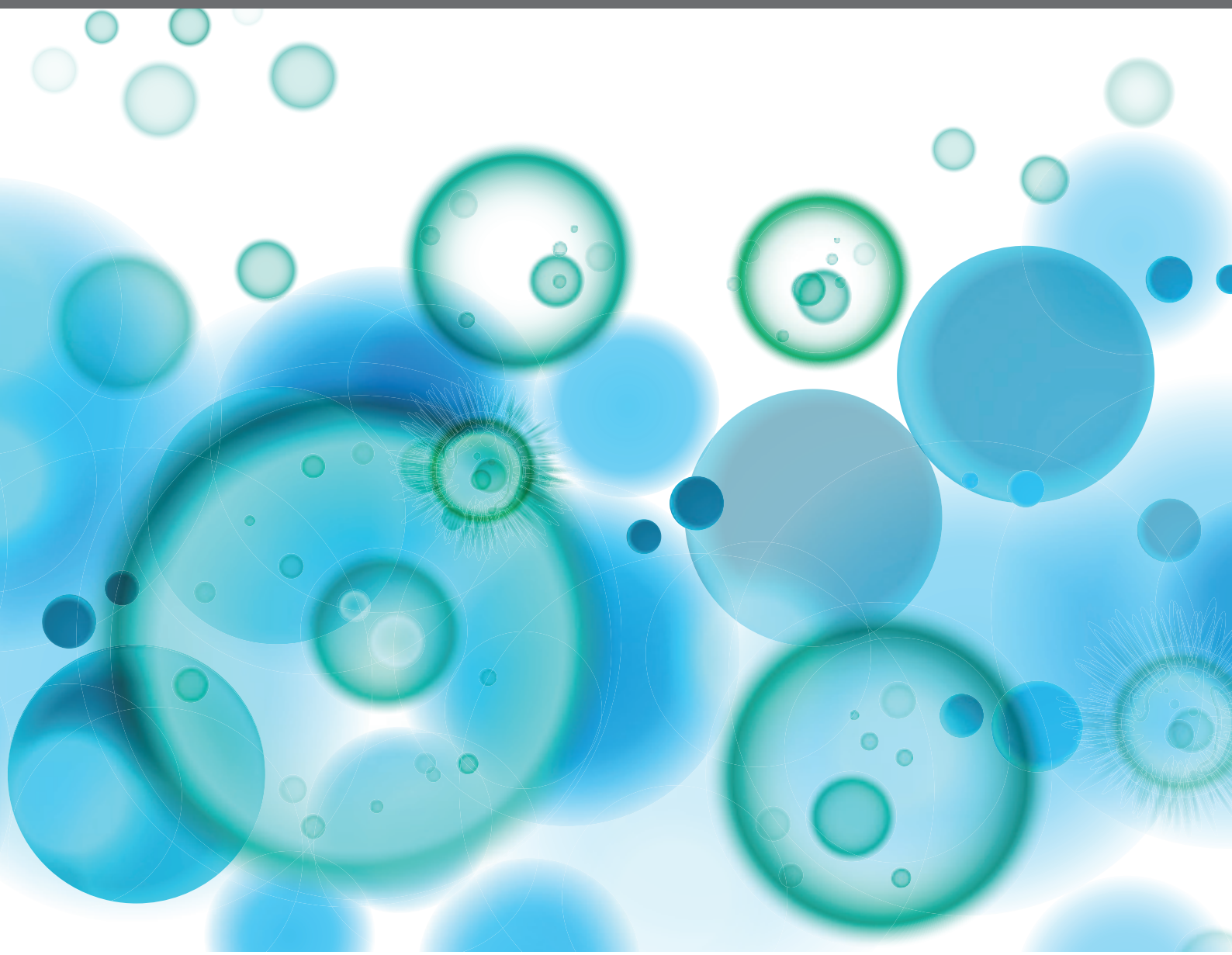

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THE AGING IMMUNE SYSTEM AND HEALTH

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The world population presents an increased percentage of individuals over 65 years old and the fastest growing subgroup is over 85 years old. The increase in life expectancy observed in the last century has not been synonymous with extra years lived in good health (disability-free years). Population studies have shown that as individuals age, they can present a great heterogeneity of ability and health. Therefore, aging has been associated for some individuals with disabilities and hospitalizations. Deaths related to infectious pathogens are increased in the aging population mainly due to pneumonia and influenza whereas Cytomegalovirus, Epstein-Barr virus, among other viruses seem to contribute to the low-grade inflammatory process observed (inflammaging).

Aging is a complex and multifactorial process in which functions of the organism are adjusted (remodelled) in order to deal with damaging events during life. One of the most important changes in aging individuals occurs in the immune system (innate and adaptive responses) with consequences such as poor response to new infections and vaccinations; increased susceptibility to cancer development and autoimmune diseases; frailty, and organ dysfunction. In addition, it has been proposed that immunosenescence not only reflects the aging of the organism but also contributes to this process. Bone marrow presents decreased hematopoiesis, the thymus undergoes involution and lymphoid organs (lymph nodes, spleen) also present reduced functionality. Therefore, cells derived, matured, or residing in these tissues decline in number and function. These changes have been identified in experimental models, in vitro conditions, peripheral blood, and biopsies via biomarkers such as cell phenotype, stimulus-induced proliferation, cytokines and antibodies levels. Telomere length and telomerase activity also decline in bone marrow-derived and peripheral blood cells and have been shown to play a role in immunosenescence. More recently, the investigation of short non-coding RNA molecules (microRNAs; miRNAs) pointed to this system as a possible control of aging-related mechanisms.

Data obtained on these markers for aging individuals could lead to the generation of a marker panel for pathology prediction, to indicate interventions, and to evaluate the efficacy of interventions. Interventions such as nutrition supplements, exercise, vaccination (different dose, concentration of antigen, adjuvants) have been proposed to circumvent age-related diseases. Considering the heterogeneity in the aging process, further investigation is vital before the indication of interventions

for aging individuals. As the extension of life expectancy is a reality, it is a challenge to understand how the aging population copes with the remodelling of the organism and how interventions could provide longevity in good health.

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Septic Shock and the Aging Process: A Molecular Comparison

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Aging is a continuous process promoted by both intrinsic and extrinsic factors that each trigger a multitude of molecular events. Increasing evidence supports a central role for inflammation in this progression. Here, we discuss how the low-grade chronic inflammation that characterizes aging is tightly interconnected with other important aspects of this process, such as DNA damage, mitochondrial dysfunction, and epigenetic changes. Similarly, inflammation also plays a critical role in many morbid conditions that affect patients who are admitted to Intensive Care. Although the inflammatory response is low grade and persistent in healthy aging while it is acute and severe in critically ill states, we hypothesize that both situations have important interconnections. Here, we performed an extensive review of the literature to investigate this potential link. Because sepsis is the most extensively studied disease and is the leading cause of death in Critical Care, we focus our discussion on comparing the inflammatory profile of healthy older people with that of patients in septic shock to explain why we believe that both situations have synergistic effects, leading to critically ill aged patients having a worse prognosis when compared with critically ill young patients.

Keywords: aging, systemic inflammation, immunity, sepsis, critical care

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INTRODUCTION

Over time, improved health conditions have led to a steady growth in the older population, resulting in a substantial increase in the number of critically ill-aged patients. In addition, advanced age is associated with a worse outcome in all of the most frequent critical care conditions (1).

Chronic, low-grade, systemic inflammation, and the deregulation of several innate and acquired immune responses have been reported in seniors (2). The signaling pathways implicated in this scenario, called inflammaging, create a complex network (3–7) that is probably triggered and perpetuated by prolonged exposure to varied exogenous and endogenous factors, such as infection, tissue injury, DNA damage, mitochondrial dysfunction, intestinal barrier failure, and dysbiosis (8–10) and may contribute to the increased risk of acute illnesses, disability, and death in this population (11). Indeed, inappropriate inflammation and metabolic stress lead to the accumulation of senescent cells, which are characterized by transcriptional and epigenetic alterations that determine cell cycle arrest, as well as aberrant mRNA production and maturation, chromatin structure changes, and impaired proteostasis (12, 13). Moreover, emergency myelopoiesis and the persistence of immature myeloid cell progenitors have been shown to be significant contributors to dysfunctional inflammation in sepsis and are emerging in aging research (14, 15).

Pathogen-associated molecular patterns are molecules shared by many microorganisms, but not found in mammals, that are recognized by the immune system and activate cell defense. Several host factors are also able to alarm the immune system, even in sterile conditions. Indeed, persistent or recurrent contact with microbes (16), as well as with non-infectious danger signals, induce the

production of damage-associated molecular patterns (DAMPs), which accumulate during aging (17). Examples include adenosine triphosphate, high mobility group box 1 protein, oxidized lipoproteins, heat shock proteins, and urate and cholesterol crystals. These DAMPs are able to activate membrane receptors and cytosolic receptors (including inflammasomes) or act directly at the nuclear level, inducing gene transcription (16, 18). Moreover, accumulating evidence suggests that mitochondrial and genomic DNA and histones also activate danger signals and induce systemic protection (19). For example, levels of circulating cell-free DNA, presumably released from damaged or dying cells, are increased in older adults (20) and are associated with both mortality and the magnitude of the inflammatory response (21–23). Similarly, critical inflammatory conditions, such as sepsis, are also characterized by high levels of circulating host DNA (24, 25).

Many nucleic acid molecular sensors have been found (26). CpG-enriched DNA, such as mitochondrial and bacterial DNA, are mostly recognized by TLR9 (21, 27), but other systems to detect not only mitochondrial and microbial DNA but also the nuclear DNA that migrates to the cytosol under pathologic conditions have been described (28–31). Furthermore, inside the nucleus, a sophisticated system of DNA sensors is able to detect DNA damage and activate immune signaling (31). In parallel, harmful DNA coming from pathogens, apoptotic cells, or DNA replication byproducts can be directly degraded by DNases to avoid the excessive activation of immune cascades (31).

Enduring Permanent Aggression: From Mitochondrial Dysfunction to Genomic Instability

Aging is accompanied by a decline in mitochondrial function in all tissues; however, some tissues, such as the muscles, are particularly affected (32). Beyond their function in bioenergetics, growing research suggests that mitochondria participate in many other mechanisms that are deregulated in senescence (33). Indeed, mitochondria are important organelles in the maintenance of stem cells (12), the activation of the unfolded protein response (34), the regulation of innate and adaptive immune pathways (35, 36), and the modulation of the metabolic profile of the cell (32). As such, mitochondria are deeply integrated into cellular homeostasis (37).

Similarly, mitochondrial dysfunction is a common finding in a wide range of patients in critical care conditions (38–41). Pro-inflammatory mediators and oxidative stress impair the function of the respiratory chain enzyme complexes and damage the mitochondrial structure, including their genes (42, 43). While the genomic DNA can be affected in a similar manner, the mitochondrial DNA is likely more vulnerable to this type of damage due to its close location to the electron transport chain, its lack of protective histones, the limited efficiency of the mtDNA repair mechanisms, and the fact that, like bacterial DNA, it exclusively contains coding regions (44, 45). However, a single-cell contains thousands of mitochondrial genomes, and mutations of all of them in the same gene are unlikely, putting the nuclear genome at a higher risk (46). An excellent publication from Patananan et al. describes the current challenges to therapeutically modifying the mitochondrial genome and the important concept of heteroplasmy (47). Furthermore, emerging evidence suggests that DNA damage activates signaling from the nucleus to the mitochondria, creating complex networks that are crucial for mitochondria maintenance (48).

The genetic lesions arising from DNA damage include point mutations, translocations, gains, losses, strand breaks, and telomere shortening. DNA lesions occur frequently, even under physiological conditions (49). These genetic changes have a well-established impact on the aging process and have been largely investigated as both a cause and consequence of chronic low-grade inflammation. The same process may occur in the critically ill; however, it seems to occur over a short-time period and be massive in this population, while it is low-grade and persistent during aging.

The occurrence of DNA damage induces further immune activation, promoting a vicious circle with disastrous consequences (50). Indeed, there is increasing evidence pointing to reciprocal interactions between DNA damage, DNA repair, and the immune system (51). In the short term, depending on the intensity of damage or the presence of deficient DNA repair responses (52–54), genotoxic stress has the potential to induce aberrant cell responses, apoptosis, organ failure, and immunosuppression (19, 43). The occurrence and magnitude of acute DNA lesions in the setting of severe systemic inflammation, however, remain to be confirmed. In the long term, these plausible DNA mutations and deletions can significantly impact patient quality of life and predispose the survivors of inflammatory catastrophes to several morbid consequences. In this regard, a recent publication from our group showed that sepsis induces telomere shortening (55), confirming that inflammation affects telomere length (56) and that stress-induced premature senescence is a telomere-dependent process (57). Despite that, however, the other evidence in the literature addressing this topic is controversial and/or indirect. The phenotype of sepsis survivors, for example, resembles accelerated aging, and sepsis survivors suffer from a higher risk of additional morbidities, such as cardiovascular disease, cognitive impairment, tumor progression, and possibly death, for years following the sepsis event (58–60).

Since genomic instability is a hallmark of aging, genetic damage secondary to severe infection or other causes of critical illnesses may have a larger impact in seniors than in young patients. We believe that this is a critical factor that partially explains the worse outcome of older people, compared with the young, when affected by overwhelming inflammatory syndromes.

Redox reactions generate oxidatively modified signaling biomolecules that are crucial for the generation of appropriate innate and adaptive cell responses (35) and for many other fundamental biological processes (61–63). Reactive oxygen and nitrogen species have important physiological effects and display various well-established specific signaling functions, but they need to be tightly regulated; otherwise, they can generate significant tissue damage (64). Oxidative damage occurs to a larger extent both in older people and in the presence of acute or chronic inflammatory processes. It can lead to substantial DNA damage and significantly contribute to genomic instability and mitochondrial

dysfunction. A DNA microarray study performed by our group found that the oxidative phosphorylation and the mitochondrial dysfunction pathways were the most-enriched pathways in septic patients of advanced age when compared with the young septic group (65).

The Epigenetic Code: An Additional Layer of Complexity

Epigenetic changes involve various histone marks, DNA methylation, nucleosome positioning, and mechanisms governed by non-coding RNAs that are able to repress or activate transcription (66). Epigenetic alterations are important aspects in the regulation of aging, linking environmental factors with the genetic profile (67). We believe epigenetic modifications may be implicated in the global modifications to the cell response that manifest during the evolution of catastrophic inflammatory processes, especially those caused by overwhelming infection. In support of this hypothesis, a recent publication reported that sepsis in humans induces selective and precise chromatin modifications in distinct promoter regions of immunologically relevant genes (68). Cellular dysfunction secondary to genetic and epigenetic changes in the course of major inflammatory syndromes may be stochastic and partially reversible, even though some organs and tissues appear to be more strongly affected. Moreover, DNA regions that are robustly activated are likely more influenced since they are less protected than the heterochromatin. Unfortunately, this topic remains obscure in the critically ill. However, it could potentially explain, for example, the long-term cognitive impairment that is frequently detected in survivors of septic shock and other intriguing findings, such as the phenomenon of endotoxin tolerance (69).

There is a significant interconnection between the DNA damage response and epigenetic changes. DNA damage is a serious threat to cell viability, compromising the integrity of both the genome and the epigenome. The DNA damage response can lead to significant alterations in chromatin structure, affecting chromatin components and epigenetic marks, with major implications for cell metabolism (70). As stated by López-León and Goya, "aging seems to be characterized by a progressive depression of the transcriptional activity of chromatin" (71); this has been partially attributed to a reduction in DNA methylation and an altered chromatin architecture (72). The end result of epigenetic changes is aberrant gene expression, reactivation of transposable elements, and genomic instability (73).

There is increasing evidence that together with the above discussed factors, microRNAs and long non-coding RNAs also play a key role in fundamental epigenetic processes, with important implications for the aging process and various morbid states (74–76).

Aging and Critical Illnesses: From Low-grade to Explosive Inflammation

The treatment of critically ill aged patients is challenging. Older people frequently exhibit atypical symptomatology, due to comorbidities and dysfunctions throughout all body systems that are related to the aging process (77).

Sepsis is a disease of the elderly. The incidence of sepsis increases exponentially with age, and sepsis-associated long-term sequelae particularly affect older patients. Sepsis survivors are at substantial risk for poor quality of life, functional disability, and cognitive impairment. As advances in medicine and quality of life extend the life expectancy worldwide, a growing number of aged patients need critical care (78). A recent study demonstrated a significant rise in survivorship after sepsis in the United States, caused by a rising incidence of sepsis rather than improvements in its case fatality rate, generating a substantial population burden of aged patients with disabilities (79).

The reason for the higher susceptibility to infection and increased mortality in older adults remains in debate (80). The basal inflammatory state found in healthy seniors suggests that aged people possess a limited capacity to control inflammation. Similarly, the critically ill are frequently affected by overwhelming inflammatory syndromes, where the host response is the major cause of damage. Examples include diseases such as septic shock, severe acute pancreatitis, burns, trauma, ischemia reperfusion injuries, and hemorrhages. As discussed earlier, the chronic low-grade inflammation in the elderly and the explosive inflammation in the critically ill share several commonalities. We propose that, together, these processes may have synergistic effects, leading to a worse outcome (**Figure 1**).

Notably, these synergistic effects have interesting peculiarities. A study performed by our group found that older people are as immunocompetent as young individuals regarding the cytokines, chemokines, and growth factors produced in response to devastating infections. After our analysis of several inflammatory mediators in the plasma of critically ill individuals, we were unable to find any reason that could serve to better explain why the aged show an increased susceptibility and mortality to septic shock (81). As detailed in the section below, this phenomenon can be partially explained by the fact that aged people probably display a prolonged inflammatory systemic response under acute stress conditions, when compared with the systemic response of the young, even though both groups share the same ability to trigger and sustain the same intensity of inflammatory signaling in the acute phase (81). Moreover, in a study performed in rats, we were able to demonstrate that despite a similar systemic response, aged rats show increased intestinal gene expression levels of TNFα, α-defensin 5, and α-defensin 7, when compared with young rats (82). Similarly, other work from our group demonstrated greater gene expression levels of COX-2 and intercellular junction proteins in the guts of aged rats with acute pancreatitis when compared with young rats in the same conditions, suggesting that, in situations of intestinal damage, the young animals are better able to restore intestinal barrier integrity (83). The results of these studies strongly suggest that the inflammatory response of the elderly is compartmentalized, with significant differences in the inflammatory profile depending on the organ under investigation.

For many years, the catastrophic systemic inflammation associated with many critical care diseases has been attributed to a massive and transient activation of the innate immune system, followed by a period of immunosuppression (84, 85). Seminal high-throughput gene expression studies performed

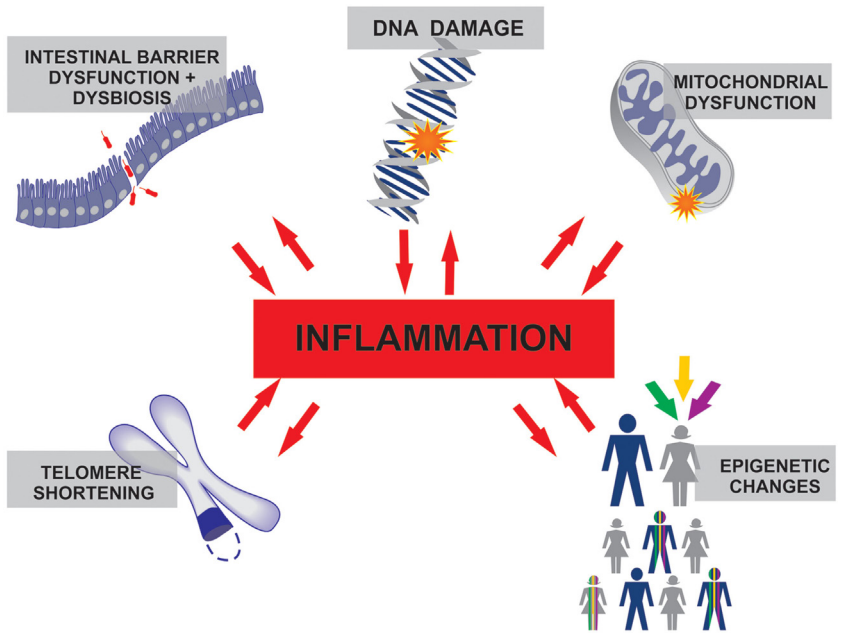

FIGURE 1 | The inflammation that characterizes healthy aging and the inflammatory processes in several critical care conditions differ in duration and intensity, but both involve similar molecular interconnections where inflammation plays a central role.

in septic patients by the Wong group, however, challenge this theory. Indeed, instead of the classical biphasic curve, they have consistently detected an elliptical curve, formed by the persistent activation of innate immune genes in conjunction with widespread repression of gene programs corresponding to the adaptive immune system (86–88). Confirming these findings, a similar pattern was found in trauma patients (89), suggesting that infectious and non-infectious systemic inflammation in the critically ill may involve analogous cell responses. Notably, more recent studies are finding subtle differences in the transcriptional program of different acute stress conditions and even in different subsets of the same morbid process (90).

Maintenance of the Intestinal Epithelial Barrier and the Human Microbiome

The intestinal mucosal barrier is a fundamental line of defense against undesirable luminal contents, such as microorganisms, toxins, and antigens, preventing their entrance into the bloodstream. It is mostly composed of epithelial cells, immune components, and mucus. Some researchers also consider the microbiome as part of the intestinal barrier (9, 91), since it helps to maintain the integrity of the intestinal barrier, providing nutrients and protecting against pathogens.

Aged people are in a persistent systemic inflammatory state that may be partially attributed to increased bacterial translocation, secondary to intestinal barrier dysfunction (92). As people age, the intestinal barrier weakens, partially due to decreased levels of tight junctions connecting epithelial cells, and the enteric immune system becomes ineffective (93). Indeed, higher plasma levels of lipopolysaccharide can be detected in the blood of older subjects, when compared with young individuals (94, 95). Furthermore, there is a shift in the intestinal microbiome after the age of 65, with an increased abundance of Bacteroidetes phyla (96) and a reduction in the capacity of the microbiota to carry out metabolic processes, such as short-chain fatty acid production (97). We propose that these previous alterations to the intestinal barrier in the elderly are probably exacerbated during systemic inflammation processes. In support of this idea, Zhang et al. recently demonstrated that neutrophil activation and aging are both affected by the intestinal microbiota and that depletion of the microbiome with broad-spectrum antibiotics significantly reduces the number of circulating aged neutrophils, ameliorating inflammation-related organ damage in a model of endotoxin shock (98). Another recent publication showed that germ-free mice do not display the increase in circulating cytokines that is a hallmark of aging and that co-housing germ-free mice with old, but not young, conventionally raised mice reconstitutes this phenotype; the authors concluded that, in mice, intestinal permeability increases with age due to microbial dysbiosis (99). Taken together, these observations suggest that the increased mortality of aged patients in critical care conditions is probably due to a prolonged systemic inflammatory response, at least partially caused by increased bacterial translocation and defective bacterial clearance (100).

Microbiome studies are challenging because there is extensive inter-individual variability, even among healthy subjects. Genetic, lifestyle, and environmental factors, such as diet, physical activity, geography, and exposure to xenobiotics, all cause substantial modifications to the intestinal microbiome. However, despite this extensive interindividual variability, specialists agree on the existence of a global human core microbiota (101, 102).

Commensal bacteria have much shorter generation times than humans and consequently undergo rapid evolutionary changes, adapting quickly to environmental changes. Unfortunately, external forces sometimes shape a microbiome that is detrimental to the host, a state called dysbiosis. Once established, dysbiosis can exert profound effects on the immune system, creating a feedback loop in which host factors and the microbiome (cell components and metabolites) regulate each other, perpetuating the dysbiotic state (103). It is well established that the intestinal microbiota is severely modified during critical illnesses. However, it remains unclear which change occurs first.

The causal mechanisms of dysbiosis in the critically ill are not completely understood, but they likely result from many intrinsic and extrinsic factors, such as widespread antibiotics use, hypoxic injury, inflammation, intestinal dysmotility, epithelial barrier disruption, vasopressors treatment, and sedation (104). The intestinal microbiome of the critically ill differs substantially from that of healthy individuals and is characterized by lower phylogenetic diversity, commensal microbe loss, and pathobiont overgrowth (105, 106).

CONCLUDING REMARKS

Older adults in critical care conditions develop a peculiar inflammatory response, which is associated with poorer outcomes. Current treatments are unspecific and mainly rely on life support techniques. Novel strategies are under investigation (99), and Personalized Medicine has been widely discussed to improve care of the critically ill (107); however, to a large extend, these proposals still remain experimental and hypothetical, without impacting clinical applications. Thus, manipulation of the inflammatory storms that are so frequent in Critical Care remains a challenging task, filled with negative results and nebulous findings. Prolonged hospital stays, recurrent infections, decrepitude, and malnutrition characterize the critically ill population as a whole, but particularly apply to the subset composed of aged adults.

By the other hand, impressive advances in the molecular biology of aging are emerging. Biomarkers of aging have been extensively investigated to guide tailored treatments of the aging process, as well as to detect individuals that age faster (108). Despite the current challenges (109–111), *in vivo* partial cellular reprogramming (112), direct reprogramming (109), and epigenetic interventions (67) are tentative highways for drug development and captivating platforms to reach this goal. Indeed, the rapid advancement of scientific knowledge in this field provides hope that, in a not-so-distant future, sophisticated medical technologies to delay and even reverse normal aging might be available.

AUTHOR CONTRIBUTIONS

FPS conceived and wrote the manuscript. MCCM contributed with suggestions and ideas.

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Aging, Obesity, and Inflammatory Age-Related Diseases

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The increase in the prevalence of obesity represents a worldwide phenomenon in all age groups and is pathologically and genetically correlated with several metabolic and cardiovascular diseases, representing the most frequent age-related diseases. Obesity superimposed on aging drastically increases chronic low-grade inflammation (inflammaging), which is an important link between obesity, insulin resistance, and age-associated diseases. Immune cells of both the innate and the adaptive immune systems infiltrate the adipose tissue (AT) and during obesity induce inflammatory responses associated with metabolic switches and changes in phenotypes and function of immune cell subsets. Obesity poses new health problems especially when it occurs in the context of other diseases, many of them frequently affect elderly subjects. An emerging problem is the decreased proportion of patients with obesity achieving clinical response to therapy. In this review, we will discuss the reciprocal influences of immune cell and AT inflammation in aging and age-associated diseases and the complex relationship of nutrient and energy-sensing homeostatic checkpoints, which contribute to shape the phenotype of the AT. We will specifically examine type-2 diabetes, rheumatoid arthritis, osteoarthritis, cognitive impairment, and dementia, where obesity plays a significant role, also in shaping some clinical aspects.

Keywords: aging, obesity, inflammation, type-2 diabetes, rheumatoid arthritis

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INTRODUCTION

The increase in prevalence of overweight and obesity represents a worldwide phenomenon, which is associated with several chronic diseases such as type-2 diabetes (T2D), cancer, rheumatoid arthritis and osteoarthritis (OA), cognitive impairment and dementia, and those affecting the cardiovascular (CV) system.

The global obesity pandemic affects all age groups. Recent studies examining body mass index (BMI) data in 68 million people in 195 countries showed both increased prevalence and disease burden of high BMI subjects globally over the past 20 years (1). Although the prevalence of obesity among children is lower than in adults, its rate of increase exceeds that of adults (2). The global burden of disease related to high BMI is calculated in individuals without underlying conditions, and it increases at a slower pace in adults mainly because of the reduction of other risk factors for CV diseases and for effective clinical intervention. However, increased BMI has been shown to be pathogenetically related to several diseases. Among these, insulin resistance (IR) and T2D have a strong link to obesity, and the metabolic syndrome represents a cluster of risk factors for severe CV events (coronary artery disease, stroke). Obesity superimposed on aging represents an additional risk factor for older age groups in which the prevalence of chronic disease as well

as the occurrence of complications increases (3–5). The disease burden of high BMI in children (≤18 years of age) has not been addressed in the same detail.

The aging process is characterized by a state of chronic inflammation, known as inflammaging. Several factors contribute to inflammaging, including polymorphisms in the promoter regions of pro-inflammatory genes, chronic stimulation of immune cells with viruses such as cytomegalovirus, changes in the gut microbiome, and increased permeability from the intestine [reviewed in Ref. (6)]. It has been recently proposed that continuous engagement of innate receptors by endogeneous signals such as damage-associated molecular patterns drives a chronic state of background inflammation, which needs to be counterbalanced by anti-inflammatory mechanisms. Cellular senescence and the acquisition of the senescence-associated secretory phenotype (SASP) by fibroblasts (7) and endothelial (8) and immune cells (9–11) has also been pinpointed as a significant contributor to inflammaging. Cell senescence induces the accumulation of terminally differentiated B, T, and NK cells with dysregulated function through the activation of pathways integrating senescence and energy-sensing signals.

Inflammaging is an important link among obesity, IR, aging, and age-associated diseases such as cognitive impairment, atherosclerosis, cancer, and autoimmunity. Elevated pro-inflammatory cytokines are associated with decreased insulin sensitivity. Chronic low-grade (sterile) inflammation causes IR, which leads to the transition from metabolically normal obesity to metabolic syndrome. This occurs through both systemic inflammation and metaflammation (12), a process whereby excess nutrients promote chronic low-grade inflammation, and whose metabolic hallmarks are high levels of lipids, free fatty acids (FFAs), glucose, and reactive oxygen species (ROS).

Immune cells of the innate and adaptive immune systems infiltrate insulin responsive tissues, such as the visceral adipose tissue (VAT) and with obesity incite inflammatory responses. Immune cells (macrophages, T, B, NK, NKT cells, and neutrophils) have been implicated in adipose tissue (AT) inflammation and IR (13–17). Inflammation leads to local and systemic increases in pro-inflammatory molecules, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, interferon (IFN)-γ, inflammatory adipokines, chemokines, and FFAs [reviewed in Ref. (16)].

LINKS OF OBESITY TO INSULIN RESISTANCE (IR) AND T2D

IR is the lack of appropriate response to circulating insulin in several tissues, including liver, muscle, and AT (18). It frequently associates with obesity, hypertension (integrating features of the metabolic syndrome), and CV disease and typically precedes the onset of T2D. In the pancreas, β-cells adapt to hyperglycemia with an expansion of the total β-cell mass and with increased secretion of insulin (hyperinsulinemia), which is able not only to control normal levels of glycemia but also can induce β-cell stress, causing β-cell failure, and then T2D (19). Poor glycemic control in individuals with T2D results in severe complications, such as renal failure, blindness, neuropathy, and CV disorders (20).

It is not completely clear how obesity causes the development of IR. Although many molecular mechanisms have been proposed, including ER stress, oxidative stress, dysregulation of lipid homeostasis, mitochondrial dysfunction, hypoxia, and impairment of the insulin signaling pathway in insulin-responsive cells, there is evidence that obesity-induced inflammation may be a key factor for IR (21). **Figure 1** summarizes the main pathways leading to inflammation in the obese AT.

Production of Pro-inflammatory Mediators in the Obese AT

High levels of TNF-α in the AT are associated with chronically elevated basal lipolysis, the process of hydrolysis of tryglycerides to release FFAs and lipids (22). These provide chronic stimulation to macrophages leading to FFA-induced TNF-α production, causing IR. It has been proposed that adipocyte-derived TNF-α contributes to elevated levels of FFAs in the blood of obese individuals (22), and neutralization of TNF-α *in vivo* in obese mice decreases circulating levels of FFAs (23). TNF-α has also been shown to reduce the expression of proteins stabilizing lipid droplet (perilipins) (24), leading to ectopic lipid deposition in insulin-sensitive tissues. Lipids and lipid-derived molecules have direct effects on insulin-sensitive tissues and induce IR (25).

Other major pro-inflammatory cytokines released by the obese AT are IFN-γ secreted by $CD8^+$ T cells (26) and NK cells (27) and IL-17 secreted by $CD4^+$ T cells (28).

Hypoxia and Release of "Self" Antigens in the Obese AT

During the development of obesity, the supply of oxygen to the expanding AT becomes inadequate, resulting in areas of hypoxia (29, 30). This phenomenon of poorly oxygenated AT not only activates the transcription factor hypoxia-inducible factor-1α (HIF-1α) and further release of pro-inflammatory cytokines (31) but also induces cell death and release of "self" antigens, which stimulate class switch and the production of IgG pathogenic antibodies. Hypoxia in the AT has been the only mechanism suggested so far for the release of "self" antigens in the obese AT.

Immune Cell Infiltration in the Obese AT

Data from obese mice and humans have indicated that the hypertrophied AT becomes heavily infiltrated by a variety of immune cells displaying a pro-inflammatory phenotype, characterized by secretion of SASP markers (32), and their numbers inversely correlate with insulin sensitivity. Cells with an anti-inflammatory phenotype have also been reported in the obese AT, but these cells are present at low frequencies. These are B1 B cells producing IL-10 (15, 33) and innate lymphoid cells type 2, which produce large amounts of Th2 cytokines such as IL-4, IL-5, and IL-13 (34). Tregs have also been reported but only in the lean AT (35).

Macrophage infiltration within the AT has been considered a major driver of inflammation, due to the secretion of pro-inflammatory cytokines and chemokines involved in the

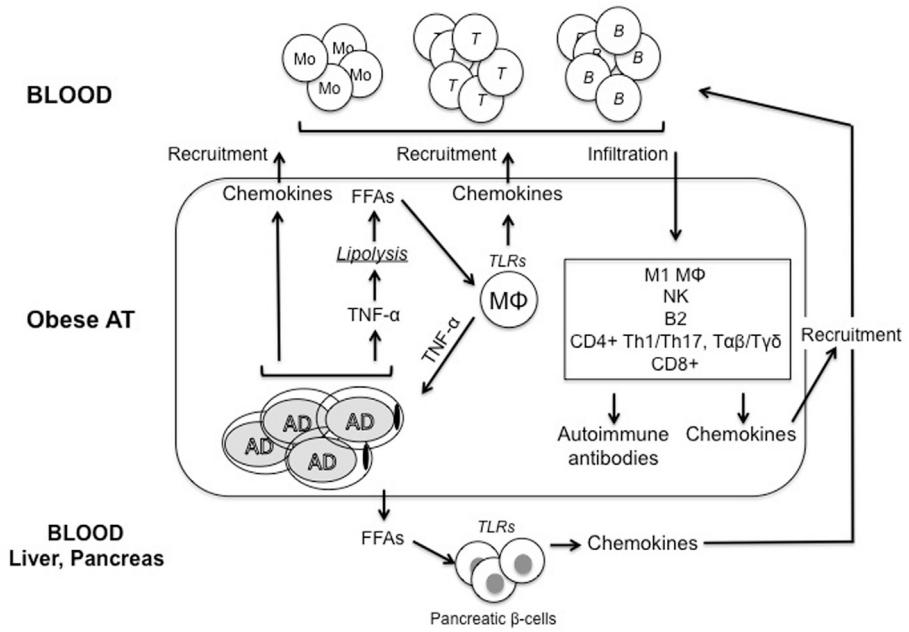

FIGURE 1 | Model for regulation of inflammatory pathways in the obese adipose tissue (AT). Adipocytes (AD) in the obese AT are highly inflammatory and secrete several pro-inflammatory cytokines and chemokines, which recruit immune cells, thus contributing to the establishment and maintenance of local and systemic inflammation. Among these inflammatory mediators, tumor necrosis factor (TNF)-α released by both AD and immune cells induces lipolysis and release of free fatty acids (FFAs), which activate tissue-resident macrophages (MΦ) to release cytokines and chemokines. FFAs are also released in blood and cause both insulin resistance and inflammation in major insulin target tissues. Immune cells recruited to the obese AT differentiate into inflammatory subsets and secrete additional pro-inflammatory mediators. We hypothesize that these cells would generate suboptimal immune responses in obese individuals by circulating to peripheral lymphoid organs. Pathogenic antibodies may be secreted by B cells in the AT. These antibodies may form immune complexes with "self"-antigens, which in turn activate complement and Fc receptors on immune cells, leading to enhanced local inflammation, remodeling of the AT, impairment of adipocyte function and nutrient metabolism, and exacerbation of obesity-associated conditions. These antibodies can also exert additional detrimental effects both locally and systemically targeting distinct clusters of self proteins. One mechanisms for the release of "self"-antigens in the obese AT is the decreased supply of oxygen, resulting in areas of hypoxia, which leads to further release of pro-inflammatory cytokines, as well as to the release of "self"-antigens, such as intracellular proteins, cell-free DNA, and lipids.

recruitment of immune cells to the AT. However, adipocytes also secrete pro-inflammatory mediators (cytokines, chemokines, and adipokines) and in larger amounts compared with immune cells (36). Therefore, with obesity, a crosstalk between adipocytes and the immune cells infiltrating the AT contributes to the establishment of chronic inflammation, a prerequisite for IR. Macrophages in the AT are almost exclusively M1, they depend on glycolysis for their inflammatory function, and their stimulation in the AT induces glucose transporter expression and glucose intake and utilization (37). Hypoxia (via HIF-1α) potentiates glycolysis and stabilizes the inflammatory phenotype (38). In M1 macrophages, the inflammasome NLRP3 activates caspase 1 and the secretion of IL-1β (39), which is directly toxic to pancreatic β-cells and induces IR (40). Increased inflammasome activity has been reported in monocyte-derived macrophages from T2D patients (41).

T cells in the AT are Th1 $CD4^+$ and IFN-γ-producing $CD8^+$ T cells (26). These promote secretion of pro-inflammatory cytokines from M1 macrophages leading to both local and systemic IR (42). Similar to macrophages, T cell subset skewing in the AT occurs through modulation of substrate metabolism regulated by hormones (leptin) and intracellular nutrient sensing kinases, such as AMPK/mTOR (43). Th1 $CD4^+$ T cells express high levels of membrane glucose transporters and are highly glycolytic (44), a trait supporting inflammatory responses.

Interferon-γ, the signature Th1 cytokine, induces macrophages and T cells to secrete chemokines, which recruit immune cells to the obese AT (45, 46). Moreover, IFN-γ facilitates the M2 to M1 polarization (47) and decreases insulin receptor signaling by reducing the expression of insulin receptors and glucose transporters (48). IFN-γ production is regulated by T-bet, a T-box family transcription factor first identified as a transcriptional inducer of IFN-γ in $CD4^+$ T cells (49). T-bet plays a critical role in the development of IR in animal models of obesity, and T-bet-deficient mice fed a high-fat diet are refractory to the induction of IR (50). These mice show improved insulin sensitivity and glucose tolerance, reduced numbers of immune cells in the AT ($CD4^+$/$CD8^+$ T cells, NK cells, and macrophages), and reduced production of pro-inflammatory cytokines per gram of fat (IFN-γ, TNF-α, IL-1β, and IL-6).

Obese and T2D patients have alterations in the composition of their microbiome, with reduced proportions of Bacteroidetes (beneficial bacteria) in obese versus lean individuals (51). Moreover, it has been reported that the gut microflora regulates the development of obesity in animal models (52). T-bet regulates mucosal T cell activation (53), and T-bet deficiency alters

the composition of microflora (54). T-bet deficiency may also alter the microbiome in individuals with obesity leading to the inflammatory and metabolic processes that regulate T2D.

B cells also accumulate in the obese AT (15, 55, 56). B cell recruitment can initiate T cell-induced M1 polarization and IR. Obesity and hyperglycemia have direct influence on antibody production, and IgG secretion from inflamed VAT modulate the function of resident macrophages. It has been reported that B cells in AT are induced to produce pathogenic IgG autoantibodies, due to the dysregulated expression of autoantigens by hypoxic adipocytes. B cells also support the activation of inflammatory T cells, which are the main pathogenic drivers in systemic inflammation and IR.

Recently, a new lymphoid tissue called fat-associated lymphoid clusters (FALCs) has been identified in the mesenteric AT of mice and humans. FALCs are rapidly induced after inflammatory stimuli and support B cell proliferation and differentiation regulating antibody production within the AT (57).

OBESITY AND RHEUMATOID ARTHRITIS (RA): EVIDENCES AND MECHANISTIC LINKS

RA is a debilitating chronic autoimmune disease that causes synovial inflammation and destruction of joints including the cartilage and the adjacent bone. It generally occurs between the fourth and sixth decades of life and affects more women than men. It is characterized by joint stiffness, pain, and swelling and is accompanied by extraarticular manifestations and systemic inflammation. RA has been associated with muscle wasting and cachexia due to uncontrolled inflammation driven by TNF-α, which fuels hypercatabolism (58). However, BMI rarely falls below normal because loss of lean tissue is compensated by increased AT, and this characterizes rheumatoid cachexia, also called "cachectic obesity" (59, 60). It has been observed that in RA patients, despite adequate nutrient intake, but inflammatory cytokine dominance and reduced activity due to pain, joint deformity, and decreased muscle strength (61), cachexia appears to be similar to that occurring in aged subjects with disability. Abnormal body composition in RA can be defined as a sarcopenic obesity (62) with characteristic changes that in the elderly contribute to frailty (63, 64). Moreover, the percentage of obese RA patients has increased (65), and the impact of obesity on RA has become a relevant issue not much for the negligible risk of developing RA (66), but for its negative effects on disease activity, response to therapy, and CV risk. Obese RA patients are indeed less likely to achieve sustained remission in response to therapy with conventional chemical (4) or biologic (TNF-α inhibitors) agents (67). Despite opposite results with some treatments (68), obesity decreases the rate of remission in RA and negatively affects disease activity (69) and patient-reported outcomes during therapy (70).

The "Obesity Paradox" in RA

Obesity represents an important link with comorbidities such as metabolic syndrome (71) and CV diseases (72); however, in some studies, increased BMI had the opposite effect of reduced mortality (70, 73), which has been described as the "obesity paradox" (74). Moreover, in overweight RA patients, progression of bone destruction was reduced (75, 76), the number of swollen joints is not increased, and better quality of life has been reported (77). Weight loss and cachexia represent major determinants for a greater risk of death (78) and worse quality of life (77), thus strengthening the paradoxical observation of lower mortality in obese patients. However, follow-up studies have demonstrated that in RA patients with a history of obesity reduced BMI is strongly associated with death. Therefore, the "obesity paradox" does not entail a biologically protective role of obesity (73), raising the question whether the use of BMI is a valid tool for assessing obesity in RA (65).

High BMI contributes to disease activity in RA by affecting both biomechanics and the metabolic status, and obese RA patients show worse subjective assessment of symptoms (79). Hyperglycemia, as a part of the metabolic syndrome, is more common in early RA (80), whereas active RA shows decreased lipid levels (81) despite an increased risk of CV events, due to the lipid-lowering effect of systemic inflammation (82). An increase in VAT, e.g., the epicardial fat (83), and the more abundant macrophage infiltrate are associated with systemic inflammation, metabolic syndrome, and IR (84). Anti-TNF-α therapy improves insulin sensitivity in RA patients who are resistant, but despite controlling inflammation, it does not achieve the same extent of improvement in obese RA patients (85). In addition, even when therapy succeeds in the control of disease activity, it fails to restore the altered body composition and improve physical function (86). Adipokines (leptin, adiponectin, visfatin, resistin, and chemerin) have been postulated to be the mediators linking AT and RA activity (87). Adipokine imbalance may underlie the higher degree of inflammation (88), the levels of autoantibodies (leptin and adiponectin differentially regulate the generation of Treg cells, which are abundant in normal VAT), and also the lower amount of bone resorption observed in obese patients (89). On another level, the association of RA with both the metabolic syndrome and atherosclerosis is probably also mediated by VAT through altered secretion of adipokines. Therefore, adipokines contribute decisively to the systemic inflammation underlying RA, which represents an independent risk factor for CV diseases.

Since weight reduction may have possible contraindications (lower BMI being associated with accelerated mortality in RA), and the assessment of the inflammatory milieu of VAT in RA patients is still incomplete, much research has been devoted to uncovering the metabolic changes occurring in the development and chronicization of RA. This field has been recently reviewed (90) and can be summarized in the two distinct stages of early and chronic RA. In the first stage, there is a high metabolic demand in all cell types involved, due to proliferative signaling, angiogenesis, cellular de-differentiation, and unbalanced bone turnover. However, in RA T cells, at variance with other types of inflammatory metabolic changes, the glycolytic pathway is reduced in favor of the pentose phosphate shunt (91), reduced ROS generation, and decreased AMPK function. In early stages, AMPK activation (e.g., by Metformin)

may be an attractive target because its activity is decreased in several tissues of obese or IR patients. In the late (erosive) stage of RA, the inflamed joint is a hypermetabolic lesion (90), T cells undergo a metabolic switch to aerobic glycolysis due to hypoxic conditions, and mitochondrial dysfunction with increased lactate production causes acidification of the synovia. The reprogramming of T cells accounts for pro-inflammatory Th1/Th17 phenotypes and premature T cell aging (92). Several aspects of immunosenescence have been found to be relevant in RA pathogenesis (93–96), and rejuvenation of the immune system has been proposed as therapy, including mTOR inhibitors (97, 98).

Role of B Cells in RA Pathogenesis

The key role played by autoreactive B cells is highlighted by the presence of diagnostic autoantibodies, and rheumatoid factor (RF) (99) and anticyclic-citrullinated peptide antibodies (ACPAs) (100) are well-established indicators of disease and disease severity and may precede the onset of disease. The role of B cells in RA pathogenesis in the context of overweight/obesity has not been addressed yet and deserves thorough attention. A primary defect in early B cell tolerance has been detected since the majority of naive B lymphocytes express polyreactive autoantibodies, including RF and ACPA. These B cells are resistant to Fas-induced apoptosis and therefore not suppressed by Treg (101). However, B cells are involved in RA by other mechanisms, in a bidirectional support of helper T lymphocytes, as self-antigen-presenting cells, with the release of inflammatory mediators, and with the promotion of lymphoid neogenesis (which is prominent in RA synovitis). RF^+ B cells are able to take up IgG-containing immune complexes and present antigen to T cells, thus activating a reciprocally reinforced response (102).

The phenotype of B lymphocytes in RA has been extensively studied in peripheral blood and in synovial tissue, with some discordant data owing to examination of different stages of the disease. The general consensus is the increased presence of memory ($CD27^+$) B cells with an activated ($CD95^+$, $CD21^{low}$) phenotype both in peripheral blood and in the synovial compartment (103). These cells increased even more significantly after B cell depletion therapy (BCDT) with rituximab. There is also agreement on the fact that response to BCDT relies on elimination of memory B cells, and their repopulation, along with transitional B lymphocytes, may predict relapse (103). The role of homeostatic lymphoproliferation of both memory B cells and the extent of BCDT in bone marrow and synovial tissue represent critical points still to be elucidated. Since it has been observed that $CD4^+$ T cell activation decrease after BCDT (104), changes in not only B cell subsets but also T cell subsets may underlie the response of RA patients to therapy. The Treg compartment is less affected by RA treatments (105), but the presence of Breg lymphocytes (decreased in untreated RA) seem to play a role in balancing immune abnormalities and predict the treatment outcome (106). Cytokine production by B cell subsets is also relevant to RA pathogenesis and disease activity [reviewed in Ref. (107)], with inflammatory cytokines predominating in untreated severe RA, as activated memory B cells preferentially secrete TNF-α, whereas BCDT induced a shift to subpopulations producing IL-10. The recent identification of a subset of B cells able to produce large amounts of RANKL (108) provides a mechanistic link between activated memory B cells and bone resorption through induction of osteoclastogenesis. It is relevant to mention that ACPAs are associated with more joint and bone damage and that therapy does not eliminate ACPA-producing autoreactive B cells in the synovial tissue. The central role of B lymphocytes in RA pathogenesis and in tissue damage makes these cells and their products attractive targets for treatment; however, there is still uncertainty about the beneficial or even protective effects of B cell subsets.

OSTEOARTHRITIS (OA), AGING, AND OBESITY

In the elderly, arthritis is frequently associated with other diseases with multiple aging or degenerative features (109). OA and RA share common features in elderly patients and significantly contribute to disability (110). OA is usually differentiated from RA by age at diagnosis, duration of morning stiffness, pattern of joint involvement, and radiographic findings. Distinguishing between the diseases can be challenging, but in the >60 years of age group, OA is by far more common. Despite the fact that OA directly correlates with age, the real cause of this association is not clear, and OA development can be separated into aging-dependent and aging-independent processes (111–114). Both increased production of matrix metalloproteinases and cytokines, reduced levels of collagen type II synthesis, and increased production of ROS induce age-related changes in chondrocytes (114). These changes alter cartilage function, and sarcopenia further leads to decreased joint stability (115). Cellular senescence, impaired regeneration, and repair are recognized factors contributing to cartilage damage with aging (115, 116).

In patients younger than 60 years of age with symptomatic OA, joint pain and disabilities are less recognized as inevitable consequences of growing old, compared to OA patients older than 70 years (117). Several factors contribute to the development of OA: acute injury (including fracture), excessive mechanical overloading (113, 118), diabetes, and chronic tobacco smoking, all playing a role in the amplification of senescence-inducing stresses (118–121). These factors develop before symptoms appearance and may cause early onset of OA; multimorbidity including OA and obesity can be seen at an adult age (122). The prevalence of arthritis is increasing, with 29.3% ever reported doctor-diagnosed arthritis in individuals aged 45–64 years versus 49.6% in individuals aged 65 or older in the United States (123). However, obesity prevalence did not change significantly over time among middle-aged and younger adults with doctor-diagnosed arthritis (124) despite increasing significantly over time among older adults with RA and remaining also higher when compared with adults without RA. Obesity impacts progression of OA and has a negative influence on outcomes (125). Exercise and loss of at least 10% of body weight can effectively lead to improvement in symptoms, pain relief, and physical function. Physical activity may reactivate a regenerative process by mobilizing stem cells and increase proteoglycan production, restoring the cartilage structure (113, 115, 126).

COGNITIVE IMPAIRMENT, DEMENTIA, AND RELATIONSHIP TO AGE-ASSOCIATED DISEASES AND OBESITY

Our summary of conditions where inflammation, obesity, and aging converge in defining particular features and outcomes of disease must also briefly mention the dementias, whose prevalence has been reported to be declining among older US adults between 2000 and 2012 (127). However, dementia rates are growing at an alarming proportion in most regions of the world and are related to population aging (128). Prevalence varies in countries with different mean population ages. However, differences persist after adjusting for age (129). The decline in the United States occurred in those older than 65 years and was related to increased number of years in education despite the age- and sex-adjusted increase in the prevalence of hypertension, diabetes, and obesity in the same years. There is a long unresolved debate on the prodromal phase of the neurodegenerative disorders with inflammatory features, such as Alzheimer's dementia, but it is undisputed that prevalence of dementias of all types increase with old age, from about 2–3% among those aged 70–75 years to 20–25% among those aged 85 years or more (130). The known risk factors include obesity, depression, diabetes, decreased physical activity, hypertension, smoking, hypercholesterolemia, coronary heart disease, and alcohol use; and assessment of these provide an estimate of the risk of developing dementia (131) despite the fact that in the oldest-old (80–97 years old), these factors did not increase the risk for dementia, so that age plays a major role.

Taken together for the two most frequent types of dementia (Alzheimer's and Vascular) (129), vascular risk factors such as dietary fat intake, high cholesterol, obesity, T2D, and hypertension have emerged as the most important determinants (132). Vascular risk is seldom isolated and is accompanied by alterations in hormonal metabolism. Overweight/obesity, due to excess AT, increase the CV risk and also for late-onset dementia. This is exemplified in the prodromal phases of dementia, as vascular and metabolic parameters decline in direct relation to cognitive impairment and in a way which seems to differ from that occurring in "normal" aging. With regard to obesity, its presence at midlife is associated with an increased risk of dementia and Alzheimer's later in life (133), and in particular central obesity in midlife increases the risk of dementia independent of diabetes and CV comorbidities (134). The risk is reversed when late-life BMI is considered: underweight persons had an increased risk of dementia, whereas being overweight was not associated and being obese reduced the risk of dementia compared with normal BMI. This has been dubbed as an "obesity paradox" also in this case (135). A recent systematic review and meta-analysis suggests a positive association between obesity in mid-life and later dementia but the opposite in late life (136). A successive study confirmed the association of mid-life obesity and dementia, but that of being underweight and dementia remained controversial (137). It is difficult to draw a clear distinction between visceral adiposity and total body fat in most studies, and this is reflected on the paucity of mechanistic hypotheses supported by experimental data. The attention has been focused on the role of several adipokines and mainly the two major hormones produced by the AT, leptin and adiponectin, that interact directly with the brain (138). They have the capability to cross the blood–brain barrier and influence dementia processes within the brain (139), but evidence for a direct role is missing. Another postulated link is through altered gut microbial flora, which may participate in the development of obesity, T2D, and subsequent initiation of AD (140). Also lacking is the evidence that weight reduction in mid-life may produce beneficial effects on dementia development. However, in older adults, regular exercise provides numerous health benefits that include improvements in blood pressure, coronary artery disease, diabetes, lipid profile, OA, osteoporosis, mood, neurocognitive function, and overall morbidity so that studies in this area should be encouraged.

CONCLUDING REMARKS

Immunity and metabolism are highly integrated factors in aging and age-related diseases. This is an expanding field of investigation. Obesity and related complications are a major global epidemic. Scientific research must be a crucial part of the solution to understand all implications of obesity, but this research is still in its initial phase. The investigation of the mechanisms whereby inflammation and immune activation disrupt a functional immune response adds novel insights to the understanding of the relationship between inflammation and long-term metabolic disease outcome and opens new ways for effective therapeutic interventions.

AUTHOR CONTRIBUTIONS

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

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End-Stage Renal Disease Causes Skewing in the TCR Vβ-Repertoire Primarily within CD8+ T Cell Subsets

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A broad T cell receptor (TCR-) repertoire is required for an effective immune response. TCR-repertoire diversity declines with age. End-stage renal disease (ESRD) patients have a prematurely aged T cell system which is associated with defective T cell-mediated immunity. Recently, we showed that ESRD may significantly skew the TCR Vβ-repertoire. Here, we assessed the impact of ESRD on the TCR Vβ-repertoire within different T cell subsets using a multiparameter flow-cytometry-based assay, controlling for effects of aging and CMV latency. Percentages of 24 different TCR Vβ-families were tested in circulating naive and memory T cell subsets of 10 ESRD patients and 10 age- and CMV-serostatus-matched healthy individuals (HI). The Gini-index, a parameter used in economics to describe the distribution of income, was calculated to determine the extent of skewing at the subset level taking into account frequencies of all 24 TCR Vβ-families. In addition, using HI as reference population, the differential impact of ESRD was assessed on clonal expansion at the level of an individual TCR Vβ-family. CD8+, but not CD4+, T cell differentiation was associated with higher Gini-TCR indices. Gini-TCR indices were already significantly higher for different CD8+ memory T cell subsets of younger ESRD patients compared to their age-matched HI. ESRD induced expansions of not one TCR Vβ-family in particular and expansions were predominantly observed within the CD8+ T cell compartment. All ESRD patients had expanded TCR Vβ-families within total CD8+ T cells and the median (IQ range) number of expanded TCR Vβ-families/patient amounted to 2 (1–4). Interestingly, ESRD also induced clonal expansions of TCR Vβ-families within naive CD8+ T cells as 8 out of 10 patients had expanded TCR Vβ-families. The median (IQ range) number of expanded families/patient amounted to 1 (1–1) within naive CD8+ T cells. In conclusion, loss of renal function skews the TCR Vβ-repertoire already in younger patients by inducing expansions of different TCR Vβ-families within the various T cell subsets, primarily affecting the CD8+ T cell compartment. This skewed TCR Vβ-repertoire may be associated with a less broad and diverse T cell-mediated immunity.

Keywords: TCR-repertoire, T cell subsets, end-stage renal disease, ageing (aging), CMV-latency

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INTRODUCTION

End-stage renal disease (ESRD) patients have a decreased vaccination efficacy (1–4), an increased susceptibility for infection (5–7) and a higher risk for the development of tumors (8–11). Loss of renal function is associated with a prematurely aged T cell system (12), most likely caused by the uremia-induced proinflammatory environment (13). These uremia-induced effects on T cells are expressed as a decline in thymic output, a severe depletion of naive T cell compartment, a shift

to more highly differentiated memory T cell subsets, attrition of T cell telomeres (14) and a defective T cell receptor (TCR)-induced ERK phosphorylation (15).

A broad TCR-repertoire capable of recognizing a wide range of foreign antigens is crucial for adequate T cell-mediated immune responses (16). Most TCRs consist of an α and β chain and each chain is composed of a variable (V) and a constant (C) region (17). The TCR Vβ-repertoire can be assessed using several approaches such as gene scan spectratyping *via* a DNA-based PCR (18), Vβ-family phenotyping by flow-cytometry (19–21), and assessment of clonal diversity *via* next generation sequencing (NGS) (22, 23). Gene scan spectratyping of the TCR Vβ-repertoire is at best a semiquantitative measurement. Both flow-cytometry and NGS result in a more accurate quantitative assessment of the TCR Vβ-repertoire. As NGS is more labor-intensive and sorting of highly pure T cells or their subsets is required, many researchers prefer to use flow-cytometry. Flow-cytometry allows for measuring percentages of TCR Vβ-families at the T cell-subset level obviating the need for cell sorting.

We recently examined the TCR Vβ-repertoire in ESRD patients using multiplex DNA-based spectratyping. We showed ESRD to significantly and independently skew the TCR Vβ-repertoire in older individuals and this skewing was predominantly present within the $CD8^+$ memory T cell compartment (24). However, details of this skewed TCR Vβ-repertoire in ESRD patients are still lacking and quantitative data related to the impact of ESRD on TCR Vβ-repertoire in the various T cell populations is rare.

During aging, the TCR Vβ-repertoire has been reported to contract (25). Aging is associated with a decline in the naive T cell compartment which possess the broadest TCR repertoire (26), and a shift toward memory T cells, developing upon encountering of an antigen and having a skewed repertoire toward particular specificities (27, 28). The prevalence of CMV-seropositivity is high amongst ESRD patients, varying from 30 to 100%, depending on socioeconomic and ethnic background (29). CMV latency profoundly affects circulating T cells resembling features of aging, including increased frequencies of more differentiated memory T cells (30, 31) and loss of telomere length (32). CMV latency may also induce contraction of the TCR Vβ-repertoire as it induces expansion of CMV-specific T cells immunocompetent donors (33) and these CMV-specific clones are stably maintained for 5 years (34). Thus, TCR Vβ-repertoire diversity may be affected by various factors.

In this study, we assessed the TCR Vβ-repertoire diversity within different T cell subsets in ESRD patients using a flow-cytometry-based taking into account the effects of aging and CMV latency.

MATERIALS AND METHODS

Study Population

A cohort of 10 stable ESRD patients, either younger individuals ($n = 5$, age < 45 years) or older individuals ($n = 5$, age ≥ 65 years) with an oligoclonal TCR Vβ-repertoire, as determined by DNA-based spectratyping earlier (24) were studied in more detail at the T cell-subset level using a flow-cytometry based assay for TCR Vβ-repertoire analysis. Patients having a glomerular filtration rate below 15 mL/min and either or not receiving renal replacement therapy (RRT) were included. Patients were excluded from the study when having a bacterial or viral infection, malignancy, a previous transplantation or taking immunosuppressive medication (except for glucocorticoids). The patient data are compared to those generated from 10 age- and CMV-matched healthy individuals (HIs) with a polyclonal TCR Vβ-repertoire, as determined by DNA-based spectratyping (24). Lithium-heparinized blood was drawn from ESRD patients and HI. Written informed consent was obtained from all individuals included. The study was approved by the local medical ethical committee (METC number: 2012-022) and conducted according to the principles of Declaration of Helsinki and in compliance with International Conference on Harmonization/Good Clinical Practice regulations.

Sample Preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from 35 mL of lithium-heparinized blood by density centrifugation as described previously (35) and then frozen at 10×10^6 PBMC per vial at −190°C until further use.

Cryopreserved PBMCs (1 vial of 10×10^6 PBMCs) were thawed, counted, washed and resuspended in Isoflow™ Sheath Fluid (Beckman Coulter B.V., Woerden, Netherlands). The PBMCs were stained with Brilliant Violet 510-labeled anti-CD3 (BioLegend, Uithoorn, Netherlands), Alexa Fluor (AF)700-labeled anti-CD4 (Beckman Coulter B.V.) and Allophycocyanin (APC)-Cy7-labeled anti-CD8 (BioLegend) to identify $CD4^+$ and $CD8^+$ within $CD3^+$ T cells. ECD-labeled anti-CD45RO (Beckman Coulter B.V.), PE-Cy7-labeled anti-CCR7 (BD, Erembodegem, Belgium), V450-labeled anti-CD31 (BD; clone WM59), peridinin chlorophyll-A protein-Cy5.5-labeled anti-CD28 (BD) and APC-labeled anti-CD57 (BioLegend) as well as fluorescence minus one controls were used to appropriately identify the different T cell subsets (illustrated in Figures S1B–D in Supplementary Material). As shown in Figure S1B in Supplementary Material, CCR7 and CD45RO are used to distinguish the different naive and memory T cell subsets, i.e., naive ($CD45RO^-CCR7^+$), central memory (CM, $CD45RO^+CCR7^+$), effector memory (EM, $CD45RO^+CCR7^-$), and terminally differentiated effector memory $CD45RA^+$ T cells subsets (EMRA, $CD45RO^-CCR7^-$). CD31-expression within naive T cells (Figure S1C in Supplementary Material) identifies T cells that recently have left the thymus, also referred to as recent thymic emigrants (RTEs) (36). Loss of CD28 ($CD28^-$ T cells) and gain of CD57 ($CD57^+$ T cells) expression is observed in relation to increased replicative history (37, 38) and allows for identification of more differentiated T cells (Figure S1D in Supplementary Material).

Subsequently, the cell suspension was divided into eight tubes (100 μL/tube) labeled A-H, corresponding to the different antibody cocktails to stain for the 24 TCR Vβ-families (IOTest® Beta Mark TCR V beta repertoire kit, Beckman Coulter B.V.). Each cocktail contains antibodies directed to three different Vβ-families, i.e., one is fluorescein isothiocyanate (FITC-), one is PE-labeled and one is labeled with both FITC and PE. **Table 1** shows the description of the antibodies directed to the different TCR Vβ-families in tube A to H. A typical example of the proportions of several TCR Vβ-families within $CD3^+$ T cells from tube

TABLE 1 | TCR Vβ-families in tube A-H.

Tube	Vβ family	Fluorochrome	Clone
A	Vβ 5.3	PE	3D11
	Vβ 3	FITC	CH92
	Vβ 7.1	PE + FITC	ZOE
B	Vβ 9	PE	FIN9
	Vβ 16	FITC	TAMAYA1.2
	Vβ 17	PE + FITC	E17.5F3
C	Vβ 18	PE	BA62.6
	Vβ 20	FITC	ELL1.4
	Vβ 5.1	PE + FITC	IMMU157
D	Vβ 13.1	PE	IMMU222
	Vβ 8	FITC	56C5.2
	Vβ 13.6	PE + FITC	JU74.3
E	Vβ 5.2	PE	36213
	Vβ 12	FITC	VER2.32
	Vβ 2	PE + FITC	MPB2D5
F	Vβ 23	PE	AF23
	Vβ 21.3	FITC	IG125
	Vβ 1	PE + FITC	BL37.2
G	Vβ 11	PE	C21
	Vβ 14	FITC	CAS1.1.3
	Vβ 22	PE + FITC	IMMU546
H	Vβ 13.2	PE	H132
	Vβ 7.2	FITC	ZIZOU4
	Vβ 4	PE + FITC	WJF24

Detailed information with respect to the different TCR Vβ-family antibodies in tube A-H, labels and clones (IOTest® Beta Mark TCR V beta repertoire kit, Beckman Coulter).

A, tube B and tube C is depicted in Figure S1A in Supplementary Material, the last three plots.

The samples were measured on a Navios flow cytometer (10-color configuration; Beckman Coulter B.V.) and at least 0.5 million $CD3^+$ T cells were acquired for each tube. Data were analyzed by Kaluza™ software (Beckman Coulter B.V.). The number of events acquired for a specific T cell subset needed to be more than 100 to allow for reliable analysis of frequencies of TCR Vβ-families within this population. The only subset that did not meet this criterion was the EMRA population within the $CD4^+$ T cells.

Gini-TCR Index and Calculation of Expanded TCR Vβ−Families

The Gini index is used to describe the distribution of income in economic statistics. As the distribution of TCR Vβ−families shows similarities to that of income, the Gini index can also be applied in TCR Vβ-repertoire analysis by flow-cytometry. It has already been used in TCR-sequencing studies (39, 40), and was recently also introduced as an accurate and reliable way for analyzing TCR Vβ-repertoire data obtained by flow-cytometry (41). The TCR (Vβ)-Gini index with scores ranging from low to high indicates TCR Vβ-families from equal distribution (broad repertoire; i.e., low score) to unequal distribution (skewed repertoire; i.e., high score). A Microsoft excel file allowing for automatic calculation of the Gini-TCR index using percentages of 24 TCR-Vβ families is provided in the supporting file (41).

An expansion in a TCR Vβ-family in ESRD patients is defined as a frequency above the mean percentage + 2 times the SD of a certain TCR Vβ-family obtained using the HI as reference population. By using this approach, finding an expansion by chance is lower than 2.5%.

TABLE 2 | Demographic and clinical characteristics of the study population.

	Healthy individuals	ESRD patients	*P*-value
Number of individuals	10	10	
Age (years; range)			
younger group (<45 years)	36 (26–42)	28 (20–29)	0.06
older group (>65 years)	68 (65–73)	70 (65–73)	0.92
Sex (% male)	60	40	0.66
CMV IgG serostatus (% pos)	60	60	1.00
RRT (%)	n.a.	70	n.a.
Duration of RRT (months; median/range)		20 (7–68)	
Hemodialysis (%)		85.7	
Peritoneal dialysis (%)		14.3	
Underlying kidney disease			
Primary glomerulopathy (%)		20	
Diabetic nephropathy (%)		30	
Reflux nephropathy (%)		10	
Polycystic kidney disease (%)		20	
Lupus nephritis (%)		10	
Unknown (%)		10	

ESRD, end-stage renal disease; CMV, cytomegalovirus; pos, positive; RRT, renal replacement therapy; n.a., not applicable.

Statistical Analyses

Gini-TCR indices or median number of expanded TCR Vβ-families/individual between two different T cell subsets within individuals were compared with Wilcoxon signed rank test and Friedman test followed by Dunn's multiple comparison *T*-test was used for comparing more than two different T cell subsets. Trend analyses were performed using two-way ANOVA, comparing different subsets between individuals or $CD4^+$ and $CD8^+$ T cells. In addition, the effect of ESRD with respect to numbers of expanded TCR Vβ-families within different T cell subsets is done using Fisher's exact test. Two-sided *P*–values <0.05 were considered statistically significant. All statistical analyses were performed with GraphPad Prism 5.

RESULTS

Study Population

Detailed information of the study population is given in **Table 2**. Ten ESRD patients (5 younger individuals: age 20–29 years and 5 older individuals: age 65–73 years) and 10 age-matched HI (5 younger individuals age 26–42 years and 5 older individuals: age 65–73 years) were recruited into this study. Sixty percent of the ESRD and HI study population is CMV-seropositive. Seven out of 10 ESRD patients received RRT.

Gini-TCR Indices Increase with T Cell Differentiation

Naive T cells expressing CD31 are considered to be RTEs and the least-differentiated T cell subset. In ESRD patients, CD31-expressing naive T cells tended to or have a lower Gini-TCR index when compared to their $CD31^-$ counterparts within $CD4^+$ (**Figure 1B**) and $CD8^+$ T cells (**Figure 1D**), respectively. For HI,

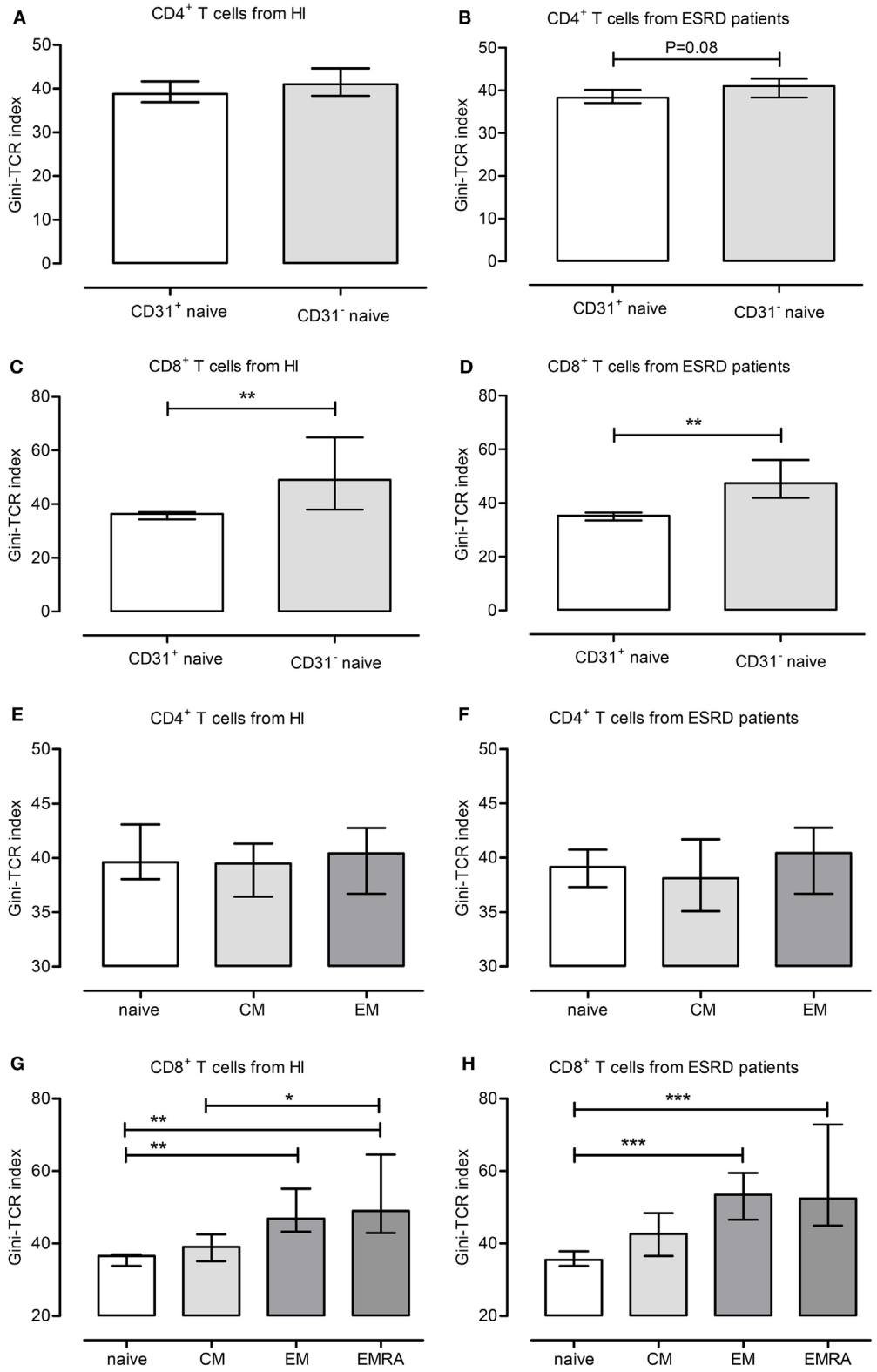

FIGURE 1 | Gini-T cell receptor (TCR) indices for different T cell subsets. The different Gini-TCR indices are depicted for healthy individual (HI) **(A,C,E,G)** and end-stage renal disease (ESRD) patients **(B,D,F,H)**. First, the Gini-TCR indices for recent thymic emigrant (CD31+ naive) and CD31− naive CD4+ **(A,B)** and CD8+ T **(C,D)** cells are given, respectively. Next, the differentiation-associated effects on Gini-TCR indices are depicted for CD4+ **(E,F)** and CD8+ **(G,H)** T cell subsets, including naive, central memory, effector memory, and terminally differentiated effector memory CD45RA+ (EMRA) T cells. *, **, and *** reflect *P*-values <0.05, 0.01, and 0.001, respectively. Data from 10 HI and 10 ESRD patients are given as median with interquartile range.

a significant lower Gini-TCR index was only observed for CD31-expressing naive CD8+ (**Figure 1C**) but not CD4+ (**Figure 1A**) T cells when compared to CD31− naive T cells. Furthermore, a T cell differentiation-associated increase in Gini-TCR indices was noted for CD8+ (**Figures 1G,H**), but not CD4+ (**Figures 1E,F**), T cells. The median value (IQ range) increased significantly ($P < 0.001$) from 36.5 (33.7–37) and 35.5 (33.8–37.9) in naive T cells to 49 (43–64.6) and 52.4 (44.9–72.8) in the highly differentiated EMRA CD8+ T cells for HI and ESRD patients, respectively.

ESRD Patients Have Increased Gini-TCR Indices within Memory CD8+ T Cell Subsets

We next analyzed the influence of ESRD, aging, and CMV latency on skewing of the TCR Vβ-repertoire by comparing Gini-TCR indices for different T cell subsets including total CD3+ T cells, as well as naive, CD31+ naive, total memory (MEM), CM, EM, EMRA, CD28−, and CD57+ populations within both the CD4+ and CD8+ T cell subsets. ESRD effects with respect to Gini-TCR indices were limited to the CD8+ T cell compartment as it tended to induce higher Gini-TCR indices ($P = 0.06$) in CD8+ memory T cells when compared to HI (**Figure 2A**). The median (IQ range) value for Gini-TCR index in CD8+ memory T cells amounted to 48.4 (45.8–63.3) and 43.8 (41.1–51.2) for ESRD patients and HI, respectively. Younger (**Figure 2B**), but not older (**Figure 2C**), ESRD patients had significantly higher Gini-TCR indices within the CD8+ CM ($P < 0.05$), EM ($P < 0.05$) and CD57+ T cell compartment when compared to age-matched HI. The median (IQ range) for Gini-TCR in CD8+ CM, EM and CD57+ T cells amounted to 47.4 (40.8–54.2) versus 35.3 (34.0–39.2), 54.4 (49.6–68.5) versus 43.7 (42.4–50.8) and 77.6 (65.8–78.4) versus 59.2 (57.2–67.1) for younger ESRD patients versus younger HI.

The following results, describing Tables S1 and S2 in Supplementary Material need to be interpreted with caution as the *P*-values were not adjusted for the number of parameters compared.

Aging effects were not visible when comparing Gini-TCR indices for the different T cell subsets between younger and older ESRD patients (Table S1 in Supplementary Material). In HI, aging effects were confined to the CD8+ T cell compartment and an aging-related increasing trend in Gini-TCR index was observed for CD8+ CM T cells ($P = 0.06$), as the median (IQ range) for Gini-TCR amounted to 40.1 (39.1–44.1) in older HI versus 35.3 (34.0–39.2) in younger HI. An increased Gini-TCR index ($P = 0.03$) was observed for older HI, within CD8+CD28− T cells (Table S1 in Supplementary Material). The median (IQ range) value for Gini-TCR in CD8+CD28− T cells amounted to 42.0 (40.6–47.4) versus 57.8 (43.7–63.7) for younger and older HI, respectively.

CMV latency did not significantly affect Gini-TCR indices apart from a CMV-related increasing trend within CD4+CD57+ T cells ($P = 0.07$) of HI, but not ESRD patients, i.e., the median (IQ range) Gini-TCR index amounted to 67.0 (57.4–72.2) for CMV-seropositive HI versus 45.6 (35.5–58.0) for CMV-seronegative ones (Table S2 in Supplementary Material). No differences were observed when comparing CMV-seronegative and CMV-seropositive ESRD patients to their CMV-serostatus matched HI with respect to Gini-TCR indices for the different T cell subsets (data not shown).

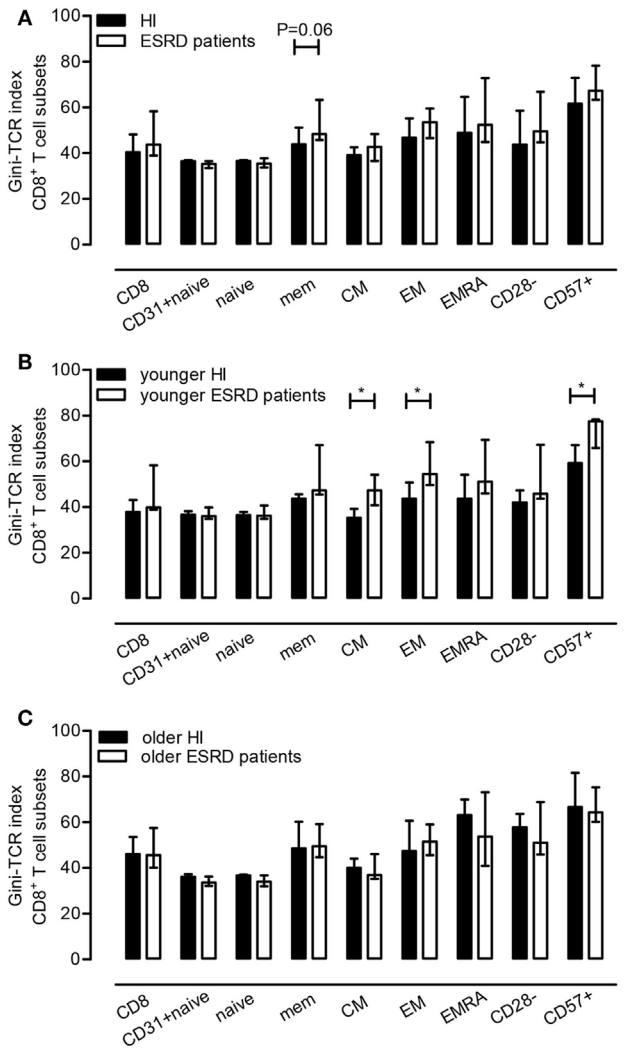

FIGURE 2 | Effect of end-stage renal disease (ESRD) on Gini-T cell receptor (TCR) indices of CD8+ T cell subsets. In **(A)**, the median and IQ range of the Gini-TCR indices for healthy individuals ($N = 10$) and ESRD patients ($N = 10$) for different CD8+ T cell subsets is depicted, whereas in **(B,C)** those for the younger and older group ($N = 5$) are given, respectively. *P* value: *<0.05.

Clonal Expansions of TCR Vβ-Families in Different T Cell Subsets

Apart from characterizing the impact of ESRD on Gini-TCR indices for the different T cell subsets, we also evaluated the impact of ESRD on clonal expansions of TCR Vβ-families by comparing frequencies to the average + 2SD obtained using HI as reference population. **Figure 3** shows a typical example of expanded TCR Vβ-families within CD8+ memory T cells of ESRD patients.

Clonal expansions of TCR Vβ-families were observed within the CD3+ T cells in 7 out of 10 ESRD patients (**Table 3**). Half versus all of the ESRD patients showed expanded TCR Vβ-families

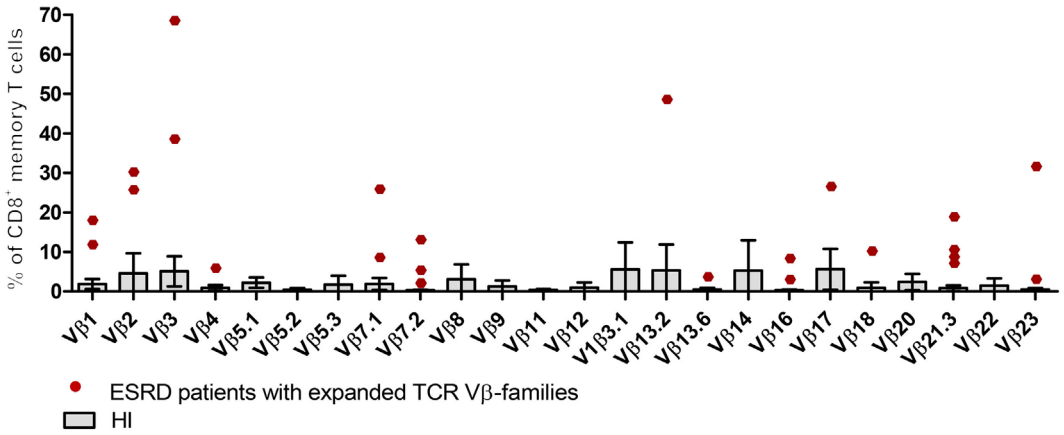

FIGURE 3 | Expansions of T cell receptor (TCR) Vβ-families within CD8+ memory T cells from end-stage renal disease (ESRD) patients. Boxes and error bars represent the mean and 95% confidence interval (mean ± 2SD) of 24 TCR-Vβ families from 10 healthy individuals (HI). Red dots correspond to expanded TCR Vβ-families from ESRD patients (frequencies > mean + 2SD from HI).

TABLE 3 | Effect of ESRD on expansions of TCR Vβ-families.

	# of ESRD patients with/without expansions	Median # of expanded TCR Vβ-families (IQ range)	Total # of expanded TCR Vβ-families
CD3+	7/3	1 (0–2)	15
CD4+	5/5	1 (0–2)	10
CD31+ naive	6/4	1 (0–1)	9
Naive	3/7	0 (0–1)	5
MEM	9/1	1 (1–3)	16
CM	6/4	1 (0–2)	11
EM	6/4	1 (0–2)	13
CD28−	9/1	1 (1–1)	12
CD57+	9/1	1 (0–2)	11
CD8+	10/0	2 (1–4)	26
CD31+ naive	7/3	1 (0–2)	15
Naive	8/2	1 (1–1)	12
MEM	10/0	2 (2–4)	28
CM	7/3	2 (0–3)	20
EM	9/1	4 (2–4)	32
EMRA	10/0	2 (1–3)	26
CD28−	10/0	3 (1–3)	28
CD57+	9/1	3 (1–4)	25

within CD4+ and CD8+ T cells ($P < 0.05$), respectively. The median (IQ range) number of expanded TCR Vβ-families per patient amounted to 1 (0–2) and 2 (1–4) families for CD4+ and CD8+ T cells ($P < 0.05$), respectively (**Figure 4A**). Interestingly, expansions were also detected within the naive T cell compartment, as 3 out of 10 and 8 out of 10 patients had expansions of TCR Vβ-families within the naive CD4+ and CD8+ T cell compartment, respectively (**Table 3**). Most clonal expansions were observed within the (more differentiated) memory CD8+ T cell subsets (**Figure 4A**). The median (IQ range) number of expanded TCR Vβ-families amounted to 1 (1–1) versus 2 (2–4) for naive and memory CD8+ T cells, respectively ($P < 0.05$). Moreover, ESRD affected different TCR Vβ-families as illustrated in **Figure 3** for CD8+ memory T cells. ESRD induced expansions within both younger (**Figure 4B**) and older (**Figure 4C**) ESRD patients when compared to their age-matched HI.

DISCUSSION

The main finding of this study is that the multiparameter flow-cytometry-based approach for evaluating the skewed TCR Vβ-repertoire diversity in ESRD patients showed that TCR skewing can be observed primarily in CD8+ T cell subsets, including naive T cells. However, higher Gini-TCR indices, indicative for an enhanced TCR Vβ-repertoire skewing, were specifically associated with more differentiated CD8+, but not CD4+, T cell subsets in both ESRD patients and HI.

Our previous data showed that ESRD may lead to a skewed TCR Vβ-repertoire as assessed by DNA spectratyping, providing at best semiquantitative information about TCR Vβ-clonality (24). The current study provided more quantitative details with respect to this skewed TCR Vβ-repertoire at the T cell-subset level using the Gini-TCR index as a tool for calculating skewness (41) and evaluating number/type of expanded TCR Vβ-families. Higher Gini-TCR indices are indicative of a more skewed TCR Vβ-repertoire. The current study confirmed several of our previous findings. Increased skewing of the TCR Vβ-repertoire was observed for more differentiated CD8+, but not CD4+, T cells, corresponding to our spectratyping data as well as findings described by others (27, 42). In addition to the Gini-TCR index, we calculated the number of TCR Vβ-families per patient that were expanded beyond the mean + 2SD values of HI. This approach yielded similar results as the Gini-index but gives detailed information at the individual patient level for the different T cell subsets. For instance some patients have a large number of expanded TCR Vβ-families while others show only a few. Moreover, ESRD did not seem to affect one TCR Vβ-family in particular, indicative of expansions of different clonal origin. Altogether, using both Gini-TCR indices as well as the number of expanded TCR Vβ-families, revealed skewing to mainly occur

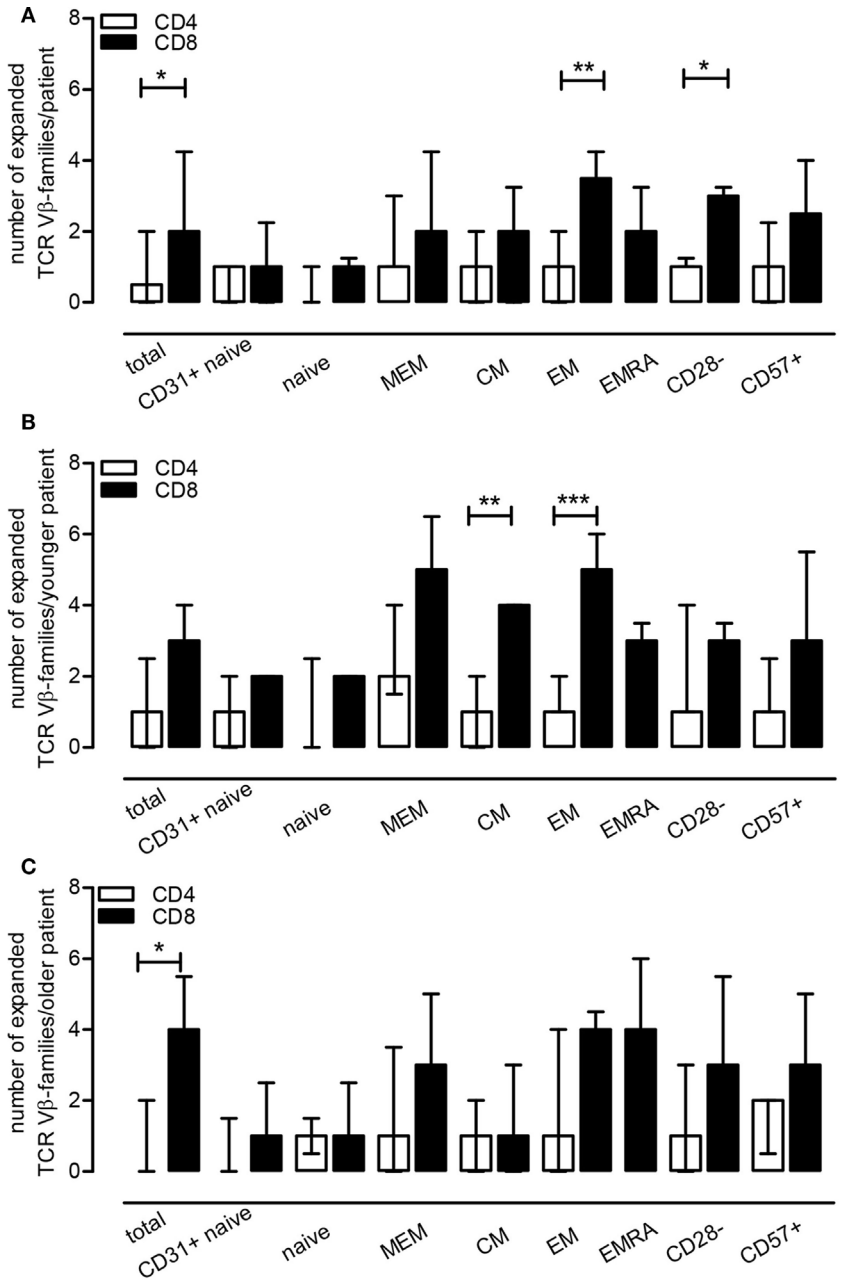

FIGURE 4 | End-stage renal disease effect on T cell receptor (TCR) Vβ-families within T cell subsets. Using healthy individual (HI) (*N* = 10) as a reference population, we evaluated the number of TCR Vβ-families that were expanded per patient for a T cell subset (frequency of a TCR Vβ-family exceeding the mean + 2SD value obtained for HI) **(A)**. In **(B,C)**, the median (IQ range) of expansions per younger and older patient for a T cell subset is depicted using the younger (*N* = 5) and older HI (*N* = 5) as a reference, respectively. The open bars represent the median and IQ range for the different CD4+ T cell subsets, whereas the closed bars represent that for the different CD8+ T cell subsets.

within the CD8+ and in particular within the CD8+ memory T cell subset similar to what was observed before using DNA-based spectratyping on sorted T cell subsets (24).

The commercially available flow-cytometry-based assay, used to characterize the TCR Vβ-repertoire, is composed of 24 different TCR Vβ-antibodies covering about 70% of the normal human TCR Vβ-repertoire (brochure Beckman Coulter). Evaluating other TCR Vβ-families as well as TCR Vα and TCR Vγ/Vδ families may contribute to a better understanding of the whole TCR-repertoire. In this respect, γδ+ T cells account for approximately 8% of CD3+ peripheral blood T cells and around 6% of γδ+ T cells were observed within CD3+CD8+, but not CD4+ T cells in HI (19). As frequencies of γδ+ T cells may also vary amongst individuals, it might be more accurate to evaluate the TCR Vβ-repertoire not within total CD3+, like performed in the current study, but within αβ+ CD3+ T cells.

Interestingly, using this multiparameter flow-cytometry-based approach, we were also able to detect expanded TCR Vβ-families within the naive T cell compartment. This characteristic has, to our knowledge never been described for ESRD patients. Uremia induces a proinflammatory environment significantly affecting T cell-mediated immunity characterized by increased risk for infections (5) and decreased vaccination efficacy (1, 43). We have observed that progressive loss of renal function is accompanied by a severe depletion of the naive T cell compartment and a relative shift toward more differentiated memory T cells (44). Naive T cells employ a mechanism referred to as homeostatic proliferation in order to maintain the naive T cell pool that is not replenished by newly developed naive T cells from the thymus due to thymic involution. Homeostatic proliferation occurs in response to homeostatic cytokines, e.g., IL-7, or low affinity self antigens presented by antigen-presenting cells (26). This mechanism has been described to be associated with a decline in TCR Vβ-repertoire diversity within naive T cells with increasing age (27, 45). ESRD enhanced homeostatic proliferation of naive T cells to a similar extent as observed in older HI (12) and as a consequence of this compensatory mechanism, loss of TCR Vβ-repertoire diversity may also be induced by ESRD within naive $CD8^+$ and/or $CD4^+$ T cells.

Naive T cells are required to mount adequate immune responses to newly encountered antigens (46, 47). ESRD patients, with a severely depleted naive T cell compartment (44), are hampered in inducing adequate protection to, for example, HBV vaccination as a result of defective generation of antigen-specific memory T cells (43). The ESRD-associated defects in T cell composition as well as function, reminiscent of aging-associated T cell defects, led to the concept of premature T cell aging introduced in 2011 (12). ESRD patients have a T cell compartment that is aged by 15–20 years compared to their chronologic age, using age-matched HI as a reference. Consistent with premature T cell aging (12), we observed ESRD-associated increases in Gini-TCR indices and TCR Vβ expansions to occur already at young age.

Aging is known to affect TCR Vβ-repertoire diversity toward a more skewed pattern, starting from roughly 600×10^3 clonotypes detected per 10^6 T cells in childhood, declining by 5×10^3 clonotypes per year (25). Age-related effects were limited within our cohort of HI and this may be a consequence of the selection procedure applied. We did select HI with a polyclonal (i.e., non-skewed) TCR Vβ-repertoire using DNA-based spectratyping (24), to ensure a relatively standard healthy population to be used as reference for comparison to ESRD patients. The ESRD patient population however only consisted of individuals with an oligoclonal (skewed) TCR Vβ-repertoire. This might have resulted in an underestimation of the effect of aging on TCR Vβ-families. Likewise, our selection procedure may also explain the minimal effects of CMV in both cohorts.

CMV latency is known to introduce skewing of the TCR Vβ-repertoire induced by expanded CMV-specific T cell clones in both HI (33, 34, 48) and ESRD patients (24). CMV latency may result in a vast and long-lasting expansion of CMV-specific T cells (33, 34). Moreover, CMV latency has additional effects mainly on circulating $CD8^+$ T cells of ESRD patients (49).

End-stage renal disease, aging, and CMV all influenced the TCR-Vβ repertoire diversity to a different extent (24), however, because of different factors present in the environment, they all have their specific effect on clonotype selection. Even though these findings need to be verified in a larger cohort without preselection using DNA-based spectratyping of TCR-Vβ-repertoire, our study already shed some light on this altered TCR-Vβ repertoire at the T cell-subset level in particular with respect to ESRD.

Relating TCR-repertoire data to functional capacities of T cells is warranted to increase knowledge on uremia-induced T cell defects in ESRD patients. Moreover, tracking TCR clones in whole blood or tissue infiltrates may provide additional information on antigen specificity important for diagnosis of infection and allograft rejection after transplantation (50–52).

In conclusion, ESRD is associated with a skewed TCR Vβ-repertoire as a result of variable TCR Vβ-family expansions, but not one TCR Vβ-family in particular. ESRD, aging and CMV latency exert their effects by influencing different TCR Vβ-families. This altered repertoire may be associated with a less broad and less diverse T cell-mediated immunity.

ETHICS STATEMENT

All individuals included gave informed consent and the Erasmus medical center medical ethical committee approved the study (METC number: 2012-022). It was conducted according to the principles of Declaration of Helsinki and in compliance with International Conference on Harmonization/Good Clinical Practice regulations.

AUTHOR CONTRIBUTIONS

LH participated in the design of the study, analyzed the data, and wrote the manuscript. MB participated in the design of the study, interpreted the data, and revised the manuscript. MK established the experimental protocol, conducted the experiments, and analyzed the data. AL participated in the design of the study, interpreted the data, and revised the manuscript. CB participated in design of the study and revised the manuscript. NL designed the study, interpreted the data, and revised the manuscript. All authors have read and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Excess of Mortality in Adults and Elderly and Circulation of Subtypes of Influenza Virus in Southern Brazil

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Purpose: In the elderly population, the influenza infection and its clinical complications are important causes of hospitalization and death, particularly, in longer-lived age. The objective of this study is to analyze the impact of influenza virus circulation on mortality in the elderly and adults, in years with different predominant virus strains.

Methods: We performed a time trend study to evaluated excess of mortality for pneumonia and influenza, respiratory disease, and all-causes in southern region of Brazil, from 2002 to 2015. After considering other models, we opted for Serfling regression. Excess of death rates per 100,000 inhabitants were analyzed in specific age groups (24–59, 60–69, 70–79, ≥80 years) and by year of occurrence. Mortality information were taken from Brazilian Mortality Information System and etiological data were accessed in Sentinel Virological Surveillance database, getting the weekly positivity of the immunofluorescence tests for influenza A (H1N1, H3N2), and B.

Results: In southern Brazil, there is an evident seasonal pattern of all death outcomes among different age groups in the dry and cold season (April–September). The highest excess mortality rates occurs among older, particularly in years of circulation of influenza AH3N2, especially among people ≥80 years, in 2003 and 2007—years of great severity of influenza activity. After 2009, with the introduction of the pandemic influenza AH1N1, we observed a lower impact on the mortality of the elderly compared to <60 years.

Discussion: A cross reactivity antibody response from past exposure probably provided protection against disease in the elderly. Despite not controlling for comorbidities, climate, and vaccination, for the >70 years, ratio of respiratory diseases excess mortality rates between AH1N1 (2009) and severe year of H3N2 (2007) shows protection in the pandemic year and great vulnerability during AH3N2 virus predominance.

Conclusion: The reduced immune response to infection, and to vaccination, and presence of comorbidities recommend a special attention to this age group in Brazil. Besides medical assistance, the timeliness of vaccine campaigns, its composition, and etiological surveillance of respiratory diseases are some of the preventive and public health measures.

Keywords: influenza, excess of mortality, influenza AH3N2, influenza AH1N1, pandemic, elderly, Serfling regression model

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INTRODUCTION

Human influenza viruses can cause diseases through many direct and indirect pathological effects. Consequences are destruction of infected cells, release of cytokines leading to fever, malaise, damage to respiratory epithelium and pulmonary parenchyma, and pneumonia. It includes secondary bacterial infections because of tissue damage and exacerbation of preexisting comorbidities such as cardiovascular and renal diseases, diabetes, or chronic lung disease (1–3).

The rates of hospitalization and mortality associated with influenza are higher among patients with chronic diseases, children under 1 year and after 65 years of age (4, 5). With the aging population in recent decades, the raw number of hospitalizations and deaths related to pneumonia and influenza tends to increase (4), this phenomenon has been observed also in Brazil (6, 7). However, the impact and severity of influenza virus circulation depend in part, on the strain that predominates in the season each year.

Due to the lack of laboratory confirmation, influenza-associated morbidity and mortality are often classified as pneumonia, other respiratory diseases, or other causes. Given the difficulty of directly measuring influenza morbidity and mortality, time series models are used to elucidate disease patterns in various age groups. Trends are usually determined by means of statistical inference, based on seasonal coincidence of the occurrence of certain diseases or death and laboratory confirmation of the viral circulation (4, 8).

Different approaches, with and without the quantification of the proportion of viral isolates, can produce average estimates of excess deaths associated with the circulation of certain viral variants (9–11). Viral surveillance data, hospitalization, or death indicators are particularly useful for the study of influenza in the tropics, as seasonality may be less evident (11–13). Serfling regression has been used to analyze excess of mortality related with respiratory virus circulation (7, 14–16). Despite some limitations (17), the inclusion of sinusoidal terms in weekly regression may reduce spurious correlation between influenza occurrence and death (18, 19). It is particularly useful when no other covariables are available, and with small samples of viral sentinel surveillance data (18). Poisson regression and the generalized linear model (GLM) can produce more specific estimates and support adjustments for variables (temperature, humidity, comorbidities, other circulations of viruses), although they require a more robust and consistent virological surveillance and cannot be used for pandemics (4).

In Brazil, surveillance for influenza syndromes was implemented in 2000, monitoring the occurrence of respiratory viruses (influenza A and B, parainfluenza 1, 2, and 3, respiratory syncytial virus, adenovirus). The Brazilian Ministry of Health provides vaccination coverage annually since 1999 for seniors and some risk groups, with vaccine coverage of the elderly population at around 80% in southern Brazil, the region with the highest coverage of the country. Despite the adequate coverage, protective titers after vaccination (HI ≥ 0) are consistently lower with poorer cell mediate and antibody responses in the elderly comparing to adults (20).

Considering the vulnerability of the elderly to influenza virus infection, and the lack of studies on its repercussion in Brazil, the objective of this study was to analyze the impact of different strains of Influenza A virus circulation. We analyzed particularly the most predominant variants (AH1N1 and AH3N2) on excess of mortality in the adults and elderly of different age groups in a region with marked seasonality of respiratory diseases in Brazil.

MATERIALS AND METHODS

Local of Study

This is a time trend study to evaluated excess of mortality from 2002 to 2015 in southern region of Brazil (states of Paraná, Santa Catarina e Rio Grande do Sul), total area is 576,774,31 km^2, population is 27,386,891 inhabitants with subtropical climate (Köppen-Geiger classification Cfa). We choose these states for analysis because of the consistent seasonal pattern of influenza, as well as the availability and quality of etiological data from the virological surveillance system in that region.

Mortality Data and Population

For the mortality rates of specific age groups (24–59, 60–69, 70–79, and $\geq$80 years) and death causes, we took data from Brazilian Mortality Information System. Causes are classified according to International Causes of Death ICD-10 revision, pneumonia, and influenza (ICD J 10 to J18.9), respiratory diseases (ICD J00 to J99), and all-cause (excluding external causes of mortality).

We obtained population of each year and age group from Instituto Brasileiro de Geografia e Estatística-IBGE from the Census-2010, and population estimates for the following years.

National Viral Surveillance Data

Etiologic information of flu-like syndrome was accessed in database of the National Sentinel Virological Surveillance System. It has data from 128 sentinel units distributed in all regions of the country—North (21 units), Northeast (26 units), Southeast (34 units), South (38 units), and Central West (9 units). Surveillance is performed through the systematic collection of weekly samples of nasopharyngeal secretions from patients who present flu-like syndrome. Reference laboratories process samples by using indirect immunofluorescence (IIF), with tests for influenza A and B, parainfluenza 1, 2, and 3, respiratory syncytial virus, and adenovirus. A portion of the samples is submitted to polymerase chain reaction tests to identify the virus genotype.

We calculated the laboratory positivity indicator using weekly positive results of IIF divided by the total of weekly valid tests, i.e., excluding the results within inadequate samples (not enough biological material, improper storage, incorrect material in the sample) or inconclusive results (no valid results).

Influenza vaccination coverage (%) of southern region from 2002 to 2015 was obtained from Brazilian National Program of Immunization data base (DATASUS).

Definition of Influenza Epidemic Periods

The criteria used to define the period of increase of influenza activity was when the positivity of the samples tested exceeded

twice the annual mean of the weekly positivity of samples processed by surveillance, during two consecutive weeks.

In the year 2009, we consider the period officially recognized by the Brazilian Ministry of Health as epidemic by the influenza AH1N1pmd2009 strain, due to irregularity of the sample collection by the sentinel surveillance system at the end of epidemic.

Statistical Analysis

We calculated the weekly mortality rates by age group using the number of deaths per group of causes divided by the estimated population in the middle of the year multiplied by 100,000.

We constructed a Serfling cyclical regression model (14) for weekly data applied to each age group and causes of death (pneumonia and influenza, respiratory diseases, and all causes), as seen in others studies (7, 15), to estimate baseline of predicted deaths in the absence of influenza epidemics.

To fit regression, we used period of 13 years (from 2002 to 2015), excluding the weeks of epidemics periods. A cyclical linear regression was adjusted with the equation:

$$\begin{aligned} Y = {} & \beta 0 + \beta 1 * t + \beta 2 * t2 + \beta 3 * t3 + \beta 4 * \sin(2 * \pi * t / 52.17) \\ & + \beta 5 * \cos(2 * \pi * t / 52.17) + \beta 6 * \sin(4 * \pi * t / 52.17) \\ & + \beta 7 * \cos(4 * \pi * t / 52.17) + e1, \end{aligned}$$

where Y is the mortality rate, β is the coefficients of regression, t is time in weeks, and t^2 and t^3 are variables for adjusting the secular trend of the disease. We used of sine and cosine for adjust of annual and semiannual periodic components.

After adjusting a linear regression and define the expected mortality rate, we delimited 95% upper confidence limit of the baseline as the reference threshold in the absence of influenza epidemics. We calculated the excess of deaths as the observed mortality minus the expected mortality in the periods when mortality was above 95% of the confidence interval during epidemics periods.

We also present ratios of excess mortality rates among years of predominant circulation of influenza strains AH3N2 (mean and years of severity), AH1N1 pre-pandemic, and AH1N1 post-pandemic for each age group.

For data compilation, we used Microsoft Office Excel 2007, and for statistical analysis, SPSS for Windows, version 24.0.

RESULTS

Table 1 shows the proportion of positivity of the IIF nasopharyngeal samples and the annual prevalence of strains of influenza in the period. Before 2009, the year of entry of the pandemic strain AH1N1pmd 2009, there was a predominance of influenza AH3N2 in the years 2003 to 2007. After 2009, there is alternation of strains in the southern Brazil. Annual elderly vaccination coverage in southern region is high and homogeneous, around 80%, and even higher in the recent years.

There is an evident seasonal pattern of deaths from pneumonia and influenza, respiratory diseases, and all-causes among the elderly in different age groups in the dry, cold months (April–September) in southern region (**Figure 1**).

We note a progressive increase in the rates of excess deaths (of all outcomes) with increasing age, especially among those older than 70 years. In the pre-pandemic years with dominance of the AH1N1 strain, the excess of mortality rates associated with influenza were relatively low, compared to years of prevalence of AH3N2 strain (**Table 2**). Among those over 80 years, the ratio of excess mortality rates between 2009 and the years with dominium of H3 strains was less than one. This ratio suggests that this age group was spared in the 2009 pandemic. However, in years of predominance of strain H3, excess of mortality rate of all causes in this group were 449.6 per 100,000 (corresponding to 1,598 obits), 5, and 8.2 times greater than the same rate in years of circulation of H1N1 in pre- and post-pandemic period, respectively.

Among adults (24–59 years), we observe a large excess of deaths rates during the 2009 pandemic (953 obits), which correspond to 7.1 excess deaths from all causes, and 99 excess mortality from respiratory diseases associated with viral infection in every 100,000 individuals of the age group. The ratio between excess mortality rates due to pneumonia/influenza in the pandemic year (2009) and the mean rate of the period was 12 times higher among the youngest (**Table 2**).

TABLE 1 | Specimens collected, positive proportion, and predominant subtypes of influenza between 2002 and 2016 in the sentinel units of southern Brazil.

Years	Samples	Positive samples	% indirect immunofluorescence positive	AH1N1	AH3N2	Not subtyped	B	Virus Predom.	% Vac. Coverage
2002	186	23	12			8	15	H1/B	75.3
2003	355	41	12			37	4	H3	73.0
2004	474	52	11			47	5	H3	75.6
2005	383	15	4			14	1	H3	72.7
2006	676	94	14			72	22	H3	84.0
2007	800	127	16			122	5	H3	71.1
2008	1,018	129	13			60	69	H1/B	71.4
2009	540	112	21			91	21	H1/B	76.9
2010	949	142	15			62	80	H1/B	77.1
2011	1,104	175	16			129	46	H3	82.9
2012	1,514	138	9			106	32	H1/B	96.5
2013	3,728	926	25	320	229	0	377	H1/B	91.8
2014	5,249	815	16	73	660	10	72	H3	91.4
2015	5,752	854	15	109	390	19	336	H3	93.7

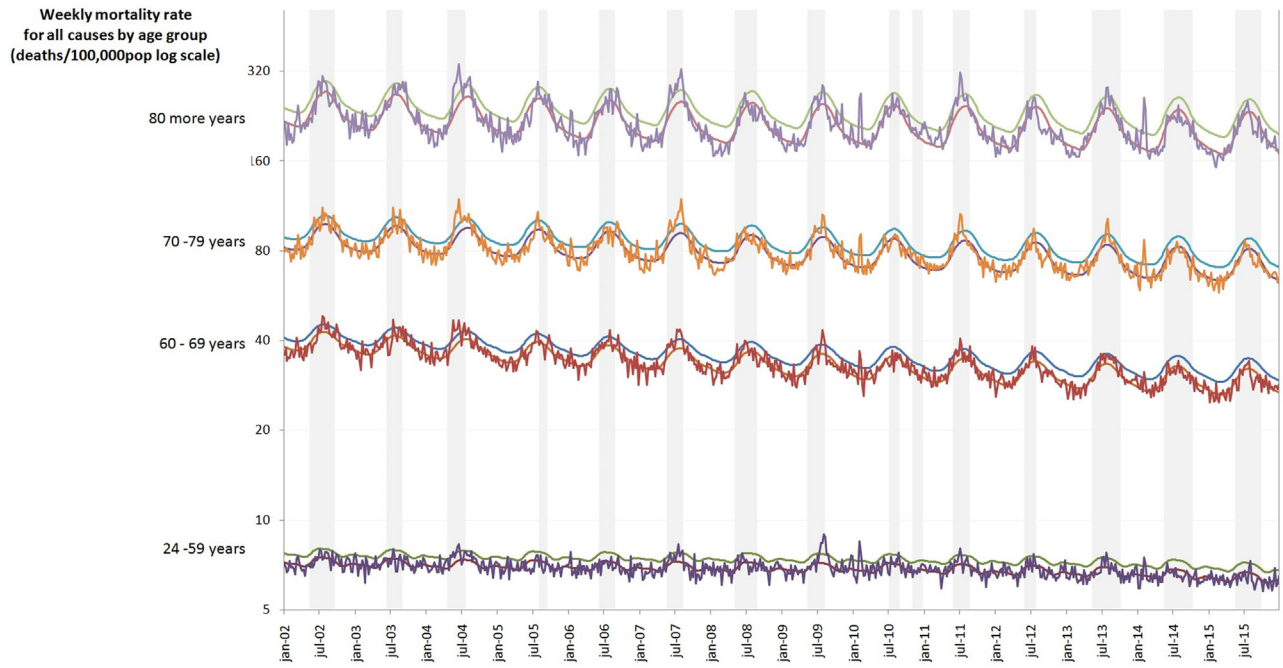

FIGURE 1 | Weekly mortality rate by age group (deaths/100,000) log scale in southern regions of Brazil, 2002–2015. Colored lines are age groups and gray columns are the seasons of influenza.

Rates of excess mortality by pneumonia and influenza and respiratory diseases are lower than all causes in all age groups, but particularly high in older than 80 years (**Table 2**).

DISCUSSION

The results highlight the great vulnerability of elderly to influenza AH3N2, especially among older than 70 years in severe years of influenza activity, like 2003 and 2007. The study also shows the lower impact of influenza AH1N1pdm 2009 in this age group compared to younger. Risk of dying among the elderly in years of circulating AH3N2 influenza has been reported in several parts of the world (9, 10, 21, 22); however, in Brazil, there are no recent estimates available. Few studies analyze the circulation and impact of influenza in tropical and subtropical regions (6, 7, 9–11). Influenza B virus is also associated with severe disease (23); however, this variant did not circulate with intensity during the study years in Brazil.

Although the elderly are the most vulnerable group to viral respiratory infections, we found relative small excess of deaths in years of circulating AH1N1 pre pandemic (2002 and 2008). Study comparing excess deaths from respiratory diseases in the elderly in Latin America shows stable rates (mean of 89.4 per 100,000 inhabitants) in southern Brazil between 1998 and 2008 (pre-pandemic Flu A-H1N1), although higher in Brazil than in other countries (24). In the USA and in European countries, influenza seasons dominated by subtype AH3N2 are typically associated with mortality two to three times higher than in seasons with predominance of AH1N1 (prior to pandemic strain 2009) and of influenza B viruses (9, 10, 19, 25).

When all causes of death are studied, the overall mortality associated with influenza among elderly exceeds that observed in younger age group. It should be considered that all causes mortality is a non-specific measure and a distant outcome of influenza infection. However, it is difficult to determine which group of causes of death could better characterize the influenza burden in mortality. By choosing only the respiratory causes, we may underestimate clinical complications of pulmonary viral infection (e.g., cardiovascular). Therefore, in this study, we analyzed all causes, respiratory, and pneumonia and influenza deaths.

The unfavorable evolution of infection in the elderly is possibly due to the prevalence of comorbidities, deficiencies in defense mechanisms, and poor antibody response to vaccination, as cell-mediated and humoral responses limit severity of disease (26). Patients with chronic diseases are more susceptible to infection due to decline of the immune function through inflammatory mechanisms, hindering the mucosal barrier, and the adaptive and innate immunological defense mechanisms (27).

The immune response to infection in the elderly tend to be delayed and weak, with prolonged inflammatory responses, which involves different types of host reaction, mainly to clearance virus. The exacerbations of these mechanisms may induce immune-mediated pathology causing tissue damage (28). Cytokine high serum levels of IL-6, TNF-a, IFN-g and sIL-2R, chemokines IP-10, MCP-1, and monokine induced by IFN-g (MIG), are associated with severe clinical cases and lung damage (29).

Immunological abnormalities in people with diabetes, chronic respiratory diseases, cardiopathy, or other chronic diseases have increased risk of severe infection and bad prognosis (19). For example, there is the consistent association of influenza infection

TABLE 2 | Excess mortality rate (per 100,000 population) and excess deaths (absolute number) according to influenza virus subtypes prevalent in southern Brazil, 2002–2015.

		24–59		60–69		70–79		≥80	
		Excess mort rate	Exc. deaths	Exc. mort rate	Exc. deaths	Exc. mort rate	Exc. deaths	Exc. mort rate	Exc. deaths
Mean of annual excess mortality during all period	Pnm and Flu	0.2	27	0.8	17	2.9	29	20.2	85
	Resp	0.4	57	3.8	64	12.3	113	37.2	143
	All causes	1.3	176	13.0	214	37.0	331	116.5	422
Mean of annual excess mortality in H1/B pre-pandemic years (2002 and 2008)	Pnm and Flu	0.0	0	0.4	7	2.1	19	2.2	9
	Resp	0.0	0	3.5	54	7.4	59	15.4	42
	All causes	0.9	118	13.1	201	35.7	287	88.3	255
Excess mortality during 2009	Pnm and Flu	2.5	331	3.2	57	12.0	113	20.5	82
	Resp	5.2	698	7.4	129	17.6	167	7.3	29
	All causes	7.1	953	21.0	368	58.6	555	125.1	501
Mean of annual excess mortality in H1/B pos-pandemic years (2010, 2012, and 2013)	Pnm and Flu	0.4	60	1.5	31	4.2	44	17.3	83
	Resp	0.7	99	3.0	62	14.3	150	21.5	102
	All causes	0.9	129	5.6	116	16.6	178	54.6	262
Mean of excess mortality during H3 years (2003, 2004, 2005, 2006, 2007, 2011, 2014, 2015)	Pnm and Flu	0.0	4	0.6	12	2.1	22	27.4	112
	Resp	0.1	13	4.1	66	12.6	111	50.6	191
	All causes	1.2	148	15.1	240	42.3	371	145.6	514
Excess mortality during severe H3 epidemics (2007)	Pnm and Flu	0.0	0	0.6	10	2.1	19	49.5	176
	Resp	0.2	24	8.5	138	32.5	288	134.2	477
	All causes	2.9	375	35.6	576	136.1	1,209	449.6	1,598
Relative risk 2009/mean of period	Pnm and Flu	12.6		3.8		4.1		1.0	
	Resp	12.4		1.9		1.4		0.2	
	All causes	5.3		1.6		1.6		1.1	
Relative risk 2009/mean of H3 years	Pnm and Flu	83.9		5.6		5.6		0.7	
	Resp	49.2		1.8		1.4		0.1	
	All causes	6.2		1.4		1.4		0.9	
Relative risk 2009/severe H3 epidemics	Pnm and Flu	#		5.5		5.6		0.4	
	Resp	28.3		0.9		0.5		0.1	
	All causes	2.5		0.6		0.4		0.3	

Exc., excess; Pnm and FLU, pneumonia and influenza; Resp, Respiratory causes International Causes of Deaths ICD chapter J (J10–J18.9).
 # - Relative risk has no valid value as denominator is zero.

with cardiovascular mortality, particularly acute myocardial infarction (30). In part, it is attributed to altering endothelial function due to an acute inflammatory and procoagulant stimulus during viral infection (31, 32). Clinical complications of diabetes triggered by influenza infection cause impairment of leukocyte function and increase post-infection colonization rates resulting in poor prognosis in the elderly (33, 34).

In young people and adults, in 2009, the emerging influenza AH1N1 strain had a notable impact on the mortality of people up to 59 years in various parts of the world, including Brazil (7, 25, 35, 36). Excess mortality of individuals aged 24–59 years in the state of São Paulo, Brazil was identified during the pandemic AH1N1 virus (7). Pregnant women adults with metabolic conditions, including obesity, chronic respiratory disease, and other chronic diseases were significantly associated with severe acute respiratory syndrome and the lethality in Brazil (37). Our study showed a 41.5-fold higher rate of mortality from pneumonia and influenza in adults (24–59 years) in the pandemic year AH1N1 than the average of years with predominance of AH3N2 circulation in southern region.

In addition to the clinical severity and the large portion of the affected population, pandemics affect age groups in different ways (38). While only 10% of deaths from seasonal influenza occur among those under 65 years of age, in the pandemics of 1918, 1957–1958, and 1968, this proportion was 95, 40, and 50%, respectively (39). Therefore, pandemics tend to affect a larger proportion of young people than seasonal influenza. In this study, higher rates of death due to pneumonia, influenza, respiratory, and all causes were observed among those aged 24–59 years in 2009.

One explanation for the higher mortality observed among the youngest is that they would be more prone to the situation known as "cytokine storm," i.e., a dysfunctional overproduction of cytokines that would lead to diffuse damage to the respiratory tract with severe and potentially lethal systemic repercussions (40). Viral replication and production of inflammatory mediators seem to be involved in the pathogenesis of infection with influenza A H1N1pmd2009, hindering the clearance of virus in lung tissue and leading to pathologic lesions (41).

Another explanation for the lower mortality in the elderly is that they were exposed previously to antigens of the pandemic virus. Hancock et al. (42) suggested a cross-reactive antibody response to 2009 pandemic AH1N1. Similarities between AH1N1 antigen from 2009 and 1918 were detected. This last virus strain has not circulated since 1958 (39), when the AH1N1 strain was displaced by AH2N2 (Asian flu). At that time, AH1N1viral circulation occurred mainly in children, the current elderly of 2009.

The emergence of the AH3N2 strain in the pandemic year 1968 (Hong Kong flu) affected several age groups. This new strain

resulted from a large genetic mutation (shift) recombining virus material of the circulating AH2N2 with the avian H3, of Asian origin, resulting in the new variant AH3N2 (38).

In 2002–2003, under selective pressure an antigenic small mutation (drift), resulted in A/Fujian/411/02(H3N2) a strains emerged after a "jump" in genes evolution of Hemagglutinin and Neuraminidase proteins of virus surface (43, 44). The circulation of the Fujian strain had a great impact on the mortality from pneumonia in several parts of the world in 2003–2004 and 2004–2005 (22) and in Brazil (45). In 2007, a new drift resulted in influenza AH3N2 detected in south Brazil (46) also affecting hospitalizations and deaths in various parts of the world (47). We observed high rates of excess mortality in the elderly, in the years of 2003 and 2007.

Limitations of this study refer mainly to the ecological analysis of pooled data. We did not analyze individual information regarding comorbidities and history of vaccination that could be important confounders influencing mortality (17). We just had the overall annual vaccination coverage which were in general, around 80% in the period. Estimates of the number of deaths (all causes, respiratory, and pneumonia-influenza) supposedly related to influenza may be inaccurate in inferring the impact of respiratory viruses. Correlations in time series studies may produce spurious associations, especially between all causes of death and influenza infection, due to the distance between cause and outcome, and to multiple components of the obits. Serfling addresses part of this limitation by introducing sinusoidal terms in equation, since non-influenza mortality is not expected to coincide exactly with sinusoidal pattern (14, 19). Moreover, excess mortality of pneumonia, respiratory diseases, and all causes can be considered as an alert to surveillance of viral respiratory diseases, such as a sentinel indicator to be investigated (4, 48). Although all causes mortality is a non-specific indicator, it does not underestimate the complications of chronic diseases associated with influenza (4). Despite the influenza component in all causes mortality is small, the indicator can be considered an indirect measure, a warning, useful in epidemiological monitoring.

Another limitation is the lack of robust etiologic data from virological surveillance in the years 2002–2012, which could lead to imprecision in the analyses; however, the data on the predominance strains in the southern region are reliable, and influenced the composition of the vaccine of each season.

Considering the option for the analysis model, Serfling linear regression may produce different estimates when compared with other models (Poisson, ARIMA, and GLM) (9, 10); Poisson and ARIMA models produce higher mortality estimates than Serfling, and Serfling higher than GLM, especially among the elderly (16, 17, 21). We chose Serfling model because we do not have robust virological surveillance data, before 2013, and the study period includes a pandemic year (4).

Besides, in this study, we did not analyze climatic variables (minimum temperatures and relative air humidity) that could also interfere with viral transmission and increase the impact of the disease, particularly in the elderly.

In conclusion, probably previous exposures to influenza AH1N1 in the past influenced the mortality of Brazilian elderly in 2009, despite the vulnerability of this age group to clinical complications. For the >70 years, we observe higher excess mortality rates (of all outcomes) in severe year of AH3N2 circulation (2003, 2007). It is also worth noting that vaccination has been associated with the prevention of death particularly at age 65 (49). Therefore, the high elderly vaccination cover in southern Brazil may have attenuated excess of mortality estimated, although the immune response is limited among those.

More attention should be given to the circulation of influenza AH3N2 in subtropical regions in Brazil. The reduced immune response to infection and to vaccination, and associated comorbidities recommend a special attention to this age group. Besides medical assistance, the timeliness of vaccine campaigns, its composition, and etiological surveillance of respiratory diseases in the region are some of the preventive and public health measures.

AUTHOR CONTRIBUTIONS

Both authors made contributions to the conception of the work, acquisition, analysis, interpretation of data, and writing the manuscript.

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REVIEW
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Immunosenescence and Inflamm-Aging As Two Sides of the Same Coin: Friends or Foes?

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The immune system is the most important protective physiological system of the organism. It has many connections with other systems and is, in fact, often considered as part of the larger neuro–endocrine–immune axis. Most experimental data on immune changes with aging show a decline in many immune parameters when compared to young healthy subjects. The bulk of these changes is termed immunosenescence. Immunosenescence has been considered for some time as detrimental because it often leads to subclinical accumulation of pro-inflammatory factors and inflamm-aging. Together, immunosenescence and inflamm-aging are suggested to stand at the origin of most of the diseases of the elderly, such as infections, cancer, autoimmune disorders, and chronic inflammatory diseases. However, an increasing number of immune-gerontologists have challenged this negative interpretation of immunosenescence with respect to its significance in aging-related alterations of the immune system. If one considers these changes from an evolutionary perspective, they can be viewed preferably as adaptive or remodeling rather than solely detrimental. Whereas it is conceivable that global immune changes may lead to various diseases, it is also obvious that these changes may be needed for extended survival/longevity. Recent cumulative data suggest that, without the existence of the immunosenescence/inflamm-aging duo (representing two sides of the same phenomenon), human longevity would be greatly shortened. This review summarizes recent data on the dynamic reassessment of immune changes with aging. Accordingly, attempts to intervene on the aging immune system by targeting its rejuvenation, it may be more suitable to aim to maintain general homeostasis and function by appropriately improving immune-inflammatory-functions.

Keywords: inflamm-aging, immunosenescence, immunometabolism, immune-adaptation, immunoremodeling, longevity, healthspan

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INTRODUCTION

Aging is one of the most intricate and complex biological phenomenon. A comprehensive understanding of aging requires an integrated approach of all physiological systems (1–3). This has captured human imagination from immemorial centuries and the search for a "Fountain of Youth" is still ongoing. Aging is often termed "senescence," which literally means to grow old. Despite

this clear and simple definition, the common interpretation of senescence and related senescent states is shadowed with a negative connotation associated with growing old, that is the only aspect which is inescapably considered is death instead of the process *per se*.

One physiological system that shows marked changes during aging is the immune system (4–7). The interest of the immune system in aging is related to the fact that this is an interacting master regulatory system that keeps the organism free of invaders, either internal or external. Since the introduction of the notion of immunosenescence, many scientists have questioned the justification for unidirectional implication of the immune system and its decreased efficiency associated with aging (8). Whereas some functions are indeed decreased, others are increased. Therefore; changes are not as uniform as the designation would suggest. In this review, we will describe recent advances in the domain of changes in the immune system with aging and outline our vision on how these changes can be dynamically reconsidered from immunosenescence to immunoadaptation/immunoremodeling.

IMMUNE CHANGES WITH AGING: IMMUNOSENESCENCE AND INFLAMM-AGING, AS THE TWO SIDES OF THE SAME COIN

The prevailing current opinion is that the most marked changes that occur with aging in the adaptive immune system determine the state of immunosenescence (9–11). It is of note that since the 1980s it has been recognized that the innate system is influenced by aging but perhaps not always in the same direction (12–14). At the turn of the century, a new concept has been put forward by Claudio Franceschi. This concept suggested that aging was associated with a chronic, sterile, low-grade inflammation called inflamm-aging (15). The first related question that arises is what are the characteristic aging-associated changes in the various compartments of the immune system? Furthermore, are these changes faithful indicators of senescence (and progressive incapacitation of the system) or an adaptation, as well as ultimately whether they have any clinical significance. The second question is what the relationship between immunosenescence and inflamm-aging is and how it can be integrated into the broader mechanism of the aging process? Finally, the third question is whether we should attempt to intervene to modulate it.

Innate Immune Changes

The innate immune response is the most phylogenetically conserved protection in the animal kingdom that allows the organism to efficiently defend against an impressive number of aggressive pathogens (16). This compartment is meant to recognize and react to the conserved pathogen-associated molecular patterns (external threats) and danger-associated molecular patterns (internal threats) by way of specific receptors that play a key role in elimination of the aggressors (17, 18). There are three classes of pattern-recognition receptors (PRRs), each one having distinct roles although all of them elicit some form of inflammation. The first class of PRR comprises the Toll-like receptors which, using various intracellular signaling pathways, results in NF-κB activation and production of various mediators such as cytokines and chemokines (19). The second class comprises the NOD-like receptors, which are able to stimulate the inflammasome complex and that results in the production of IL-1, IL-18, and IL-33 (20). The third class includes the Rig-like receptors that act through the interferon response elements (21). There are also other receptors which are crucial for the functionality of innate immune cells, such as various Fcγ receptors, chemokine receptors such as fMLP receptors, and complement receptors (14). One of the most important observations of these last years, besides the discovery of PRR, is the fact that the innate immune system possesses some sort of memory which has been termed trained innate immune memory (22, 23). As described by Franceschi et al., the innate immune system may be viewed as possessing the "bow tie" architecture where many signals converge to a few sensors but result in many effectors (24). These coordinated events help to elicit a precise and efficient response.

Whereas many immune changes have been described with aging, we will not describe in details all the aging-associated changes for each immune cell type, as this topic has been comprehensively reviewed recently (25, 26). Collectively, the main characteristics of aging with respect to the innate system are immune stimulation at the basal level on the one hand and, immune paralysis when specific functions such as free radical production are needed, on the other hand (8). This dichotomy was initially proposed to be at the basis of the inflamm-aging concept, which stated that the relatively maintained innate response overrode the more altered adaptive immune response, resulting in higher production of pro-inflammatory mediators. Since that original observation, it became obvious that other processes may contribute to inflamm-aging, such as cell senescence, mitochondrial dysfunction, and microbiota changes (dysbiosis) (27, 28). Furthermore, under some circumstances the effects of oxidative stress were included as part of the process (oxy-inflamm-aging), emphasizing the role of the oxidative stress in the complex mechanisms of aging (29). Whatever the nature and the involvement of the components of inflamm-aging are, it is a fact that the phenomenon results in subclinical low-grade inflammation. For instance, life-long antigenic stimulation by pathogens would maintain this quiescent state of innate immune system activation. The innate immune system can also be stimulated by the so-called internal GARBage system (30). Thus, a heightened inflamm-aging state is produced as a consequence of (1) dysfunctional mitochondria, (2) defective autophagy/mitophagy (disposal of dysfunctional organelles), (3) endoplasmic reticulum stress, (4) activation of inflammasome by cell debris and misplaced self molecules, (5) defective ubiquitin/proteasome system (misfolded/oxidized proteins), (6) activation of DNA damage response, (7) senescent T cells and their senescence-associated secretory phenotype (SASP), and (8) age-related changes in the composition of gut microbiota (dysbiosis) (27–29).

The demonstration of trained immune memory may explain, at least partially, some of the immune aspects of aging (22, 23). Following their response, innate immune cells return to a

quiescent state due to epigenetic changes and modulation of cell metabolism alternating between (aerobic) oxidative phosphorylation (OX-PHOS) and anaerobic glycolysis (Warburg effect) (31). A subsequent stimulation (e.g., by the same or different type of pathogen) elicits a faster and higher response than the first one due to trained innate memory (32).

The hypothesis of trained innate memory may, at least in part, explain why aging innate immune cells are in a state of sustained activation (14). This concept is relatively novel and was first observed after a specific stimulation, such as the Calmette–Guérin bacillus (BCG). Even after 3 months following challenge, innate cells (monocyte/macrophages) were still able to sustain a certain "memory" of the initial infection and to react in the absence of BCG to any other stimulation (22). This observation has led to the concept that the innate immune system has a certain "memory," which was not foreseen from the previous paradigm of the immune system function.

Within the context of the aging innate immune system, it can also be suggested that the sustained trained immune memory is (or may be) leading to a sustained state of activation even in the absence of a specific challenge (6). This memory is likely due to a shift in the epigenetic landscape (epigenome) of the innate cells and fueled by an energetic shift of these cells. As yet, there is no formal proof for the contribution of these phenomena to the basic activation of innate immune cells, but this seems strongly probable. If this was the case, the epigenome and pathways involved in energy production, use, and conservation by immune cells could be targets of choice for immune modulation in the elderly. This possibility may explain the suggestions that macrophages are at center stage of inflamm-aging (15). Macrophages are able to modify their phenotype, produce pro- and anti-inflammatory mediators, and orchestrate many vital functions. Moreover, this phenomenon may to some extent resemble hormesis, providing a possibility to better react after each repeated stimulation (33, 34). Finally, it has recently been reported that not only does a high low-grade controlled inflammation was present in aged individuals (centenarians) but also that inflammation showed a better correlation with longevity than any other parameters, according to two longitudinal studies (35, 36).

The epigenetic clock notion in aging whole-organism has been proposed recently (37). ELOVL2 (elongase of omega 3 and 6 fatty acids) was found to be the most powerful single epigenetic biomarker of aging (38). Furthermore, Franceschi's group has shown that centenarians and their offsprings are epigenetically younger than one could deduct from their chronological age. According to this study (39), semi-supercentenarians are on average 8.7 years younger than expected based on chronological age, and offsprings of aged greater than 105 years are 5.2 years younger than age-matched controls where DNA methylation age and chronological age overlap. These findings reinforce the idea that epigenome control of the innate trained memory and its possible dysregulation with aging lead to DAMAge by inflamm-aging. Can this be reconciled with the heightened inflamm-aging in centenarians? Perhaps trained immune memory is the key regulated by epigenetic changes. The methylation age observed in centenarians suggests that the heightened inflamm-aging is either not connected to it or, paradoxically, methylation age should be younger. These observations may represent a trade-off between a potentially harmful process which, when under tight control, may remain beneficial. It is tempting to suggest that inflamm-aging may be considered the essence of life and the real "Fountain of Youth." In this context, centenarians may be considered as the standard and not the exception and may serve as model for the better understanding the role of inflammation and epigenetics in aging.

Chronic challenges during aging are paralleled by intracellular changes such as mitochondrial dysfunction, altered autophagy and changes in DNA repair mechanisms. However; immune cells are constantly maintained in an alert state due to chronic low-grade inflammation. However, this state may be counter-balanced by anti-inflammatory molecules as shown in the case of centenarians (40–42). Chronic low-grade inflammation (inflamm-aging) is a physiological response to the life-long antigenic stress and represents an efficient defense mechanism as long as it is under control. Without the essential counter-regulation by anti-inflammatory molecules as seen in aging, it is now clear how damaging this physiological state may be to the whole organism (43). Centenarians seem to represent an exception to the inflamm-aging effects on physiological aging or, perhaps, they present the physiological dynamics of aging. Thus, we may suggest that the norm (successful) aging is exemplified by centenarians whereas individuals that do not reach this age are the biological exceptions that lack the individual epigenetic history and machinery to reach that pinnacle.

The corollary of chronic low-grade inflammation is the down-regulation of the innate immune functions or immune paralysis or eventually a sort of innate immune tolerance (8). This physiological condition protects the organism against further self-induced damage even if it is at the expense of the defense from pathogens or from GARBAge. However, it is a reductionist view to assume that this immune paralysis is equal to a non-functional state. Although there are functional alterations in elderly individuals when compared to young subjects, a straightforward assumption that immune cells of elderly humans lose their protective functions. For instance, most elderly humans are able to defend against many types of infections even if the adaptive immune response is somewhat less functional. However, there are relatively few longitudinal studies concerning innate immune function changes with age. Therefore, we do not know whether this is a continuous phenomenon or whether they remain stable during aging. We can speculate that incongruent results of cross-sectional studies suggest that there is not a uniform decrease either related to cell type or to immune cell functions.

Could there be any advantage associated with innate immune paralysis? It can easily be conceptualized that maintenance of identical intensities in the innate cell functions in elderly subjects to the level of young individuals would be energetically very difficult. For instance, this is illustrated by the situation of the large amounts of energy needed for maintaining a M1 (pro-inflammatory and anti-cancer) phenotype in the case of macrophages (44). On the other hand, the M2 phenotype (healing, promoting angiogenesis and cancer growth) consumes much less energy and, therefore, is not as much affected. A decreased

production of free radicals can be considered harmful for the eradication of pathogens. However, low free radical production may protect the whole body against further age- and oxidative stress-related damages. Decreased chemotactic activity may also be suggested to be harmful for pathogen destruction, although a sterile inflammatory process may protect against excessive tissue damage. Thus, the question is within the context of evolution, what is most rewarding for the body in an aged organism? On the one hand, is it to destroy pathogens at any cost? On the other hand, is it to maintain physiological integrity by way of chronic inflammation? This dichotomy can mirror recent findings on mitochondria where a certain degree of dysfunction was linked to successful aging and longevity, in contrast to normal or excessively altered functioning in unsuccessful aging (45). This mild impairment may work as a hormetic signal (46). Although there is at present no definitive answer to this question, it illustrates the fact that a broader perspective is needed to understand changes in the innate immune system with aging.

The innate immune system influences the adaptive immune response in many ways. One of these cases is antigen presentation by dendritic cells (DCs). There are conflicting results in this domain and it seems that DCs are less able to prime $CD4^+$ T cells in the elderly (47). It is not clear whether the problem is related to antigen presentation or to reaction to antigen presentation. In likelihood; both aspects may be affected by aging. The processing of antigenic peptides by the immune proteasome may not be so efficient and perhaps either the T cell receptor (TCR) itself or TCR-dependent-signaling could be altered (48, 49). The interaction may also occur through an interplay with the cytokines that are secreted by the innate immune system cells. Increased levels of pro-inflammatory cytokine production by the innate cells during aging may also influence the reactivity of the $CD4^+$ T cells; e.g., the increased amounts of TNFα may downregulate the expression of CD28 which will negatively affect clonal expansion (50). Moreover, these cytokines may elicit increased free radical production in T cells, which will paralyze their function by increasing inhibitory events of signaling (51). In sum, alterations in the innate immune system may also impact adaptive immune changes with aging.

Adaptive Immune System

The adaptive immune system is composed of the cellular and the humoral immune response. T cells are orchestrating the cellular immune responses. These cells are basically divided into $CD4^+$ and $CD8^+$ T cell populations, which possess very clearly defined functions. $CD4^+$ T cells are helper cells that regulate the functions of all the other immune cells. They also possess effector functions (52). $CD8^+$ T cells are effector and memory T cells responsible for clearing the aggressors (53). The $CD4^+$ compartment may be subdivided, taking into account functionalities, in Th1, Th2, Th17, and regulatory T cell (Treg) subpopulations (54). Phenotypically, $CD4^+$ and $CD8^+$ T cell compartments are subdivided into four functionally distinct subpopulations, which are naïve, central memory, effector memory, and T effector memory cells re-expressing CD45RA (TEMRA) (55).

Many alterations in the adaptive immune system have been described in aging (11, 56, 57). With respect to T cell subpopulations, aging is characterized by two main changes: a decrease in naïve T cells that leads to the shrinking of the TCR repertoire and an increase in memory T cells that is primed by different aggressors. Recent thymic emigrants of new naïve cells are vanishingly rare in the elderly because of thymic involution at puberty and acute and chronic antigenic stress over the lifetime and, age-associated hematopoietic stem cell insufficiency (9, 10), This phenomenon is considered as one of the most basic changes in the adaptive immune system with aging. How does this situation happen? This seems to be the main explanation for the increased incidence of infections, cancers and the failure of vaccination in elderly (58–60). These observations mean that elderly individuals are less able to respond to neo-antigens than young individuals. However, this idea has been seriously challenged in recent years, mainly on the basis that there may not be a dramatic shrinkage of the TCR repertoire involving the remaining and slowly produced new emigrants, as supposed for decades (60). Moreover, the newly reconsidered homeostatic proliferation of naïve T cells under IL-7 stimulation may replace the failing thymus, at least partially. The recently discovered Stem Cell-like Memory T cells may also participate in incomplete replenishment of the naïve T cell compartment (61). Overall, the alterations may not be so dramatic and even the T cell repertoire may be relatively sufficient to supply the demand. Indeed, centenarians do not present more cancer, as its prevalence plateaus after the age of 90. Furthermore, there is no tendency for these individuals to suffer from unknown new pathogen-induced infections. Findings of two longitudinal studies led to the conclusion that having more $CD8^+$ naïve T cells was not considered a survival advantage (62, 63). These new data shed serious doubts on the present concept of "immunosenescence," at least in the adaptive compartment. However, the debate between immunologists and gerontologists is far from being settled.

There could be some evolutionary reasons for thymic involution. First, the maintenance of an organ so metabolically active may be very resource-demanding in the situation where the whole organism tends to reduce energy consumption during aging. This phenomenon can parallel the other two very energy-demanding organ shrinkage situations seen in aging, namely those of muscles and bone marrow. Second, during life, the organism has already encountered most of the pathogens typically active in the temporal and spatial region of its dwelling. Thus, resources must be allocated preferentially to combat these "usual," cognate pathogens by the memory part of the immune system rather than spend energy for a useless fight, which may be terminated in any event by destruction of the invading organism.

Thymic involution is a double-edged sword. On the one hand, it may indirectly be responsible for the death of the organism which would then lack the right TCR to mount an effective response against neo-antigen(s). On the other hand, it results in lower energy consumption which becomes available for other survival-supportive functions and activities of the organism. Given the relative rarity of direct infectious causes of death in the elderly, it would appear that downregulation of capacity to respond to novel pathogens during aging does not come at an excessive cost.

The increase in the number of memory T cells may be very rewarding for the aging organism as this will continuously assure survival against attacks by cognate pathogens that may threaten the survival of the organism. T cells are all directed against specific internal and external aggressors. The body hosts many latent infections which can re-activate from time to time under specific conditions (55). One well characterized pathogen of this type is the cytomegalovirus (CMV) (62). CMV was once considered the main cause of age-related immune changes in the elderly. Although accumulating data are still quite contradictory, the current belief is that the presence of CMV infection does not seem to be only detrimental (63–66). On the contrary, CMV infection may be considered a recurrent stimulation that maintains a sustained immunological alertness that favors a better immune response, e.g., to vaccination (67). The global response to the many various CMV antigens has even been linked to better survival (68). Thus, the increased number of committed memory T cells may not be considered unequivocally as detrimental or related only to aging.

One of the most important features of aging is the notion of senescent cells (69). This idea has re-gained popularity in recent years as a way to explain the decreased functionality of the immune system with aging (70). Senescent cells conform to the model of Hayflick replicative senescence as they are not proliferating but remain metabolically active and secrete several pro-inflammatory substances (SASP) (71, 72). Formerly, accumulated memory T cells were considered "senescent" (70). However, experimental evidence suggests that these cells are still able to function when pathogens such as CMV are re-activated (64, 65). Furthermore, there are no universally accepted markers of cell senescence (7). Finally, cell senescence is also a double-edged sword as these cells are needed in the case of some physiological functions, for instance repair and fight against cancerous transformation, whereas they are detrimental—to other cell functions (73, 74).

There is also a large confusion in the field of aging with respect to the number and distinction between senescent and exhausted cells (75–77). Senescent and exhausted immune cells are to be distinguished as the former may be functionally inert, whereas the latter may be functionally "dormant." This distinction is crucial when considering immune functions in relationship to aging. Exhausted T cells can be awakened by modulation of some surface receptors called the immune checkpoint inhibitors and, they can then resume function (78, 79). The most important of these receptors are PD-1, CTLA-4, LAG-3, TIM-3, and TIMIN. This distinction has gained considerable importance since it has been reported that a number of cancers in some elderly subjects could be successfully modulated and T cells engineered to be immunotoxic toward such cancers, namely melanoma and NSCLC (80–82).

One additional aspect where the literature has not, in our view, paid enough attention is the age-associated impairment of metabolic regulation of immune cell functions which is of vital importance for an adequate immune response. Quiescent cells compared to activated cells require different metabolic responses (83–87). Whereas quiescent cells use the OX-PHOS pathway for their functions that generates 36 ATP per metabolized glucose, activated cells use anaerobic glycolysis, which generate two ATP. Why is it so? When cells are activated, they need energy very quickly which cannot be provided by the OX-PHOS pathway, but only by aerobic glycolysis (Warburg effect). This is another example where the body is trading efficiency for rapidity, as in many circumstances in the aging immune system. Not only do the quiescent and activated states have different metabolic requirements but also the differentiation of the various subtypes of T cells is dependent on the specific metabolic pathways used (88). The master of cellular metabolism is the mTOR pathway that regulates clonal expansion, whereas its inhibition drives (*via* autophagy) the reconstruction of damaged cells (89). Very few studies in aging have addressed the metabolic changes that occur in immune cells with aging (90). However, one of these studies has emphasized the metabolic differences in T cells of young and elderly subjects (91). T cells of elderly individuals suffer from insufficient substrate to feed mitochondrial respiration and, consequently, are energy deprived. Instead of breaking down glucose, they shunt it into the pentose phosphate pathway, promoting an anabolic state. One of the metabolic consequences is the accumulation of reductive elements, particularly NADPH and reduced glutathione, and the scavenging of reactive oxygen species (91). Energy-deprived T cells upregulate activation of the energy sensor 5′-AMP-activated protein kinase (AMPK). A downstream target of inappropriately activated AMPK in aging T cells is the dual-specificity protein phosphatase 4 (DUSP4) (92), which negatively regulates members of the MAPK superfamily, in particular ERK1, ERK2, and JNK. ERK is also subject to increased negative regulation by another dual-specificity protein phosphatase, DUSP6 (93). ERK is a key regulator of the T-cell receptor signaling cascade, and its dephosphorylation by DUSP4 and DUSP6 functions as a suppressive mechanism, weakening the TCR-induced signal and dampening T-cell function. Thus, it may be very important to take into account age-related changes to determine how the extrinsic nutritional availability of glucose, amino-acids, and lipids will modulate age-related changes in immune system functioning.

Once immune cells are stimulated as a result of recognition of cognate antigen presentation, they initiate signaling pathways that result in the transcription of the appropriate molecules required for the expected functions (94–96). In elderly subjects, this cascade of events has been found to be altered, from impaired immune synapse formation to defects associated with translocation of transcription factors (49). Interestingly, detailed investigations of these alterations have revealed that they were mostly related to factors which could be considered modulable. One of the key factors that are involved in these changes are the composition and the organization of components of the plasma membrane, which orchestrates assembly of signaling molecules in cholesterol/ganglioside-containing nanoclusters (97). Thus, changes that were once considered a part of the aging process could be viewed as only the manifestation of some environmental interference and modulated by lifestyle factors such as exercise and nutrition (98).

WHAT IS THE RELATIONSHIP BETWEEN IMMUNOSENESCENCE AND INFLAMM-AGING?

According to the original concept of inflamm-aging, a consequence of immunosenescence, the relatively conserved innate immune system overtakes the more altered adaptive immune system in aging. However, recent data are more in line with the interpretation that this is not a unidirectional relationship, but a mutually maintained state where immunosenescence is induced by inflamm-aging and *vice versa*. The main changes in the aging adaptive immune system occur in the T cell compartment (57, 91). There is an increase in the number of memory $CD8^+$ T cells, which were originally considered relatively non-functional (99). These cells are characterized by the loss of naïve T cell surface markers, such as CD28, CD27, and the emergence of new senescent markers such as KLRG1. It has been found that the increase in the number of memory T cells and, later on, that of B cells may be due to a continuous chronic antigenic stimulation similar to the phenomenon of inflamm-aging. Infection by CMV emerged above all the large variety of potential stimulating agents (62). However, some data indicate that CMV infection could not be differentiated from inflamm-aging between seropositive and seronegative individuals (100). It is conceivable that the body devotes a huge part of its immune resources to contain this specific infection throughout life. Consequently, the immune space becomes filled with CMV-specific memory $CD8^+$ T cells. These cells have been previously considered to be inactive but recent data have shown that they are metabolically active and their senescent phenotype (SASP) can participate in the development of inflamm-aging (76). Thus, chronic antigenic stimulation leads both to the phenomenon of inflamm-aging and the increase of the number of senescent T cells. One additional consequence of chronic stimulation is the phenomenon of exhaustion, characterized by the emergence of inhibitory receptors, such as PD-1, CTLA-4, and many others (75). Other cell types of the adaptive immune system are also affected by aging but to various extents. For instance, the $CD4^+$ T cell population also undergoes similar changes to $CD8^+$ T cells but to a different extent (55). The Treg population also increases with aging as well as the pro-inflammatory Th17 subpopulation (101). Finally, the B cell compartment is also altered with aging (102). The functional consequences of these overall changes result collectively in the decreased ability to fight new challenges. Thus, clonal expansion, cytokine production, and specific antibody production are compromised. This situation leads to increased infections, cancer, and chronic diseases in the elderly (43). It appears that inflamm-aging and immunosenescence progress in parallel and form a vicious cycle. Increased production of inflammatory mediators characteristic of inflamm-aging contributes to the decrease of the adaptive immune response and, eventually, to immunosenescence. In contrast, the decrease of the adaptive immune response reinforces the stimulation of the innate immune response (as the means to protect organism from infections in the circumstances when adaptive immunity fails) leading to inflamm-aging. Both processes are important not only as causes of immune changes in the elderly but also (or even mainly) because of their consequences in the aging organism.

IMMUNOSENESCENCE/INFLAMM-AGING; WHY DOES IT MATTER?

One can ask why all these changes in the immune system with aging do matter. The paradigm for many years has been that immunosenescence and inflamm-aging are the fertile soil for the development of diseases mostly considered as age-related, either acute such as infections, or chronic such as cancer, frailty, Alzheimer's disease (AD), and cardiovascular diseases (CVD) (43). The bulk of these observations has led to the field of geroscience (103–105). The consequence of this new field leads to a novel approach that consists in targeting the aging process as the single most important risk factor instead of treating each disease separately. This notion should be nuanced by individual aging, suggesting that all individuals do not age in the same way and perhaps the underlying mechanisms may be different (6).

Alterations in T cell functions, more precisely the decrease in the number of naïve T cells and the increase in number of memory T cells, has been considered the main explanation for increased incidence of infections and cancers in the elderly (43). However, there is still no direct evidence from experimental observations or longitudinal studies, which could really support this hypothesis. It is of note to mention that the overall incidence of malignancies decreases after the age of 90 (106). However, would the incidence of infections, which is claimed to increase; still be true if one would only take into account elderly individuals with healthy aging? It is also of note that relatively few elderly subjects die of infections, even if severe and requiring hospital treatment. For example, in Canada in 2013, only 4.1% of deaths in individuals aged more than 65 years could be directly attributable to infectious causes (International Classification of Diseases A00-A99, B00-B99, G00-G03, J09-J21) (Statistics Canada: http://www5.statcan.gc.ca/cansim/pick-choisir?lang=eng&searchTypeByValue=1&id=1020561, accessed Oct 23, 2017).

It has also been commonly believed for decades that elderly individuals responded poorly to vaccination, which sometimes led to generalized doubts about the efficacy of vaccination in old age in general. Among the many vaccines which were considered less efficient (107), influenza vaccination was generally cited as the gold standard (108, 109). It is now established that there are many factors besides immunosenescence, which influence effectiveness of this vaccine and others. In fact, knowledge and better understanding of these factors has already led to enormous enhancement of efficacy of vaccination (e.g., against herpesviruses) (110).

Inflamm-aging could play a role in the late manifestation of diseases such as AD, frailty syndrome (FS), and CVD. It is of note that, besides FS, the onset of these diseases start in young or middle age when the immune system is still efficient, that is before signs of immunosenescence or inflamm-aging are detectable. Frailty can be considered a manifestation of (unsuccessful) aging and may represent the clinical sign of biological age, in contrast to chronological age (111). Furthermore, in a longitudinal

study, inflammation was found to be the most important factor to account for longevity, especially in semi-super centenarians (36). In conclusion, there is no doubt that immunosenescence and inflamm-aging contribute to the increased incidence of age-related diseases. However, their exact role is not yet well defined, and in some case, it may be even doubtful. There has been little exploration of the possibility that there are optimum levels of immunosenescence and inflamm-aging and that too much or too little could exacerbate the risks of various diseases in the elderly.

HOW CAN SUCCESSES IN VACCINATION AND IMMUNOTHERAPY BE INTERPRETED IN LIGHT OF IMMUNOSENESCENCE?

There has been recent reports of therapeutic successes in domains which were strongly considered to have the potential to overcome immunosenescence, namely the decreased immune response of the elderly to vaccination and the failures in treatment of some cancers. For example, in one study a new vaccine was tested for herpes zoster (shingles) prevention. This study showed that the administration of the antigen in combination with an adjuvant induced a strong immune response even in subjects older than 80 years of age (110). Furthermore, protection by this vaccine was high even after the 3.2 years of follow-up. This report should serve as a lead to question whether reported lack of vaccine efficacy in the elderly is due to immunosenescence or to improperly designed vaccines. Alternatively, even if a decrease in the immune response occurs it may be overcome by a well targeted vaccine (110). Recently, there has been a rise of immune checkpoint inhibitors as effective therapeutics in cancer treatment. While still sparse, the observations of effectiveness in elderly subjects are very encouraging. For example, in the case of metastatic melanoma, the use of Nivolumab® and Ipilimumab® either alone or in combination had survival effects in elderly subjects similar to young patients (80–82). This is a remarkable result that suggests that the exhausted T cells of the elderly are still able to respond to inhibition of their inhibitory receptors with a recovery of cytotoxic activity. It is to be mentioned that immunotherapy often works well in the elderly, but the treatment has to be adapted to the patients and be different than for young people (112). What works for one does not necessarily work for the other, but this does not mean that the elderly will not respond. The longevity advantage in some cases of CMV infection may also militate against the exclusively detrimental effect of the immunosenescence and inflamm-aging (68).

HOW SHOULD WE INTERPRET IMMUNOSENESCENCE AND INFLAMM-AGING?

We suggest that there is a need for a complete reconsideration of immune changes with aging to gain access to a better understanding of their mechanisms and to ensure that eventual interventions do more good than harm. The question does follow: which immune changes in the elderly may be beneficial? The answer to this question would require careful reinterpretation of current data, but it could also be highly useful to cope with an extended perspective of aging. There are several potential changes in the adaptive immune system with aging. First, increased proportions of adaptive memory cells may be beneficial to fight cognate pathogens more efficiently. Thymic involution may be considered as needed for reduction of energy consumption by an organ which is not absolutely necessary for survival and the maintenance of which is energy-costly. Finally, the increased proportions of Tregs observed in the elderly may prevent autoimmune onslaught.

There are some alterations that may not be solely detrimental in the innate immune system. For instance, increased proportions of innate ("trained") memory cells may help to efficiently fight cognate—and some not so cognate—pathogens. Increased asymptomatic pro-inflammatory state (conceptualized as increased "readiness" of innate immunity to pathogen challenge) may have some evolutionary advantages and could even be considered necessary if it is well regulated, i.e., not excessive. However, too much of a good thing could ultimately lead to disastrous consequences at any age, e.g., free radicals.

Accordingly, we can propose a new paradigm for dynamic immune changes with aging (**Figure 1**). We suggest that aging leads to modified/modulated responses of the immune system, making it more adapted to cope with challenges (pathogens) in a given (local) environment, and not just to an eventually terminal deterioration of the immune system. From an evolutionary perspective, this is a simple optimization of the resources of the aging body, even if it ultimately leads to pathologies and death. From this perspective, many or most age-related changes in the immune system may be desirable adaptations to the aging process, and thus no need for rejuvenation seems to be necessary.

TWO IMPORTANT RECENT APPROACHES TO BETTER UNDERSTAND IMMUNE CHANGES WITH AGING, SUPPORTING THE NEED FOR A CHANGE OF THE CURRENT PARADIGM

There are two new approaches which can be adopted to re-conceptualize immune changes with aging. These integrate almost all aspects mentioned above. Immune systems of the elderly are remodeled with fewer naive cells and dysfunctional (exhausted vs. senescent) memory cells, due to chronic antigenic stimulation (including, but not limited to, CMV and neo-antigens from emerging malignant cells) and thymic involution, with altered innate immune response resulting in inflamm-aging eventually contributing to some age-related disease development (**Table 1**).

How do Inflamm-Aging and Immunosenescence Stand from an Evolutionary Perspective?

Considering all the alterations in the immune system with aging, the question arises whether and how inflamm-aging and immunosenescence can be the cause of these numerous age-related alterations and pathologies attributed to them. Recent

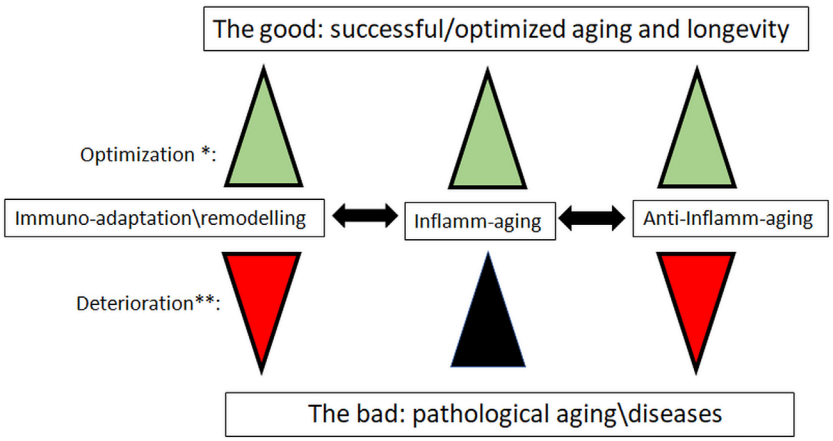

FIGURE 1 | The new paradigm for the role of inflamm-aging and immunoadaptation/remodeling in the aging process. *Optimization: all three processes increase in concert, balancing each other. **Deterioration: inflamm-aging increases, and is not balanced by opposite processes of anti-inflamm-aging and immune-adaptation/remodeling, which are decreasing. We mean by anti-inflamm-aging all compensatory mechanisms which emerged to compensate the chronic inflamm-aging. The most important diseases that could have an inflamm-aging component are cancers, cardiovascular diseases, and neurodegenerative diseases.

TABLE 1 | Summary of some immune changes associated with aging in innate and adaptive immune systems.

Features	Increase	Decrease	No change
Innate immunity			
Phagocytosis	–	√	√
Free radical production	√	√	–
Chemotaxis	–	√	–
Cytokine production	√	–	–
Myeloid cell number	√	–	–
Adaptive immunity			
Naïve cell number	–	√	–
Memory cell number	√	–	–
T regulatory cell number	√	–	–
T regulatory cell function	–	√	–
Proliferation	–	√	–
IL-2 production	–	√	–
B regulatory cell number/function	–	√	–
B cell immunoglobulin production	–	√	–
B cell autoantibody production	√	–	–

Changes are indicated with a checkmark (√) and, absence of changes with a horizontal bar (–).

observations tend to challenge the established view of the role of immune changes with aging. The publication of Lal et al. cited above that reported a positive response to a new herpes zoster vaccine even in the very old raises the question of the role of immunosenescence and inflamm-aging in the decreased response to vaccination (110). An additional recent report has further suggested that inflammation is a driving force for longevity in super semi-centenarians (36). In another study (113), Franceschi's group determined HCMV prevalence in 132 centenarians, 245 centenarian offspring, and 101 offspring of non-long-lived parents. These authors found that infection did not impact on the longevity of these elderly individuals. Finally, the studies concerning the diversity of the microbiota in centenarians may also support this changing paradigm as dysbiosis is not always the equivalent of dysregulated inflamm-aging, particularly in centenarians and semi-super centenarians. The changes in the composition of the gut microbiota with age in subjects ranging from 22 to 109 years can be mentioned as one of the best example of remodeling, with possible large influence on inflamm-aging and immunosenescence, taking into account how important is the gut microbiota for the immune system. An increase in sub-dominant species and among them, species which are considered very "good," was observed in Italian, Japanese, and Chinese centenarians despite the differences of diet and genetics in aged subjects, particularly in semi-supercentenarians (114, 115).

This situation did not involve a chronic uncontrolled inflammation, but the well-balanced inflammatory and anti-inflammatory equilibrium. Immune changes observed during aging may thus only represent an adaptation to a challenging environment (containing mostly the cognate pathogens, with the exception of cancer cells generated by semi-random mutations) that results in maintenance of homeostasis *via* hormesis. However, under conditions of not previously encountered pressure (i.e., contact with a novel, previously unknown pathogen), the aging immune system either can adapt by using the available reserves. Conversely, if it is unable to do so, that leads to a maladaptation manifested by age-related diseases or a lethal outcome.

From this perspective, it is interesting to consider the putative role of inflamm-aging in frailty. The definition of frailty is already controversial as two main designations exist, namely the phenotypic definition (116) and the multiple composite burden (deficit accumulation) (117). Frailty in fact can be conceptualized from an evolutionary point of view as the decrease of the physiological/biological/molecular reserves of the aging organs/organism, leading to less efficient responses to stresses and,

therefore, producing deleterious effects, even death (4, 111). This could be considered normal (usual) aging, in contrast to successful aging on the one hand or pathological aging on the other hand. Independently of its definition, one of the most accepted causes of frailty is inflamm-aging (4, 118) that represents the biological threshold between successful and pathological aging. This event suggests a dynamic process that could still be reversed if the underlying causes such as inflamm-aging were contained. However, this situation may also progress to death through diseases when it becomes uncontrolled and hyperinflammatory, as would be predicted by the trained innate memory process (8). From this perspective, the extent of symptoms included in the clinical picture of frailty may be considered as a surrogate measure for biological age independently of chronological age, indicating whether inflamm-aging tends toward health or disease/vulnerability. Then an "optimal inflamm-aging" may be defined for longevity and health (optimal aging). Eventually, as is the case with all biological processes, an equilibrium is needed with functional checkpoint gatekeepers. If, for any reason, this equilibrium is perturbed the pathological pathway may prevail. The aim of optimization efforts and approaches would be to help maintain this very complex equilibrium to achieve an adequate functional longevity for optimal aging.

A Dysregulatory Approach Integrating the Immune System and Other Systems

Clearly, the immune system does not exist in isolation but is influenced by and, in turn, influences many other systems such as the central and the peripheral nervous system, the endocrine system and others (3, 4, 119). This fact fits perfectly with the new approach of the study of aging which states that aging is the sum of results of the dysregulation of different system(s) from a normal regulatory level (i.e., a homeostatic state). This state is not necessarily identical between young and old subjects and may well reflect adaptations to intrinsic (GARBAge) and extrinsic changes (mainly pathogens and adverse environmental influences) related to aging. Thus, dysregulations are neither obligatorily detrimental nor beneficial but indicate a state of dyshomeostasis. This statement leads to the important insight that the pro-inflammatory state cannot be considered separately from the anti-inflammatory state (41). In this respect, this possibility will likely be expanded and nuanced by inclusion of other systems and cellular subsystems, e.g., mitochondrial metabolism. More broadly, this approach suggests that there are clear limits to the relatively linear, pathway-based, reductionist approaches to understanding physiology in general and immunology in particular. "Optimal" levels of various cell types, surface markers, and cytokines are unlikely to be very high or very low, but often intermediate, suggesting non-linear associations with risk. These optimal levels are also likely to vary depending on many other factors, such that it will be quite tricky to identify generally healthy or unhealthy states. Because of the possibility that changes with age or health state represent adaptations rather than aspects of pathology, substantial care must be exercised when interpreting changes in the system.

This way of thinking is a completely innovative approach which can be studied by various statistical analyses using smaller or larger databases obtained in the studies of elderly subjects. This approach can also lead to the discovery of new biomarkers and their use in clinical settings. Such a broad approach would allow the integration of the omics, single cell assessment and systems biology. Different statistical approaches may be warranted both in cases when the dysregulation(s) follow a single unidirectional pathway (as appears to be the case for inflamm-aging and likely metabolic syndrome) (119, 120), as well as in cases where dysregulation can produce a wide array of phenotypes sharing little beyond their departure from a homeostatic state (121–123). An appealing hypothesis is that canalized dysregulations occur as a result of adaptations to the aging process and thus reflect an optimized response to an imperfect situation. In contrast, non-canalized dysregulations reflect a true loss of homeostatic control and may themselves be the imperfect situation causing the canalized responses.

Identification of cell types in both the innate and adaptive immune systems that are affected by age-related changes would be an additional application of these kinds of integrative statistical approaches. Immunology has generally considered individual cells to belong to discrete types that can be distinguished based on their surface markers. Certainly, this is a valid paradigm for many of the major classes of immune cells (T-cells vs., B-cells, $CD4^+$ vs. $CD8^+$, etc.), but many examples of less distinct, partially overlapping classes are starting to emerge: classes based on the levels of surface receptors rather than just their presence or absence, or classes confounded by some subpopulations that are simultaneously expressing two markers that were supposed to distinguish populations (e.g., $CD45RA^+$ vs. $CD45RO^+$). It would thus appear that variation in cell surface receptors can happen in different ways. Discrete variation produces populations of cells with distinct functions and properties ("classes" or "types"), whereas continuous variation produces cells with functions and properties that vary along a gradient. This distinction is important because if one wrongly considers cells varying along a gradient as being from discrete classes, one is likely to (a) misidentify many cells with intermediate phenotypes and (b) to fail to understand the true biological processes driving cell diversity, thus wrongly interpreting the functional consequences of this diversity. For example, one can suppose that a population of cells varies along a gradient that determines their affinity for two types of pathogens (say, A and B). The cells with high affinity for A would have low affinity for B and *vice versa*. If there were a true gradient, a reasonable strategy would be to have a large population of cells with an intermediate affinity for both, maximizing the flexibility of the response. This may be a particularly good strategy during aging when the total cell population declines and the ability to maintain large numbers of cells at both extremes of the gradient is compromised. If one wrongly divides cells into A-affinitive and B-affinitive types, one may obtain uninterpretable or confusing results, depending on where along the gradient the threshold is set. It would then be not possible to understand any strategies that involve the use of intermediate values along the gradient.

Should We Intervene and How?

If we consider immune changes related to aging as an adaptation/remodeling, interventions are presently very difficult to foresee. In particular, a single, generalized immune intervention does not appear to be likely. Anti-inflammatory interventions may depend on the state (level) of inflammation and its duration and, on interactions of the innate immune system with other systems, as well as on the appropriate inflammatory state of the individual given age and disease status. If one assumes that the immune/inflammatory system in the elderly/aged organism is adapted/remodeled in order to provide the best possible anti-pathogen protection when the adaptive immune system fails, the rejuvenation approach as currently proposed (e.g., IL-7/IL-15) seems likely to cause potential long-term harm in the aged organism.

Perhaps, more general, however, purposeful interventions may be necessary, such as lifestyle interventions with personalized exercise and nutrition. Specific epigenetic diets may have their role in this modulation (124, 125). Some drugs with global action have been suggested to decrease the (over)activation of the immune/inflammatory system. One such drug is metformin, suspected for a long time to be a powerful modulator of aging (126). Still, its effects in aging immune/inflammatory system are not yet clear. In any case, any interventions will need to be personalized and the immune history (immunobiography) of the individual will have to be taken into account.

CONCLUSION AND FUTURE PERSPECTIVES

Aging is a highly complex process but an increased understanding of the process should lead to the efficient treatment of the many age-related diseases. The immune system interacts with many other systems in the organism (mainly the neural, metabolic, and the endocrine systems) and is, therefore, one of the most ubiquitous master systems of the organism. As such, it orchestrates health when it functions well but, when maladapted, it leads to diseases in the aging organism. Many changes in the immune system with age have been described and most of them have been considered deleterious and causes of many age-related diseases. Changes occur in both the innate and the adaptive immune arms of the immune system, but perhaps not to the same extent or with the same consequences. There is an intricate interrelationship between inflamm-aging and immunosenescence, which are nearly identical in some ways but very different in other aspects and, occurring in concert, mutually influencing each other. Future studies are obviously necessary to elucidate these interactions and raise targets for new interventions to decrease the deleterious effects of aging and use the beneficial effects for a better health and functionspan in the elderly.

Therefore, the phenomenon traditionally termed "immunosenescence" may be considered an immunoremodeling/adaptation as a result of chronic aggressions and time. Immunosenescence may be necessary for an adequate response to known antigens, but detrimental for responses to new antigens in most circumstances. The discovery of new processes, new immune cell subtypes, and the integration of genetic/epigenetic/metabolic and environmental factors (nutrition) will nuance our «evil» and apparently mistaken perception that aging-associated immune changes are only detrimental. Immunosenescence/inflamm-aging may contribute to diseases such as cancer but its role during aging is still controversial. Elderly in clinical settings are doing much better than predicted from experiments thus, human studies in particular are badly needed.

In view of the successes of cancer immunotherapy and vaccination in the elderly, no such intervention should be refused to an elderly subject based on a dogmatic assumption that aging-related immune changes are detrimental. Thus, time is of the essence; and the future is already now for the elderly.

AUTHOR CONTRIBUTIONS

TF has written and conceptualized the article; AL, GD, AP, EF, AC, JW, and CF have contributed to the writing and critically read it.

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An Explorative Biomarker Study for Vaccine Responsiveness after a Primary Meningococcal Vaccination in Middle-Aged Adults

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Introduction: Prevention of infectious diseases in the elderly is essential to establish healthy aging. Yet, immunological aging impairs successful vaccination of the elderly. Predictive biomarkers for vaccine responsiveness in middle-aged adults may help to identify responders and non-responders before reaching old age. Therefore, we aimed to determine biomarkers associated with low and high responsiveness toward a primary vaccination in middle-aged adults, for which a tetravalent meningococcal vaccine was used as a model.

Methods: Middle-aged adults (50–65 years of age, $N = 100$), receiving a tetravalent meningococcal vaccination, were divided into low and high responders using the functional antibody titers at 28 days postvaccination. A total of 48 parameters, including absolute numbers of immune cells and serum levels of cytokines and biochemical markers, were determined prevaccination in all participants. Heat maps and multivariate redundancy analysis (RDA) were used to reveal immune phenotype characteristics and associations of the low and high responders.

Results: Several significant differences in prevaccination immune markers were observed between the low and high vaccine responders. Moreover, RDA analysis revealed a significant association between the prevaccination immune phenotype and vaccine responsiveness. In particular, our analysis pointed at high numbers of CD4 T cells, especially naïve CD4 and regulatory T subsets, to be associated with low vaccine responsiveness. In addition, low responders showed lower prevaccination IL-1Ra levels than high responders.

Conclusion: This explorative biomarker study shows associations between the prevaccination immune phenotype and vaccine responsiveness after a primary meningococcal vaccination in middle-aged adults. Consequently, these results provide a basis for predictive biomarker discovery for vaccine responsiveness that will require validation in larger cohort studies.

Keywords: biomarkers, vaccine responsiveness, middle-aged adults, regulatory T cells, CD4 T cells, primary vaccination

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INTRODUCTION

Prevention of infectious diseases in the elderly is essential to establish healthy aging in the rapidly growing aging population. Yet, immunological aging impairs successful vaccination in the elderly (1–3). Timely vaccination of middle-aged adults may be an alternative option to strengthen the memory immunity before reaching old age. Previously, we showed that a primary meningococcal vaccination, containing antigens toward which no or very low prevaccination immunity exists, was highly immunogenic in middle-aged adults (4). Moreover, we described the induction of T cell responses by the tetanus toxoid (TT) carrier protein that are in favor of efficient T cell help (5). Current research focusses on the identification of immune markers in older individuals to be able to predict the vaccine responders and non-responders (6, 7). At present, the discovery of these predictive immune markers at advanced age is challenging and results are not unambiguous.

Potential biomarkers for vaccine responsiveness may relate to shifts in the immune phenotype from naïve to more memory cells during aging. This phenomenon occurs especially in the T cell compartment and is caused by thymus involution (8–13). Accordingly, the responsiveness to a yellow fever vaccine was found positively associated with the numbers of circulating naïve CD4 T cells that had recently left the thymus (14). Infection with persistent viruses, such as cytomegalovirus (CMV), enhances the numbers of late-differentiated T cells and consequently may accelerate immunological aging (15, 16). High numbers of these late-differentiated T cells were negatively associated with influenza and varicella zoster (VZV) vaccine responses (17, 18). In addition, increased numbers of regulatory T (Treg) cells are observed at old age (19, 20) which may underlie the lower responsiveness to the influenza and VZV vaccinations (18, 21).

Age-associated changes in the B cell compartment have also been reported and include a decrease in naïve B cells and a subsequent increase in late-differentiated and exhausted B cells, as well as B cells with inflammatory characteristics (22–24). Several vaccination studies described a positive correlation between the frequencies of prevaccination Ig switched memory B cells and the responsiveness to influenza and hepatitis B vaccines (17, 23, 25–27), whereas late/exhausted (CD27−IgD−) memory B cells were negatively correlated with the response to the influenza vaccine (23). Moreover, B cell expression levels of activation-induced cytidine deaminase (AID) and TFN-α after *in vitro* stimulation were found predictive for humoral responses after influenza vaccination (23, 26, 28–30).

In addition, several innate immune functions, gene signatures, or miRNA expressions were associated with influenza vaccine responsiveness (25, 31, 32). Moreover, the age-associated increase in inflammatory mediators, also known as "inflammageing" (33–36), as well as modified expression of biochemical markers, such as dehydroepiandosterone sulfate (DHEAs) (37) and vitamin D (38), might affect the immune function at advancing age (39). Also, a range of vaccination responses, for example, to diphtheria, tetanus, and influenza, are substantially influenced by vaccine-specific prevaccination immunity (27, 31).

The studies mentioned provide some promising predictive biomarkers that require validation in other cohort studies. In the present study, we aimed to explore differences in the prevaccination immune phenotype between low and high vaccine responders toward a primary immune response upon a meningococcal vaccination in middle-aged adults.

MATERIALS AND METHODS

Study Design

Data from 100 middle-aged (50–65 years of age, 50% males) adults who received the tetravalent meningococcal vaccine conjugated to TT were used in this explorative biomarker study. These participants were included in a larger cohort study, of which exclusion criteria and study procedures are described elsewhere (4). In short, prevaccination blood samples were drawn from all participants as well as 28 days, and 1-year postvaccination blood samples. Serum samples were collected at the different time points using serum clotting tubes (BD Biosciences) and were immediately kept cold and stored within 4 h in aliquots at −20 and −80°C before further use. Blood samples were collected in tubes containing lithium heparin (BD Biosciences) for detailed cellular immune phenotyping prior to vaccination. Subsequently, different immune parameters, i.e., absolute immune cell counts, serum cytokines, CMV-specific antibodies, and biochemical markers were measured in the prevaccination blood samples of these participants. Meningococcal-specific functional antibody titers were measured in the prevaccination, as well as 28 days and 1-year postvaccination samples. A schematic overview of the study outline is depicted in **Figure 1**. In addition, all participants filled in a short health questionnaire.

Participant Selection

Functional antibody titers for the three different meningococcal groups (Meningococci C, W, and Y) were measured with the serum bactericidal antibody assay in 100 middle-aged adults, as previously described (4, 40, 41). Meningococci-A-specific analysis was left out, due to interference of cross-reactive antibodies in the antibody assays. A functional antibody titer of 8 was considered to be protective, whereas a functional antibody titer of 128 was applied as a more conservative long-term correlate of protection (4, 40).

The quartiles of the functional antibody titers 28 days postvaccination were calculated. Participants with a functional antibody titer matching the corresponding titer of the first quartile or below were considered low responders, whereas those matching the titer of the third quartile or above were considered high responders. Since part of the participants showed antibody titers equal to the cutoff value, the lowest and highest quartile do not include 25% of the participants. In total, 25, 46, and 40 low responders and 27, 35, and 34 high responders were defined for MenC, MenW, and MenY, respectively.

Flow Cytometric Analysis

At the prevaccination time point, the absolute numbers of a broad range of immune cell subsets were determined as described previously (42–44). In brief, the absolute numbers of lymphocytes, T cells, B cells, NK cells, monocytes, and granulocytes

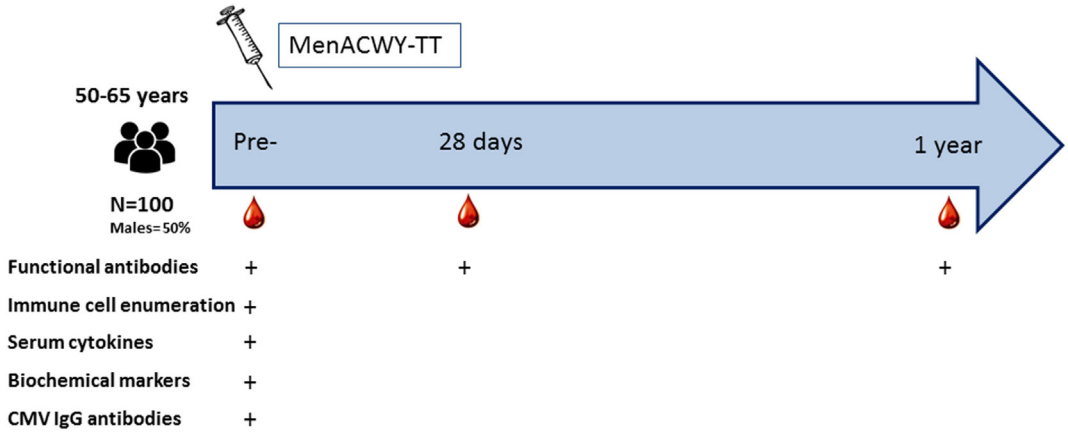

FIGURE 1 | Study outline.

were measured in fresh whole blood samples (within 18 h after collection) using TruCOUNT tubes. Gating strategies as well as a detailed description of the antibodies used were as published previously (42). Example gating strategies are shown in Figure S4 in Supplementary Material. An overview of the phenotype definitions of the different cellular subsets measured is depicted in Table S1 in Supplementary Material. These absolute cell numbers were also used to calculate the ratios between the (memory) Treg cells and the $CD4^+CD45RO^+$ effector memory T (TemRO) cells as well as between the CD4 naïve or $CD4^+CD45RA^+CD25^{dim}$ cells and the CD4 memory cells. The CD4 memory cells were defined as the sum of the CD4 central memory (CM), CD4 TemRO, and $CD4^+CD45RA^+$ effector memory T (TemRA) cells.

Serum Cytokines

A set of inflammatory (TNF-α, MCP-1, soluble CD40L, and IL-6) and one anti-inflammatory (IL1 receptor antagonist (IL-1Ra)) serum cytokines were measured in serum samples that were kept cool right after harvest and stored at −80°C within 4 h and prevented from freeze-thaw cycles. Multiplex immunoassays (MIAs) were used to measure the serum cytokine levels as described previously (45, 46). Since serum levels of IL-6 were below detection limit, IL-6 was left out of the analysis.

Serum Biochemical Parameters

Serum levels of C-reactive protein (CRP; mg/L), Rheumatoid factor (RF; IU/mL), reactive oxygen metabolites (ROM; IU/L), and total thiol (TTT; μmol/L) were measured with a clinical autoanalyzer (Dx5, Beckman-Coulter). Dehydroepiandrosterone sulfate (DHEAs; μmol/L) and 25-hydroxyvitamin D (VitD; nmol/L) were measured using the immuno-analyzer Acces-2 from Beckman Counter.

Statistical Analyses

The functional antibody titers were compared between the high and low responders with the Mann–Whitney *U*-test. The geometric means with the 95% confidence intervals (CIs) are indicated in the graphs. The chi-square test was used to determine significant differences in patient characteristics between the high and low vaccine responders.

The different immune markers were compared between the high and low responders using the Mann–Whitney *U*-test. Furthermore, the group-specific geometric mean values of the different immune markers were normalized to *z*-scores using the geometric means and standard deviation of the total group of 100 participants. The normalized *z*-scores were displayed on a color scale in the heat maps, ranging from red (below the geometric mean of the total group) to blue (above the geometric mean of the total group). The color darkness is representative of the deviation from the total group geometric mean. For these analyses, SPSS V22.0 and Graphpad Prism V7 were used.

Multivariate redundancy analysis (RDA) was used to asses associations between vaccine responsiveness and the prevaccination immune phenotype. The absolute numbers of immune cells as well as the levels of serum cytokines and biochemical markers were imported in the analysis as biological variables, whereas vaccine responsiveness, age, sex, and CMV were included as explanatory variables. Significance of the explanatory variables was assessed by Monte Carlo permutation testing (MCPT). The *p*-values as well as the false discovery rates (FDRs) are given. Biological variables with the highest variation explained by the explanatory variables are depicted in the plots (FitE > 15). Canoco5 software for Windows (47) was used to perform this analysis. A value of p of < 0.05 was considered statistically significant.

RESULTS

Participant Characteristics

The functional antibody titers of the low and high vaccine responders 28 days postvaccination are depicted in **Figure 2A**. Although the low responders possess a functional antibody titer below or matching the first quartile of that of the total group, most of these values were above the protection level (functional antibody titer of 8). The fold differences in functional antibody

titers between the low and high vaccine responders at 28 days postvaccination is 141.5, 15.2, and 16.7, for MenC, MenW, and MenY, respectively. No difference in prevaccination functional antibody titers was found between the low and high responders (**Figure 2B**). The functional antibody titers of part of the low vaccine responders had declined below the protection level at 1-year postvaccination, whereas all high responders were still highly protected (**Figure 2C**).

Participant characteristics were compared between the low and high vaccine responders (**Table 1**). Only for MenC, the

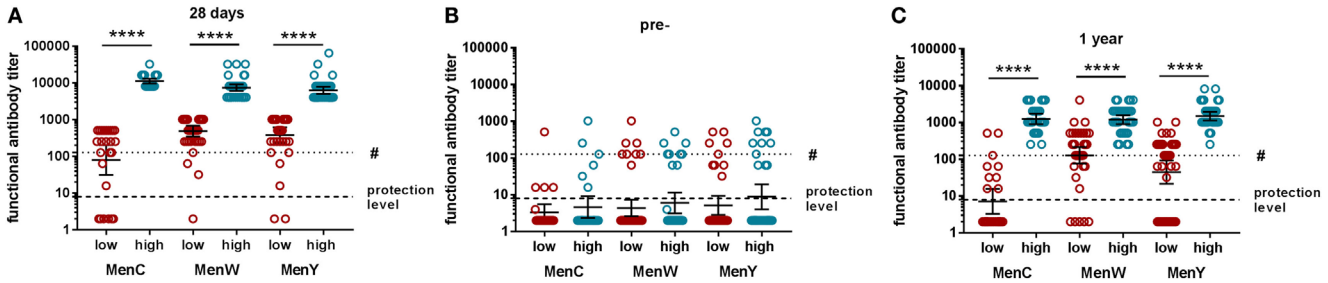

FIGURE 2 | The meningococcal group-specific functional antibody titers of the low (red) and high (blue) vaccine responders. The functional antibody titers 28 days **(A)** postvaccination, prevaccination **(B)** and 1-year postvaccination **(C)** of the low (red) and high (blue) vaccine responders. The protection level is indicated by the lowest dotted line. A more conservative long-term protection level is indicated by the # in the figures. The functional antibody titers were compared between the low and high responders using the Mann–Whitney *U*-test. MenC: low $N = 25$, high $N = 27$; MenW: low $N = 46$, high $N = 35$; and MenY: low $N = 40$, high $N = 34$. ****$p < 0.0001$.

TABLE 1 | Participant characteristics.

	MenC		MenW		MenY	
	Low	High	Low	High	Low	High
	N = 25	*N* = 27	*N* = 46	*N* = 35	*N* = 40	*N* = 34
Age (95% CI)	**59.1* (57.5–60.7)**	**56.1* (54.5–57.7)**	58.3 (57.1–59.6)	56.9 (55.5–58.4)	57.7 (56.4–59.1)	56.6 (55.0–58.1)
Males (%)	13 (52%)	16 (59%)	21 (45.6%)	19 (54.3%)	20 (50%)	17 (50%)
CMV seropositive (%)	10 (40%)	17 (63%)	21 (45.6%)	18 (51.4%)	15 (37.5%)	17 (50%)
BMI (95% CI)	24.9 (23.5–26.3)	24.8 (23.5–26.2)	25.2 (24.2–26.4)	25.8 (24.7–27.0)	24.8 (23.7–26.0)	26.5 (25.2–27.8)
TT-specific prevaccination IgG Geomean (95% CI)	0.72 (0.47–1.11)	1.20 (0.77–1.88)	0.84 (0.5–1.28)	0.87 (0.55–1.37)	1.08 (0.76–1.53)	0.78 (0.51–1.17)
Disease in last year (number, %)						
Diabetes type II	0 (0%)	1 (3.7%)	2 (4.3%)	3 (8.6%)	1 (2.5%)	2 (5.9%)
High blood pressure	5 (20.0%)	3 (11.1%)	8 (17.4%)	5 (14.3%)	6 (15%)	8 (23.5%)
Vascular diseases	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (2.5%)	0 (0%)
Lung diseases	1 (4.0%)	1 (3.7%)	0 (0%)	4 (11.4%)	2 (5%)	2 (5.9%)
Rheumatic diseases	1 (4.0%)	1 (3.7%)	2 (4.3%)	0 (0%)	2 (5%)	0 (0%)
Gastro-intestinal diseases	0 (0%)	2 (7.4%)	2 (4.3%)	1 (2.9%)	2 (5%)	2 (5.9%)
Other diseases	1 (4.0%)	1 (3.7%)	1 (2.2%)	3 (8.6%)	1 (2.5%)	3 (8.8%)
No serious disease	18 (72%)	19 (70.4%)	35 (76.1%)	26 (74.3%)	31 (77.5%)	24 (70.6%)
Medication last 6 months (number, %)						
Medication for infection[a]	0 (0%)	4 (14.8%)	3 (6.5%)	4 (11.4%)	2 (5%)	2 (5.9%)
Cholesterol lowering medication	2 (8.0%)	2 (7.4%)	5 (10.9%)	5 (14.3%)	3 (7.5%)	4 (11.8%)
Diabetic medication	0 (0%)	1 (3.7%)	1 (2.2%)	3 (8.6%)	0 (0%)	2 (5.9%)
Blood pressure lowering medication	7 (28.0%)	3 (11.1%)	9 (19.6%)	6 (17.1%)	7 (17.5%)	8 (23.5%)
Immunosuppressive medication	0 (0%)	1 (3.7%)	1 (2.2%)	2 (5.7%)	1 (2.5%)	1 (2.9%)
No medication	18 (72%)	19 (70.4%)	32 (69.6%)	23 (65.7%)	30 (75%)	23 (67.6%)
Infections (number, %)						
Influenza < 4 weeks	0 (0%)	1 (3.7%)	1 (2.2%)	0 (0%)	1 (2.5%)	0 (0%)
Cold < 4 weeks	6 (24.0%)	3 (11.1%)	7 (15.2%)	10 (28.6%)	7 (17.5%)	6 (17.6%)
No infection < 4 weeks	19 (76.0%)	25 (92.6%)	39 (85.8%)	26 (74.3%)	33 (82.5%)	29 (85.3%)
Smoking (number, %)						
Cigarette smoking	6 (24.0%)	3 (11.1%)	6 (13%)	3 (8.6%)	6 (15%)	4 (11.8%)
Cigars, pipe smoking	1 (4.0%)	0 (0%)	3 (6.5%)	1 (2.9%)	3 (7.5%)	1 (2.9%)
No smoking	18 (72%)	25 (92.6%)	38 (82.6%)	32 (91.4%)	32 (80%)	30 (88.2%)
Physical activity (number, %)						
Weekly or more	17 (68.0%)	19 (70.4%)	31 (67.4%)	24 (68.6%)	27 (67.5%)	25 (73.5%)
Less than weekly	4 (16.0%)	2 (7.4%)	8 (17.4%)	4 (11.4%)	6 (15%)	4 (11.8%)
No activity	4 (16.0%)	7 (25.9%)	7 (15.2%)	8 (22.9%)	7 (17.5%)	6 (17.6%)

[a]*Medication used more than 3 months ago and mainly consisting of corticosteroids and antibiotics.*

**p < 0.05, significant differences are depicted in bold and underlined. The chi-square test was used to determine statistical significances.*

low responders were significantly older as compared with the high responders. All participants possessed prevaccination TT-specific antibodies. Sex distribution, the number of CMV seropositive participants, and BMI were comparable between the low and high responders. Moreover, no significant differences in disease incidence (within the last year), medication use (within the last 6 months), the incidence of recent infections (within the last 4 weeks), smoking, or physical activity were observed. Not all participants were identified as low or high responder consistently for all meningococcal groups.

Differences in Prevaccination Immune Markers between High and Low Vaccine Responders

At first, the absolute numbers of immune cells were compared between the high and low responders for the different meningococcal groups separately (**Figure 3A**). Low responders to MenC possessed significantly higher absolute numbers of naïve Treg ($p = 0.033$), CD45RA$^+$CD25^{dim} ($p = 0.005$), CD4 naïve ($p = 0.021$), CD4 TemRA early ($p = 0.024$), and CD8 CM ($p = 0.038$) cells as compared with the high responders (**Figure 3A**; Figures S1A,C,D,F,G in Supplementary Material). Moreover, trends toward higher absolute numbers of memory Treg ($p = 0.084$) and CD4 TemRA ($p = 0.057$) cells were found in the low responders (**Figure 3A**; Figures S1B,E in Supplementary Material). In addition, the low responders to MenC showed lower serum levels of IL-1Ra ($p = 0.035$) as compared with the high responders (**Figure 3B**; Figures S1H,I in Supplementary Material), as well as a trend toward lower VitD levels ($p = 0.075$) (**Figure 3B**; Figure S1J in Supplementary Material).

Low responders for MenW possessed significantly higher absolute numbers of memory Treg cells ($p = 0.039$) (**Figure 3A**; Figure S2A in Supplementary Material) as well as a trend toward lower levels of IL-1Ra ($p = 0.057$) (**Figure 3B**; Figure S2B in Supplementary Material) as compared with the high responders.

Finally, the low responders for MenY had significantly higher absolute numbers of CD45RA$^+$CD25^{dim} cells ($p = 0.022$) as well as lower absolute numbers of natural effector (CD27$^+$IgD$^+$) B cells ($p = 0.008$), T cells ($p = 0.043$), CD4 TemRO intermediate cells ($p = 0.011$), CD8 TemRO cells ($p = 0.027$), and CD8 TemRO late cells ($p = 0.036$) than the high responders (**Figure 3A**; Figures S3B,D,E,H,I,K in Supplementary Material). Moreover, trends toward lower absolute numbers of total B cells ($p = 0.060$), CD27$^+$memory B cells (Bmem) ($p = 0.062$), CD4RO T cells ($p = 0.070$), CD4RO early T cells ($p = 0.081$), and CD8 TemRO intermediate T cells ($p = 0.090$) (**Figure 3A**; Figures S3A,C,F,G,J in Supplementary Material) as well as a trend to a lower BMI ($p = 0.053$) (**Figure 3B**; Figure S3I in Supplementary Material) were observed in the low responders.

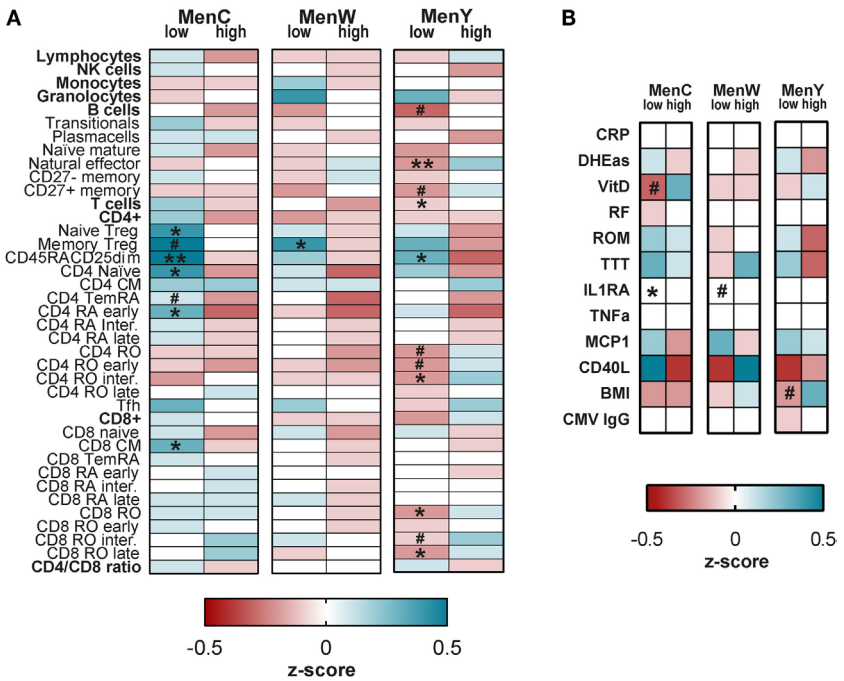

FIGURE 3 | Comparison of prevaccination immune markers between the high and low vaccine responders. Heat maps comparing the absolute immune cells numbers **(A)**, serum cytokines and biochemical markers **(B)** between the low and high vaccine responders for the different meningococcal serotypes separately. The absolute immune cell numbers as well as the concentrations of the serum markers were normalized to *z*-scores using the geometric means. The geometric means of the two groups were compared with the overall group geometric mean and standard deviation. The normalized *z*-scores are displayed on a color scale, ranging from red (below the geometric mean of the total group) to blue (above the geometric mean of the total group). The white color indicates values that are equal to the group geometric mean. The stronger the deviation from the group geometric mean, the darker the color. The different immune markers were compared between the low and high responders using the Mann–Whitney *U*-test. $^{\#}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$. MenC: low $N = 25$, high $N = 27$; MenW: low $N = 46$, high $N = 35$; and MenY: low $N = 40$, high $N = 34$.

Multivariate RDA Revealing Significant Associations between the Prevaccination Immune Phenotype and Vaccine Responsiveness

In order to determine whether high or low vaccine responsiveness at day 28 postvaccination was significantly associated with all measured prevaccination immune markers combined, hereafter called the immune phenotype; a multivariate RDA was performed for the three meningococcal groups separately (**Figures 4A–C**). Overall the included variables (age, sex, CMV, BMI, and vaccine response) explained 14.5, 10.4, and 12.0% of the total variation in immune phenotype for MenC, MenW, and MenY, respectively.

For MenC and MenY, the variable "vaccine response" was significantly associated with the immune phenotype (MenC: $p = 0.012$, FDR = 0.07 and MenY: $p = 0.028$, FDR = 0.098) (**Figures 4A,C**). As expected, based on the heat map depicted in **Figure 3A**, for MenW no significant association between vaccine response and immune phenotype was observed ($p = 0.068$, FDR = 0.16) (**Figure 4B**).

For MenC group, higher levels of DHEA, TTT, and ROM as well as higher absolute numbers of memory Treg cells, naïve Treg cells, CD45RA$^+$CD25^{dim} cells, CD4 TemRA early cells, CD4 naïve cells, lymphocytes, and CD4 T cells were strongly associated with low responsiveness, whereas higher levels of IL-1Ra were related with high responsiveness (**Figure 4A**). For MenY group, higher levels of DHEA and higher absolute numbers of CD4 naïve, CD45RA$^+$CD25^{dim}, CD8 naïve, and naïve Treg cells were strongly associated with low vaccine responsiveness, while high absolute numbers of Bmem cells were linked to high vaccine responsiveness (**Figure 4C**).

In addition, CMV seropositivity was significantly associated with the immune phenotype (**Figure 4A**: $p = 0.098$, FDR = 0.23, **Figure 4B**, $p = 0.002$, FDR = 0.014, and **Figure 4C**, $p = 0.002$, FDR = 0.014). In these analyses, CMV seropositivity was associated with higher absolute numbers of CD4RO late, CD4RA late, and CD8RA late T cells, and not related to either low or high vaccine responsiveness. Of note, the explanatory variables BMI, age, and sex were not significantly associated with the immune phenotype.

Differences in Immune Cell Ratios in the CD4 T Cell Compartment between the High and Low Vaccine Responders

In relation to the mentioned findings of higher naïve CD4 T cells and memory Treg cells in the low vaccine responders, we determined whether the ratio of naïve to memory cells, as well as the ratio of Treg to effector cells in the CD4 T cell compartment was different between the low and high vaccine responders

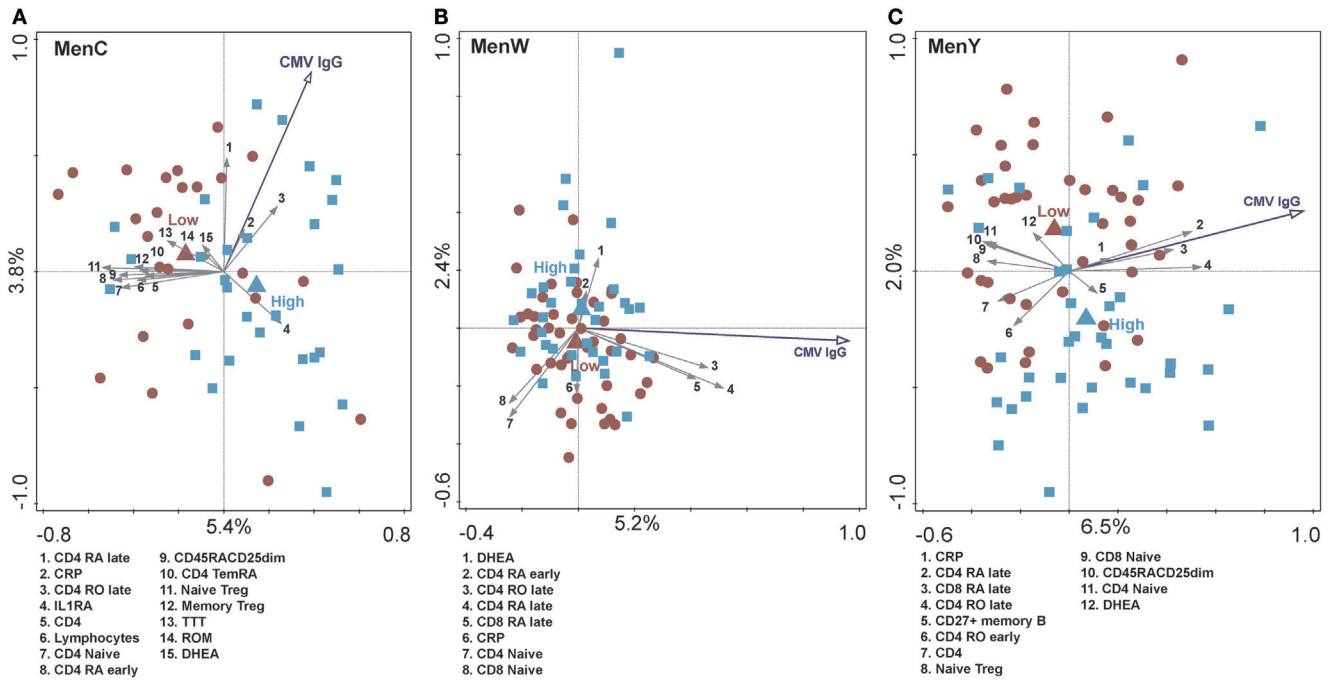

FIGURE 4 | Redundancy analysis (RDA) assessing the association between the prevaccination immune phenotype and the meningococcal vaccine response. RDA of samples collected 28 days postvaccination for MenC **(A)**, MenW **(B)**, and MenY **(C)**. Low vaccine responders are shown in red circles; High responders are shown in light blue squares. The first and second ordination axes are plotted, including the percentages of explained variation. Overall, 14.5, 10.4, and 12.0% of the variation in the datasets was explained for MenC, MenW, and MenY, respectively. The vaccine response variable (i.e., low or high responder) was significantly associated with the immune phenotype for MenC ($p = 0.012$, FDR = 0.07) and MenY ($p = 0.028$, FDR = 0.098), but not for MenW ($p = 0.068$, FDR = 0.16). CMV was significantly associated with the immune composition for MenW ($p = 0.002$, FDR = 0.014) and MenY ($p = 0.002$, FDR = 0.014) but not for MenC ($p = 0.098$, FDR = 0.23). Other environmental variables tested (i.e., BMI, age, and sex) did not significantly influence the variation in the dataset. The biological variables with the highest variation explained by the explanatory variables are depicted in the plots (FitE > 15). The length of the arrows relates to the strength of the association.

(**Figure 5**). No difference in the ratio between the total Treg cells and the number of CD4 TemRO T cells was observed (**Figure 5A**), whereas the ratio of memory Treg cells to CD4 TemRO T cells was significantly higher in the low responders for MenC and MenY as compared with the high responders (**Figure 5B**). In addition, the low responders for MenC and MenY possessed a significantly higher ratio of the naïve CD4 T cells and the total CD4 memory cells (**Figure 5C**), as well as a higher ratio of the post thymically expanded $CD45RA^{+}CD25^{dim}$ cells and the total CD4 memory cells (**Figure 5D**). Thus, the higher numbers of both naïve CD4 T cell subsets and Treg cells as seen in low responders at baseline (prevaccination) give rise to clear compositional changes in the peripheral CD4 T cell compartment.

DISCUSSION

In this explorative study, we investigated whether the prevaccination immune phenotype was significantly different between the middle-aged adults being either low or high responder after a primary meningococcal vaccination. Interestingly, the numbers of several CD4 T cell subsets differed between the low and high vaccine responders. More specifically, low vaccine responders possessed higher numbers of naïve Treg, memory Treg, naïve CD4 cells, and the subset of post thymically expanded $CD4^{+}CD45RA^{+}CD25^{dim}$ T cells, whereas high responders showed high levels of serum IL-1Ra. These results suggest that the prevaccination CD4 signature may be used to identify middle-aged adults who are potential non/low responders to a primary meningococcal vaccine. Identification of these middle-aged adults may help improve timely vaccination strategies, since vaccination schemes, doses, and adjuvant use might be adapted to improve the vaccine responsiveness in these adults.

Numbers of Treg cells are known to increase with advancing age and suggest elevated immune suppression in older adults, although the exact functionality of these Treg cells in aging individuals is still under investigation (19, 48). Accordingly, high numbers of Treg cells were previously associated with low VZV vaccine responses in nursing home elderly (18). Within our study, high absolute numbers of both naïve and memory Treg cells were associated with low vaccine responsiveness. Naïve Treg cells express CCR7 enabling these cells to migrate to lymphoid organs, whereas memory Treg cells home to the sites of inflammation along with effector T cells (49, 50). Accordingly, our results suggest enhanced suppression of the vaccine response both in the lymphoid organs as well as the site of vaccination in low responders. Elevated numbers of Treg cells might suppress T cell responses toward the tetanus carrier in this conjugated meningococcal vaccine and/or inhibit B cell responses directly (51). Our results confirm previous findings of high numbers of memory Treg cells in low vaccine responders to influenza (21), whereas we are the first showing an association between low vaccine responsiveness and high numbers of naïve Treg cells. Of importance, we observed a higher ratio of memory Treg to effector CD4 T cells in the low responders, suggesting a shift in the Treg/Teffector balance, as previously observed with advancing age (21).

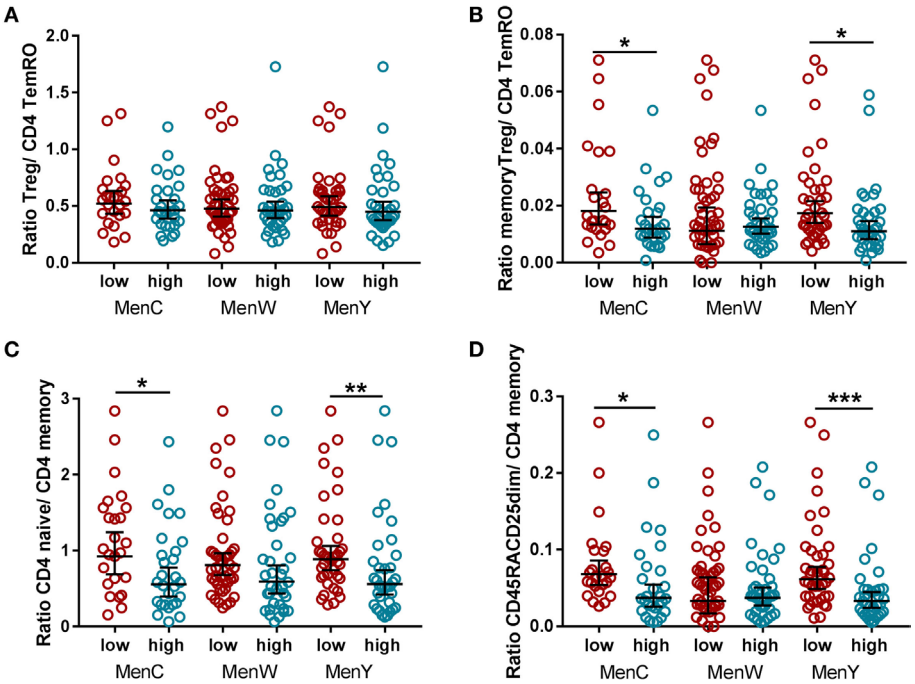

FIGURE 5 | Comparison of immune cell ratios in the CD4 T cell compartment between the high and low vaccine responders. Comparison of the Treg/TemRO ratio **(A)**, memory Treg/TemRO ratio **(B)**, CD4 naïve/CD4 memory ratio **(C)**, and $CD45RA^{+}CD25^{dim}$/CD4 memory ratio **(D)** between the low (red) and high (blue) vaccine responders for the different meningococcal groups separately. The total CD4 memory cells were determined as the sum of the CD4 CM, CD4 TemRO, and CD4 TemRA cells. The low and high responders were compared with the Mann–Whitney *U*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

The consistent association found between high numbers of CD4 naïve and the post thymically expanded $CD4^+CD45RA^+CD25^{dim}$ cells and a higher ratio of these naïve cells to the memory CD4 T cell compartment with low vaccine responsiveness was unexpected, since a naïve T cell repertoire is generally accepted to be beneficial in older adults (9, 11). Previously, the $CD4^+CD45RA^+CD25^{dim}$ subset was found to represent a broad and functional reservoir of naïve CD4 T cells, although some contraction in certain TCR Vβ families was observed in comparison to naïve CD4 T cells that recently left the thymus (52). Since no prior studies were available that linked the numbers of $CD4^+CD45RA^+CD25^{dim}$ cells to vaccine responses, our findings indicate the necessity for further research into repertoire size and functionality of these cells. However, the increase in memory Treg cells might be the dominant factor in predicting vaccine response, overruling the presence of a capable naïve CD4 T cell repertoire.

In line with the different studies investigating biomarkers for influenza and hepatitis B vaccine responses in the elderly (17, 23, 25), we observed trends toward lower numbers of switched memory $CD27^+$ B cells in the low responders. In contrast, we did not find increased numbers of CD27-memory B cells in the low responders, as reported by others in vaccine recipients aged over 65 years (23). In addition, low numbers of natural effector $CD27^+IgD^+$ B cells that were previously found to decrease with age (24) were observed in the low vaccine responders. Since we previously described that IgM is essential in the antibody functionality against the meningococcal groups (4), lower numbers of these natural effector cells, mainly producing IgM, likely explain the lower functional antibody titers.

Currently, effects of latent CMV infection on vaccine responses are controversial (53). Despite the clear associations between CMV seropositivity and higher numbers of late differentiated T cells, we did not find an association between CMV seropositivity and meningococcal vaccine responsiveness. As this meningococcal vaccine response is primarily B cell mediated, the effect of CMV might be limited. Hence, the effects of CMV on T cell-mediated vaccine responses, i.e., to influenza and VZV vaccination should be further elucidated. Although frequently suggested by others (54–57), we did not observe any effects of sex and BMI on the vaccine responses. Moreover, the effect of chronological age was inconsistent, although low responders to MenC were significantly older than high responders.

Of note, high levels of IL-1Ra were found in the high responders. IL-1Ra is known as the receptor antagonist of the IL-1 family, executing anti-inflammatory functions (58, 59). These results possibly suggest that IL-1Ra acts as an anti-inflammatory counterpart of the "inflammageing" process. Of note, a trend toward high levels of MCP-1 was found in the low responders. MCP-1 is classified as a pro-inflammatory cytokine, attracting monocytes to the site of inflammation, and serum levels were shown to increase with age (60). Consequently, our findings may suggest a higher pro-inflammatory state in the low responders (33, 34). Nevertheless, serum levels of other inflammatory cytokines, such as IL-6 and TFN-α were still low in all participants. Remarkably, trends toward higher levels of sCD40L were found in the low responders for MenC, as compared with lower levels in the low responders for MenW and MenY, which needs further evaluation.

Of importance, associations with the immune phenotype were primarily found between the extremes in the vaccine response, being either low or high responders. The intermediate group showed high variability of immune markers. In addition, our results may imply meningococcal group-specific associations between the prevaccination immune phenotype and vaccine responsiveness. Noteworthy, the difference in functional antibody titers between the low and high vaccine responders was largest for MenC, possibly explaining the higher numbers of immune parameters found associated with the vaccine response for this meningococcal group. Moreover, the participants that were classified as low or high responder did not completely overlap between the different meningococcal groups. This might be explained by the structural differences in the meningococcal polysaccharides by which different meningococcal epitopes will induce distinct immune responses (61), also shown previously for several pneumococcal conjugated polysaccharides (62). Possible structural differences will affect the B cell processing and subsequently the quality and quantity of the T cell help provided by the carrier, that might cause differences in antibody functionality to the various polysaccharides. Currently, differences between meningococcal group-specific polysaccharides conjugated to TT are not known (63). Furthermore despite similar prevaccination functional antibody titers in the low and high vaccine responders, differences in numbers of meningococcal group-specific memory B cells in the bone marrow could have been present due to historical contacts (64) and affect the vaccine response. Nevertheless, the finding of meningococcal group-specific associations between vaccine responsiveness and immune phenotype is remarkable and requires further research. Of note, we previously found that most participants possessed high prevaccination TT-specific antibody levels (4). Importantly, no direct correlation between the antibody responses to TT or the different polysaccharides and the strength or classification of the T cell response induced by the TT-carrier protein was observed (5). In this study, the similar prevaccination TT-specific antibody levels in the low and high responders, suggests that the prevaccination immunity against the TT carrier protein did not largely affect the immune responses.

An important strength of this study is the ability to compare multiple antigens and multiple immune parameters within the same group of participants. Also, the primary nature of the vaccination allowed us to explore the use of biomarkers, without the strong interference of prevaccination meningococcal immunity, as often seen in other studies. Unfortunately, the presence of prevaccination immunity in some participants did interfere with the long-term functional antibody titers (after 1 year). Consequently, we were not able to investigate the associations between prevaccination immune phenotype and the long-term vaccine responsiveness, since exclusion of participants with detectable prevaccination functional antibody titers dramatically reduced the power of the statistical analysis. In addition, information on the genetic background of the participants could have added to the predictive factors in our analysis, since several studies found associations between genetic signatures and vaccine responsiveness (25, 31, 32).

Future studies, analyzing large cohorts, using different vaccines, and using similar biomarker analyzing techniques are warranted to validate the use of the suggested biomarkers. Hence, systems vaccinology, combining data on genetic background, and environmental factors such as diet, stress, and infections, and even microbiome composition is a promising tool to discover these predictive biomarkers (65). Moreover, future research should compare the suitability of biomarkers in cohorts of different ages, in order to determine the predictive values of these markers over the entire lifespan.

In conclusion, our explorative biomarker analysis suggests several associations between the prevaccination immune phenotype and vaccine responsiveness after primary meningococcal vaccination in middle-aged adults. In general, an altered CD4 T cell signature, involving high absolute numbers of naïve Treg, memory Treg, naïve CD4 T cells, and $CD45RA^{+}CD25^{dim}$ T cells might be used as a predictive immune phenotype for low vaccine responsiveness in middle-aged adults. Accordingly, these findings support the development of vaccination strategies to enhance the memory immunity before reaching old age, in the rapidly aging population.

ETHICS STATEMENT

Written informed consent was obtained from all participants prior to enrollment and all procedures were in accordance with the Declaration of Helsinki. The medical ethical committee: Medical Research Ethics Committees United (MEC-U) approved the study and the study was registered at the Dutch trial register (Protocol no. NTR4636).

AUTHOR CONTRIBUTIONS

MH, GB, MZ, A-MB, and AB conceptualized the study. MH planned and performed the clinical work and executed the laboratory experiments. MH and SF performed the statistical analysis. MH, GB, MZ, MD, SF, A-MB, and AB interpreted the data and wrote the manuscript. All authors critically revised the manuscript.

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SUPPLEMENTARY MATERIAL

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Impairment of Several Immune Functions and Redox State in Blood Cells of Alzheimer's Disease Patients. Relevant Role of Neutrophils in Oxidative Stress

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Since aging is considered the most risk factor for sporadic Alzheimer's Disease (AD), the age-related impairment of the immune system (immunosenescence), based on a chronic oxidative-inflammatory stress situation, could play a key role in the development and progression of AD. Although AD is accompanied by systemic disturbance, reflecting the damage in the brain, the changes in immune response and redox-state in different types of blood cells in AD patients have been scarcely studied. The aim was to analyze the variations in several immune functions and oxidative-inflammatory stress and damage parameters in both isolated peripheral neutrophils and mononuclear blood cells, as well as in whole blood cells, from patients diagnosed with mild (mAD) and severe AD, and of age-matched controls (elderly healthy subjects) as well as of adult controls. The cognitive decline of all subjects was determined by Mini-Mental State Examination (MMSE) test (mAD stage was established at 20 ≤ MMSE ≤ 23 score; AD stage at <18 MMSE; elderly subjects >27 MMSE). The results showed an impairment of the immune functions of human peripheral blood neutrophils and mononuclear cells of mAD and AD patients in relation to healthy elderly subjects, who showed the typical immunosenescence in comparison with the adult individuals. However, several alterations were only observed in severe AD patients (lower chemotaxis, lipopolysaccharide lymphoproliferation, and interleukin (IL)-10 release; higher basal proliferation, tumor necrosis factor (TNF)-α release, and IL-10/TNF-α ratio), others only in mAD subjects (higher adherence), meanwhile others appeared in both mAD and AD patients (lower phytohemaglutinin lymphoproliferation and higher IL-6 release). This impairment of immune functions could be mediated by: (1) the higher oxidative stress and damage also observed in blood cells from mAD and AD patients and in isolated neutrophils [lower glutathione (GSH) levels, high oxidized glutathione (GSSG)/GSH ratio, and GSSG and malondialdehyde contents], and (2) the higher release of basal pro-inflammatory cytokines (IL-6 and TNF-α) found in AD patients. Because the immune system parameters studied are markers of health and rate of aging, our results supported an accelerated immunosenescence in AD patients.

We suggest the assessment of oxidative stress and function parameters in peripheral blood cells as well as in isolated neutrophils and mononuclear cells, respectively, as possible markers of AD progression.

Keywords: Alzheimer's disease, immunosenescence, immune function, oxidative stress, inflammation, blood cells, neutrophils, mononuclear cells

INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder and one of the major causes of senile dementia in later life. Epidemiological studies have revealed that AD is a multifactorial disease, with a complex interplay of environmental and genetics factors, which helps explain its variable clinical presentation (1, 2). Traditionally, AD has been classified into hereditary and sporadic forms. The hereditary form is linked with several genes and typically presents an earlier age of onset. By contrast, the sporadic form, which is the most common cause of AD (>95% of cases), has a later age of onset and a stronger association with aging, the major risk-incurring variable (1, 2). Due to the increase in mean life span, this pathology is exponentially increasing and is estimated that the prevalence of AD may reach >115 million worldwide by 2050 (3). Despite the enormous social, economic and health care impact, public health care systems do not have the medical therapies necessary to address AD. Moreover, the diagnosis of individual with early AD is not easy, and it is usually diagnosed in late stages (even postmortem), by which time the available treatments are not effective (4). For this reason, it is crucial to identify biomarkers of early diagnosis of AD that can help in the development of effective therapeutics and prevention methods.

Neuropathologically, AD is a progressive and irreversible brain disorder characterized by the extracellular accumulation of amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein, associated with neuronal cell death and synaptotoxicity (5). Additional changes such as brain atrophy, mitochondrial dysfunction, increase in oxidative stress, and neuroinflammation can also occur in the brains of people with AD (6, 7). However, the mechanism of AD pathogenesis and progression still remain unclear. During the last few years, a higher number of studies have demonstrated that AD is also accompanied by a systemic disturbance, reflecting the damage in the brain (8–10). Thus, several studies support the involvement of an immune-related systemic alteration in AD, which includes changes in both innate and adaptive immune systems (11–15). Given the bidirectional communication between the immune and the nervous systems, and due to how the mediators of peripheral immune cells can influence the central nervous system (CNS) (16), it is not surprising that the age-related immunological variations can modify this neuroimmune communication network, contributing to the progressive cognitive impairment in AD (16). Indeed, numerous studies have shown changes in the distribution and reactivity of immune cells in the blood of AD patients (12, 17–21). This seems to cause a chronic inflammation in the periphery, which can be propagated through CNS immune cells, leading to neuroinflammation, which contributes to the cognitive deficits associated with AD (16, 22, 23).

Given that aging is accompanied by a decline of the nervous and the immune systems, as well as of their communication (24), which contributes to the deterioration of homeostasis and health, AD can be understood in the context of aging (25). Since advanced age is considered the most consistent risk factor for sporadic AD (1), the age-related dysregulation of the immune system, which is denominated immunosenescence, should be considered to understand the development of this pathology (11, 16, 17, 25–27). Immunosenescence involves restructuring changes in both innate and adaptive immune functions, which negatively affect the health of older adults, increasing susceptibility to infections and mortality (24, 28). Thus, there is an age-related decrease in several leukocytes functions, such as phagocytosis, chemotaxis, or stimulated proliferation, as well as an increase in other functions, such as adherence capacity to tissues and spontaneous lymphoproliferations, among others (24, 28–30). In AD patients, several immune functions are hampered in relation to healthy individuals with the same chronological age (8, 11, 19, 31–34), suggesting that this immune deterioration may be considered as a pathogenically relevant factor in this disease (33). Thus, it is assumed the advanced immunosenescence in AD patients, with remarkable immunological changes compared to healthy elderly subjects, which could contribute to AD pathology (11, 16, 17, 26, 33). Among these changes in AD patients the most relevant have been observed in the adaptive immune system. In fact, as also occurs in immunosenescence, several authors have reported changes in T and B lymphocyte differentiation and subpopulation distribution in peripheral blood of AD subjects, as well as an altered proliferative T lymphocytes response (11, 33, 34). Nevertheless, the results are inconsistent since increased or decreased lymphoproliferation and percentages in T and B lymphocytes, as well as no alterations in these immune parameters have been detected in AD patients (11, 33, 34). Regarding innate immunity, abnormalities in the function of natural killer (NK) cells (31, 32), a decreased phagocytosis of Aβ in peripheral macrophages (19), and an altered release of pro-inflammatory cytokines [e.g. interleukin-(IL)-6, tumor necrosis factor (TNF)-α, etc.] (8) have been shown in AD patients in relation to age-matched controls. However, the data of human studies are still scarce, preliminary, and contradictory.

According to the oxidation–inflammation theory of aging, the basis of immunosenescence is a chronic oxidative-inflammatory stress situation (a progressive imbalance between higher endogenous levels of oxidant and inflammatory compounds and lower antioxidant and anti-inflammatory defenses) (24, 28). In fact, immune cells continuously generate oxidants and inflammatory compounds to carry out their defensive functions; however, if the overproduction of oxidant compounds is not well-controlled by the antioxidant defenses, an alteration occurs in the redox

balance, leading to an oxidative stress situation, which causes oxidative damage in biomolecules (e.g., lipids, proteins, etc.), inducing remarkable negative consequences on cellular functioning (24, 28). In addition, the chronic inflammation, which is characterized by excessive production and release of pro-inflammatory cytokines and chemokines, also leads to signaling cascades that trigger the production of oxidant compounds and depletion of antioxidants (35, 36). Therefore, since oxidation and inflammation are interlinked processes, an active inflammatory response by immune cells can lead to cellular damage due to oxidant overproduction, which can also recruit other inflammatory cells amplifying the cellular damage (35, 36). In this context, there is much evidence that indicates the strong involvement of inflammation and oxidative stress and damage in the onset, progression, and pathogenesis of AD (37–40). Thus, as occurs in aging, an enhanced oxidative stress has been observed in the brain (39, 40), as well as in peripheral tissues and cells (e.g., blood cells) (27, 41, 42) from AD patients. Indeed, many studies show both overproduction of oxidant compounds and impairment of antioxidant systems, as well as increased of oxidative damage (e.g., lipid peroxidation, proteins and DNA oxidation) in several brain regions of patients with both severe AD and mild cognitive impairment (MCI) (40, 43, 44). These markers of oxidative damage were also detected in high levels in peripheral blood and cerebrospinal fluid (CSF) obtained from both MCI and AD patients, together with decreased plasma levels of non-enzymatic antioxidants and impaired activity of antioxidant enzymes. Moreover, peripheral levels of the pro-inflammatory cytokines (e.g., IL-6 or TNF-α) have been described to be higher, whereas anti-inflammatory cytokine levels (e.g., IL-10 and IL-4) are lower in patients with AD compared to age-matched controls (27, 42, 43, 45–47). Interestingly, the fact that high levels of peripheral markers of oxidative-inflammatory stress were also detected in individuals with MCI, support the hypothesis that oxidative stress and inflammation are early events in the pathogenesis of AD, and precede Aβ deposit and onset of AD symptoms, as has been seen in experimental models of AD (48, 49). However, although several studies have evaluated oxidative stress parameters in both the early and advanced stages of human AD, the findings are controversial, as well as the relation between oxidative stress and cognitive performance, in mild and severe AD subjects, not yet being fully understood.

The use of animal models is particularly useful to study the molecular mechanisms involved in the development of AD and to identify potential therapeutic targets. In the last few decades, several transgenic animal models of AD have been developed to reproduce the neuronal pathology and behavioral symptomatology of human AD. The triple-transgenic mouse model (3xTg-AD) represents a unique animal model that closely mimics neuropathological manifestations, Aβ-plaques, and NFTs, in an age-dependent and region-specific manner like those in the human AD brain (50). Interestingly, as occurs in aging, it has been described that 3xTg-AD mice also suffer a pronounced and accelerated impairment in the neuroimmune network (16, 26), as well as a marked deterioration of several immune functions and an increased oxidative stress in different types of immune cells (e.g., peritoneal leukocytes) in comparison to control mice (16, 26, 51, 52). These alterations were observed in both early and advanced stages of the AD neuropathology, as well as before the onset establishment of AD (16, 26, 51, 52). Given that these changes are characteristics of prematurely and chronologically aged subjects (28, 30, 53), the premature immunosenescence observed in the 3xTg-AD mice at early stages of AD could explain the shorter life span also observed in these animals (52). Interestingly, the immune functions of peritoneal leukocytes of mice have been found to possess similar age-related evolutions to those observed in human circulating immune cells (30). Therefore, since immune functions are good markers of the rate of aging, and their analysis allows the early identification of premature and accelerated aging in humans (28, 30), the assessment of these parameters could be useful peripheral markers of the prodromal and preclinical states of AD (52).

It is also important to note that the most of studies performed in peripheral blood cells, have been carried out using mainly isolated peripheral mononuclear cells (95% lymphocytes and 5% monocytes), which are the most commonly used cells in the clinical setting. However, there are a few studies in which changes in immune function and redox status have been assessed in isolated phagocytes. Since these cells (e.g., neutrophils and macrophages) have been suggested to be the main cells responsible for the chronic oxidative-inflammatory stress associated with immunosenescence (28, 54), they could provide a helpful sample to clarify the molecular mechanisms underlying the impairment of the immune system throughout of AD progression. Moreover, blood, and particularly circulating leukocytes, reflects the major physiological changes in various body organs and systems (47). Therefore, the use of blood cells seems to be more useful in assessing markers that are analyzed in CSF, as well as identifying new markers that could be predictive of AD. Thus, it is possible that the assessment of these immune function parameters in the different types of peripheral blood immune cells, together with the evaluation of their oxidative-inflammatory state, could be useful early peripheral markers of the progression of AD in humans. However, this kind of study in different stages of human AD is still a subject that has scarcely been explored.

With the above in mind, the aim of the present work was to study the changes in several immune functions and inflammatory-oxidative stress and damage parameters in different types of human blood immune cells at early and advance stages of AD progression. To address this study, we performed assays on both isolated peripheral polymorphonuclear (PMN) and mononuclear blood leukocytes, as well as in whole blood (WB) cells, from patients diagnosed with mild and severe AD, and age-matched controls (elderly healthy subjects) as well as adult controls.

MATERIALS AND METHODS

Subjects and Clinical Classification

For this cross-sectional study, a total of 102 volunteers were selected and divided into four experimental groups: adult healthy subjects ($n = 20$), elderly healthy individuals ($n = 38$), mild AD (mAD) patients ($n = 26$), and severe AD patients ($n = 18$). All subjects were recruited by the Neurology Department of the Hospital, 12

Octubre of Madrid, and were tested by a standardized neuropsychological battery. The AD diagnosis was established according to the guidelines of the National Institute on Neurological Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (55). Disease severity and normal cognitive function was determined by a clinician's judgment based on a structured interview with the patient and the results of the Clinical Dementia Rating and the Mini-Mental State Examination (MMSE) tests (56). The mAD stage was established at $20 \leq \text{MMSE} \leq 23$ score and AD stage at <18 MMSE score. Inclusion criteria for cognitively normal elderly healthy subjects were MMSE scores > 27, no history or clinical signs of neurological or psychiatric disease or cognitive symptoms. Demographic details and MMSE test results of the different study groups are summarized in **Table 1**. All subjects were subjected to a clinical survey and physical examination. Those with a history of cardiovascular disease, cancer, or chronic inflammatory diseases, as well as individuals with current inflammatory alterations (findings of clinical significance in general laboratory parameters) were not included in this study. The consent of the subjects was obtained according to the Declaration of Helsinki, and approval was obtained from the corresponding Research Ethic Committees. Written informed consent was obtained from all participants or representatives.

Collection of Peripheral WB Cells and Isolation of Blood Neutrophil and Lymphocytes Cells

Human samples (10 mL) of peripheral blood were collected using vein puncture and sodium citrate-buffered Vacutainer tubes (BD Diagnostic, Spain). Blood extraction was performed between 9:00 a.m. and 10:00 a.m. to avoid circadian variations in immune parameters. On the one hand, 8 mL of peripheral blood was used for isolation of both PMNs (mainly neutrophils) and mononuclear (mainly lymphocytes) leukocytes following a previously described method (57). Thus, neutrophil and lymphocyte cells were isolated using 1.119 and 1.077 g/cm^3 density Histopaque (Sigma-Aldrich, Spain) separation, respectively. Collected cells were counted (95% of viability determined using trypan blue staining) and adjusted to the corresponding final concentrations for the development of the different assays of redox state and immune functions. The immune functions assays were performed with fresh cells, whereas the redox state assays were assessed in aliquots of frozen cells. These aliquots were stored at −80°C until used. On the other hand, samples of WB cells, which contain the total red blood cells (RBC) together with the total leukocyte populations, were obtained following a previously described procedure (58). For this, 500 μL of the peripheral blood sample was diluted with 500 μL of RPMI 1640 medium without glutamine (Gibco, Burlington, ON, Canada) and 10 μL of gentamicin (0.1 mg/mL in tube). The samples were incubated for 4 h at 37°C in a saturated atmosphere of CO_2 and humidity. Then, samples were centrifuged at $900 \times g$ for 10 min to obtain the WB cell pellets after plasma removal. RPMI 1640 with glutamine (Gibco) was added to the blood cells to make 1 mL, and then several aliquots were prepared for the determination of redox state parameters. These aliquots were stored at −80°C until used.

TABLE 1 | Demographic data and neuropsychological test results of adult and elderly healthy subjects, as well as of mild Alzheimer's disease (mAD) and severe Alzheimer's disease (AD) patients.

	Adult	Elderly	mAD	AD
n (F/M)	20 (10/10)	38 (28/10)	26 (15/11)	18 (13/5)
Age (years)	40.32 ± 8.54	74.34 ± 9.22	76.08 ± 7.46	79.10 ± 6.53
MMSE	n.e.	29.02 ± 0.7	21.02 ± 0.86	16.44 ± 0.84
CDR	n.e.	0	2	3

F, female; M, male; MMSE, Mini-Mental State Examination Score; CDR, Clinical Dementia Rating; n.e., not evaluated.
The data of age and of MMSE score are expressed as mean ± SD, but those of CDR scores are presented as range values.

Adherent Capacity Assay

For the measurement of adherence capacity of human peripheral blood neutrophils and lymphocytes, we followed a method previously described (59) with some slight modifications. This method mimics, *in vitro*, cellular adherence to endothelium *in vivo*. Briefly, 500 μL of WB diluted 1:1with Hank's medium was placed in adherence columns consisting of a Pasteur pipet, in which 50 mg of nylon fibers was packed to a height of 1.25 cm. After 10 min, the effluent had drained by gravity, and neutrophils and lymphocytes were counted in this effluent using the Neubauer hemocytometer (microscope, 40×). Aliquots of 100 μL of 50% diluted WB effluent were mixed with 900 μL of Türk's solution, which has the ability to lyse RBC and differentiate the morphology of PMNs and mononuclear leukocytes. Results were expressed as the number of cells per mm^3. In addition, another aliquot of 500 μL of WB diluted 1:1 with Hank's medium was used to count the total number of neutrophils and lymphocytes present in WB, as described earlier. The difference between the number of leukocytes present in the initial mixture and in the effluent, after passing through the adherence column, gives the number of adherent cells. The percentage of adherent neutrophils and lymphocytes, expressed as Adherence Index (AI), was calculated according to the equation:

$$\text{IA} = [\left(\text{leukocytes / mm}^3\text{total} - \text{leukocytes / mm}^3\text{effluent}\right) / \left(\text{leukocytes / mm}^3\text{total}\right]\times 100.$$

Chemotaxis Assay

The induced mobility or chemotaxis of peripheral blood isolated neutrophils and lymphocytes was carried out following a method previously described (57). Boyden chambers with two compartments separated by a polycarbonate filter (3 μm of diameter; Millipore, Ireland) were used to evaluate the chemotactic index (CI). Aliquots of 300 μL of neutrophil and lymphocyte suspensions (10^6 cells/mL) in Hank's medium were deposited in the upper compartment, and aliquots of 400 μL of the chemoattractant agent *N*-formylmethionine-leucyl-phenylalanine (Sigma-Aldrich) at a concentration of 10^{-8} M in the lower compartment of the chambers. The chambers were incubated for 3 h at 37°C, and the filters were fixed and stained with Giemsa's solution (Sigma-Aldrich). The number of neutrophils and lymphocytes

on the lower face of the filter was counted in 20 microscope fields using an immersion objective (×100) and recorded as CI.

Phagocytosis Assay

Phagocytosis of inert particles (latex beads, 1.1 μm diameter, Sigma-Aldrich) was assayed in phagocytes (isolated blood neutrophils) following a method previously described (57). Neutrophils adjusted to 10^6 cells/mL were incubated on migration inhibition factor plates (Kartell, Noviglio, Italy) for 30 min at 37°C in a humidified atmosphere. The adherent monolayers obtained were washed with prewarmed PBS solution, and then 200 μL of Hank's solution and 20 μL of latex beads (1.1 μm diluted to 1% PBS, Sigma-Aldrich) were added. After 30 min of incubation under the same conditions, the plates were washed, fixed with methanol (50%), and stained with Giemsa's solution (Sigma-Aldrich). The number of particles ingested by 100 neutrophils was counted using an immersion objective (×100) and this was expressed as phagocytic index, while the number of ingesting neutrophils per 100 neutrophils was expressed as phagocytic efficiency.

NK Cytotoxicity Assay

The NK cell cytotoxicity, which is the main antitumoral protection of the organism, was measured by an enzymatic colorimetric assay (Cytotox 96 TM Promega, Boehringer Ingelheim, Germany) based on the determination of lactate dehydrogenase (LDH) released by the cytolysis of targets cells (human K562 lymphoma cells), using tetrazolium salts (57). Briefly, target cells were seeded in 96-well U-bottom culture plates (Nunclon, Denmark) at 10^4 cells/well in 1640 RPMI without phenol red (Gibco). Effector cells (mononuclear leukocyte suspensions adjusted to 10^6 cells/mL) were added at 10^5 cells/well, obtaining an effector/target rate of 10/1. The plates were centrifuged at 250 × *g* for 5 min to facilitate cell-to-cell contacts and were incubated for 4 h at 37°C. After incubation, LDH activity was measured in 50 μL/well by addition of the enzyme substrate with absorbance recording at 490 nm. The results were expressed as the percentage of tumor cells killed (% lysis).

Lymphoproliferation Assay

The proliferation capacity of lymphocytes was evaluated by a standard method, previously described (57). The assay was assessed in basal and stimulated conditions using the mitogens phytohemaglutinin (PHA) and lipopolysaccharide (LPS). Aliquots of 200 μL of isolated mononuclear leukocyte suspensions adjusted to 10^6 cells/mL of complete medium [containing RPMI 1640 enriched with L-glutamine and phenol red and supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (Hyclone, GE Healthcare, USA) and gentamicin (100 mg/mL, Sigma-Aldrich)] were dispensed into 96-well plates (Nunclon, Denmark). 20 μL of complete medium (basal lymphoproliferation), PHA, or LPS (1 μg/mL, Sigma-Aldrich) was added to each well. After 48 h of incubation at 37°C in a sterile and humidified atmosphere of 5% CO_2, 2.5 μCi ^{3}H-thymidine (Hartmann Analytic, Germany) was added to each well. Previously, 100 μL of culture supernatant from each well was collected and stored at −80°C until used for cytokine analysis. After another incubation of 24 h, cells were harvested in a semiautomatic harvester (Skatron Instruments, Norway), and thymidine uptake was measured in a beta counter (LKB, Uppsala, Sweden) for 1 min. The results were calculated as ^{3}H-thymidine uptake (counts per minute, cpm) for basal and stimulated (with mitogens) conditions and also were expressed as lymphoproliferation capacity (%) giving 100% to the cpm in basal conditions.

Glutathione Content Assay

Both reduced [glutathione (GSH)], the main non-enzymatic reducing agent of the organism, and oxidized [oxidized glutathione (GSSG)] forms of glutathione were determined using a fluorometric assay previously described (60). This method is based on the capacity of reaction that GSSG and GSH show with *o*-phthalaldehyde (OPT, Sigma-Aldrich), at pH 12 and pH 8, respectively, resulting in the formation of a fluorescent compound. The assay was evaluated in WB cells (containing total RBC and leukocyte populations), as well as in isolated peripheral blood neutrophils and mononuclear leukocytes. For this, aliquots of frozen WB cells (50 μL) were resuspended in phosphate buffer 0.1 M, pH 7.4 (200 μL) (Sigma-Aldrich), whereas aliquots of isolated neutrophils and lymphocytes adjusted to 10^6 cells/mL in Hank's solution were centrifuged at 1,200 × *g* for 10 min at 4°C. All samples were resuspended in phosphate buffer 50 mM containing EDTA 0.1 M, pH 8 (600 μL) (Sigma-Aldrich). Then, samples were sonicated, and after the addition of $HClO_4$ (60%, Sigma-Aldrich; 7.5 μL), they were centrifuged at 9,500 × *g* for 10 min at 4°C. Aliquots of supernatants (10 μL) were dispensed into 96-well black plates (Nunc). For GSH measurement, 190 μL of phosphate buffer and 20 μL of OPT (1 mg/mL in methanol) were dispensed in the wells, and the plate was incubated for 15 min in the dark and at room temperature. For the measurement of GSSG, 8 μL of *N*-ethylmaleimide (0.04 M; Sigma-Aldrich) was added to each well to prevent interference of GSH with GSSG. The plate was incubated for 30 min under the same conditions. Then, 186 μL of NaOH (0.1 N) and 20 μL of OPT were incorporated, and the plate was incubated for 15 min in similar conditions. In both GSH and GSSG measurements, the fluorescence emitted by each well was measured at 350 nm excitation and 420 nm emission. Protein content of the samples was determined following the bicinchoninic acid protein assay kit protocol (Sigma-Aldrich), using serum albumin (BSA, Sigma-Aldrich) as standard. Results were expressed as nanomoles of GSH or GSSG per milligram of protein. Moreover, the GSSG/GSH ratio was calculated for each sample.

Lipid Peroxidation (MDA) Assay

The estimation of MDA, in both blood cells and isolated peripheral blood neutrophils and mononuclear leukocytes, was evaluated using the commercial kit "MDA Assay Kit" (Biovision, Mountain View, CA, USA), which measures the reaction of MDA with thiobarbituric acid (TBA) and the MDA-TBA adduct formation. For this, aliquots of frozen WB cells (100 μL) were resuspended in phosphate buffer 0.05 M, pH 7.4 (200 μL), whereas aliquots of isolated neutrophils and lymphocytes adjusted to 10^6 cells/mL in Hank's solution were centrifuged at 1,200 × *g* for 10 min at 4°C. All samples were resuspended in lysis buffer (300 μL)

containing butylated hydroxytoluene (0.1 mM, 3 μL), sonicated, and centrifuged again at 13,000 × *g* for 10 min. The supernatants (200 μL) from each sample were added to TBA (600 μL) and incubated at 95°C for 60 min. Samples were cooled in ice for 10 min, and 200 μL of reaction mixture was mixed with 300 μL of *n*-butanol (Sigma-Aldrich) to create an organic phase in which the MDA molecules were to be placed. Samples were centrifuged 10 min at 13,000 × *g* at room temperature, and 200 μL of the supernatants (upper organic phase) was collected and dispensed into a 96-well microplate for spectrophotometric measurement at 532 nm. MDA supplied in the kit was used as standard, and MDA levels were determined by comparing the absorbance of samples with that of the standards. Protein concentration of the samples was measured as described earlier. Results were expressed as nanomoles of MDA per milligram of protein.

Cytokine Measurement

The IL-6, TNF-α, and IL-10 release was measured in culture supernatants of WB in the absence or presence of LPS following a method previously described (58). Briefly, 500 μL of blood was diluted 1:1 with RPMI 1640 medium without L-glutamine (Gibco) and incubated for 4 h with 10 μL gentamicin (1 mg/mL, Sigma-Aldrich) and 10 μL LPS (250 ng/mL, Sigma-Aldrich) or 10 μL RPMI 1640 medium (basal conditions). Samples were centrifuged, and supernatants were collected and frozen at −20°C until assay. Levels of IL-6, TNF-α, and IL-10 were measured simultaneously by multiplex luminometry (Beadlyte human multiplex cytokine detection system, HCYTOMAG-60K, Millipore, Billerica, MA, USA), with minimum detectable doses of IL-6, TNF-α, and IL-10 under 0.9, 0.7, and 1.1 pg/mL, respectively. The results were expressed as picograms per milliliter.

Statistical Analysis

Statistical analysis was performed in SPSS IBM, version 21.0 (SPSS, Chicago, USA). All tests were two-tailed, with a significant level of $\alpha = 0.05$. Data are presented as mean ± standard deviation (SD). Normality of the samples and homogeneity of the variances were checked by the Kolmogorov–Smirnov test and Levene test, respectively. Differences due to age and AD pathology were studied using a one-way analysis of variance followed by *post hoc* tests analysis or the non-parametric Kruskal–Wallis test. The Tukey test was used for *post hoc* comparisons when variances were homogeneous, whereas its counterpart analysis Games-Howell was used with unequal variances when they were not homogeneous. Figures were built using GraphPad Prism 6.

RESULTS

Immune Functions in Peripheral Blood Neutrophils and Mononuclear Leukocytes of mAD and AD Patients As Well As Healthy Controls

The results obtained in the immune functions studied in isolated human blood neutrophils and mononuclear cells obtained from adult and older healthy subjects, as well as from mAD and AD patients are shown in **Figure 1** and **Table 2**. In general, all the immune parameters analyzed in the present work (adherence, chemotaxis, and phagocytosis of neutrophils as well as adherence, chemotaxis and proliferative response of lymphocytes in both basal and stimulated conditions, and the antitumor cytotoxic activity of NK cells) have been shown to deteriorate in aging and AD pathology. However, a different pattern of impaired immune function has been observed between the early and advanced stages of AD.

In elderly healthy subjects, in comparison with healthy adults, statistically significant lower values in neutrophil chemotaxis ($P < 0.001$; **Figure 1C**) and phagocytosis (176 ± 13 and 488 ± 25 P.I., older vs adult, respectively; $P < 0.001$), as well as in the activity of NK cells ($P < 0.01$; **Figure 1E**), lymphocyte chemotaxis ($P < 0.05$; **Figure 1D**) and PHA- and LPS-lymphoproliferative response ($P < 0.001$; **Figures 1G,H**; **Table 2**) were obtained. In contrast, higher values were also shown in old subjects in neutrophil and lymphocyte adherence ($P < 0.01$; **Figures 1A,B**), as well as in basal proliferation ($P < 0.01$; **Figure 1F**).

Regarding AD pathology, there were significant differences in AD patients relative to elderly healthy controls of the same chronological age, as well as in mAD compared to the same controls. Thus, AD patients exhibited higher basal proliferation ($P < 0.05$; **Figure 1F**) and lower neutrophil and lymphocyte chemotaxis ($P < 0.05$; **Figures 1C,D**), as well as PHA- and LPS-lymphoproliferative response ($P < 0.05$; **Figures 1G,H**; **Table 2**) in comparison to elderly subjects. Interestingly, at the early stage of the disease, mAD patients also showed lower PHA-lymphoproliferation ($P < 0.01$; **Figure 1G**; **Table 2**) than elderly subjects. However, mAD exhibited a significant increase in neutrophil ($P < 0.01$; **Figure 1A**) and lymphocyte ($P < 0.001$; **Figure 1B**) adherence in relation to elderly subjects, which was not observed at the advanced stage of AD. Finally, it should be noted that immune function differences between the early and advanced stages of AD were also observed. Thus, AD patients showed significantly lower neutrophil and lymphocyte adherence ($P < 0.05$; **Figures 1A,B**), as well as lower chemotaxis of lymphocytes and lower NK cytotoxic activity ($P < 0.05$; **Figures 1D,E**) than mAD patients.

Cytokine Production in Response to LPS Stimulation in Blood Leukocytes of mAD and AD Patients As Well As Healthy Controls

Cytokines are major mediators of the complex interactions among immune cells, being responsible for the development and resolve of immune response. Aging is characterized by a chronic low-grade inflammatory state (61), and the maintenance of health relies on the adequate balance of anti-inflammatory and pro-inflammatory compounds (28). For this reason, we analyzed the levels of pro-inflammatory (IL-6 and TNF-α) and anti-inflammatory (IL-10) cytokines secreted *ex vivo* by blood cells incubated for 4 h under LPS-stimulated conditions, in mAD and AD patients, as well as in adult and elderly healthy subjects. The IL-10/TNF-α ratios, which are a good indicator of successful aging and longevity (30), were also calculated.

In general, the results of our study showed that the release of these cytokines suffers impairments with aging. Moreover,

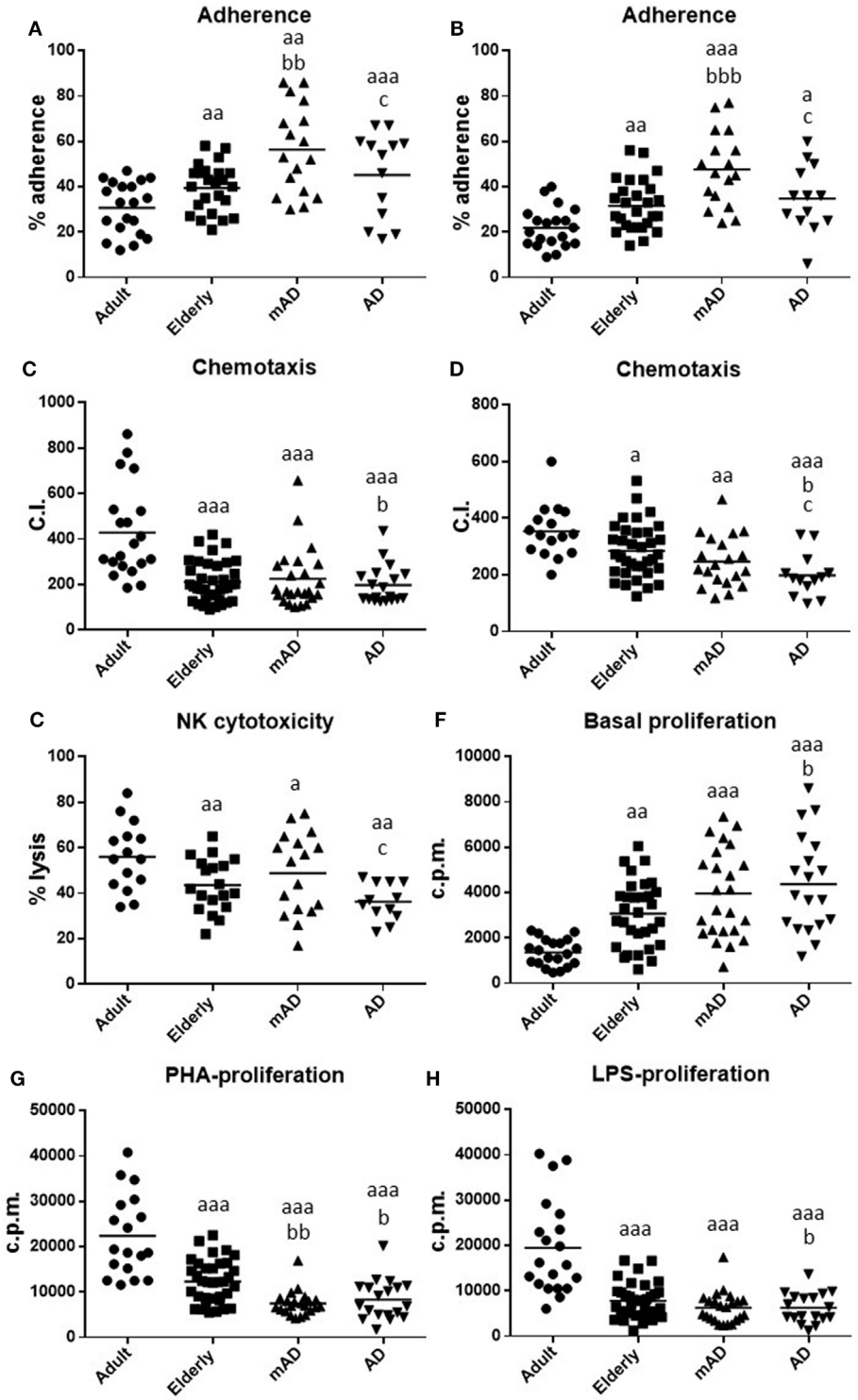

FIGURE 1 | Function parameters in isolated peripheral blood neutrophils and mononuclear cells of mild Alzheimer's disease (mAD) and Alzheimer's disease (AD) patients, as well as of adult and elderly healthy subjects. Adherence (%) of neutrophils **(A)** and lymphocytes **(B)**; chemotaxis index (C.I.) of neutrophils **(C)** and lymphocytes **(D)**; natural killer (NK) cytotoxicity (% lysis) **(E)**; proliferation of lymphocytes in basal conditions [48 h incubation; counts per minute (cpm)] **(F)**; proliferation of lymphocytes (48 h incubation; cpm) in response to phytohemagglutinin (PHA, 1 μg/mL) **(G)** and in response to lipopolysaccharide (LPS, 1 μg/mL) **(H)**. Data are shown as the mean (horizontal bar) of 18–38 values corresponding to the number of subjects analyzed in each group (20 adult, 38 elderly, 26 mAD, and 18 AD). Each value is the mean of duplicate assays. a: $P < 0.05$, aa: $P < 0.01$, and aaa: $P < 0.001$ with respect to the value in adult subjects; b: $P < 0.05$, bb: $P < 0.01$, and bbb: $P < 0.001$ with respect to the value in elderly subjects; c: $P < 0.05$ with respect to the value in mAD patients.

a similar pattern of altered secretion was observed for all investigated cytokines, in AD patients. As shown in **Figure 2**, under LPS-stimulated conditions, increased levels of IL-6 and TNF-α ($P < 0.05$ and $P < 0.001$, respectively; **Figures 2A,B**) were observed in the elderly subjects in comparison to adults. This was also accompanied by a marked decrease in IL-10 and the IL-10/TNF-α ratio ($P < 0.001$; **Figures 2C,D**). A similar cytokine profile was also observed in AD patients in relation to older subjects. Thus, AD patients had higher levels of IL-6 and TNF-α ($P < 0.01$; **Figures 2A,B**), as well as lower IL-10 release and IL-10/TNF-α ratios ($P < 0.05$; **Figures 2C,D**) than elderly controls. However, mAD patients only showed a significant increase in the levels of IL-6 ($P < 0.05$; **Figure 2A**). No differences were observed in TNF-α and IL-10 release and IL-10/TNF-α ratios between mAD and older subjects. Regarding the differences between the different stages of AD, it is important to note that AD patient exhibited a higher IL-6 LPS-induced release ($P < 0.05$; **Figure 2A**) and a lower IL-10/TNF-α ratio ($P < 0.05$; **Figure 4D**) than mAD patients.

TABLE 2 | Stimulation of proliferation (%) in response to phytohemagglutinin and lipopolysaccharide (1 μg/mL), in isolated peripheral mononuclear cells of adult and elderly healthy subjects, as well as of mild Alzheimer's disease (mAD) and severe Alzheimer's disease (AD) patients.

Lymphoproliferation (% stimulation)	Adult	Elderly	mAD	AD
PHA (%)	1,950 ± 232	391 ± 56[a]	198 ± 19[a,b]	218 ± 36[a,c]
LPS (%)	346 ± 25	176 ± 13[d]	156 ± 12[c,e]	167 ± 17[e]

Data are expressed as mean ± SD of the values corresponding to 20 adult, 38 elderly, 26 mAD, and 18 AD subjects. Each value is the mean of duplicate assays.
[a]P < 0.001 with respect to the value in adult subjects.
[b]P < 0.01 with respect to the value in elderly subjects.
[c]P < 0.05 with respect to the value in elderly subjects.
[d]P < 0.05 with respect to the value in adult subjects.
[e]P < 0.01 with respect to the value in adult subjects.

Oxidative Stress and Damage Parameters in Isolated Peripheral Blood Neutrophils and Mononuclear Cells of mAD and AD Patients As Well As Healthy Controls

A good maintenance of the redox state is vital for the proper functioning of immune cells, and thus the oxidative stress, which

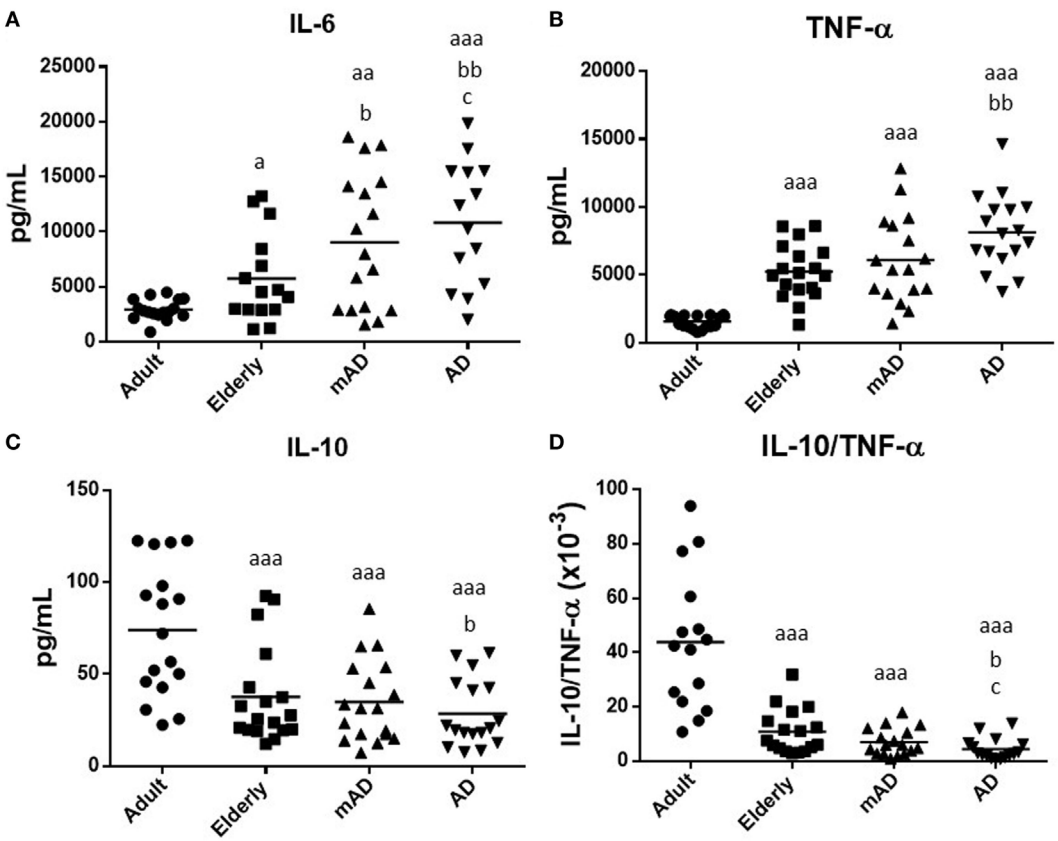

FIGURE 2 | Levels (pg/mL) of cytokines released after 4 h of culture in the presence of lipopolysaccharide (1 μg/mL) by peripheral whole blood cells of mild Alzheimer's disease (mAD) and Alzheimer's disease (AD) patients, as well as of adult and elderly healthy subjects. Interleukin (IL)-6 **(A)**, tumor necrosis factor (TNF)-α **(B)**, and IL-10 **(C)**, as well as IL-10/TNF-α ratios **(D)**. Data are shown as the mean (horizontal bar) of the values corresponding to the number of subjects analyzed in each group (20 adult, 18 elderly, 18 mAD, and 18 AD). Each value is the mean of duplicate assays. a: $P < 0.05$, aa: $P < 0.01$, and aaa: $P < 0.001$ with respect to the value in adult subjects; b: $P < 0.05$ and bb: $P < 0.01$ with respect to the value in elderly subjects; and c: $P < 0.05$ and cc: $P < 0.01$ with respect to the value in mAD patients.

is the basis of aging and age-related diseases, is detrimental for leukocyte functions (28). Hence, the decline in immune response observed in mAD and AD patients could be mediated by an increase in oxidative stress and the consequent oxidative damage. To test this possibility, the intracellular content of GSH and GSSG, the GSSG/GSH ratio (one of the best markers of oxidative stress), as well as the MDA levels (one of the major lipid peroxidation products) were assessed in isolated human blood neutrophils and mononuclear cells obtained from mAD and AD patients, as well as from adult and elderly healthy subjects. The results are shown in **Figure 3**. In general, in both leukocyte populations a higher oxidative stress and damage have been shown in elderly subjects and AD patients than in adult controls. However, a different pattern of impaired redox state has been observed between the early and advanced stages of AD, the neutrophils showing a greater oxidative stress and damage than the lymphocytes.

Regarding aging, elderly healthy controls showed in their neutrophils and lymphocytes significantly lower GSH contents ($P < 0.05$ and $P < 0.01$, respectively; **Figures 3A,B**), as well as higher GSSG amounts ($P < 0.01$; **Figures 3C,D**), higher GSSG/GSH ratios ($P < 0.01$ and $P < 0.05$, respectively; **Figures 3E,F**), and MDA levels ($P < 0.01$ and $P < 0.05$, respectively; **Figures 3G,H**) in relation to adult subjects.

Regarding the AD pathology, AD patients had in their neutrophils a marked increase in both GSSG and MDA contents ($P < 0.01$; **Figures 3C,G**), as well as in GSSG/GSH ratios ($P < 0.05$; **Figure 3E**) in comparison to elderly subjects, accompanied also by a marked decrease in the levels of GSH ($P < 0.05$; **Figure 3A**). However, in the case of lymphocytes, AD patients showed higher GSH contents ($P < 0.05$; **Figure 3B**) than elderly controls, whereas no differences were observed in the other parameters analyzed. Likewise, similar results were observed in mAD patients, who showed higher GSSG contents, GSSG/GSH ratios, and MDA levels ($P < 0.05$; **Figures 3C,E,G**) in neutrophils as well as higher GSH contents ($P < 0.01$; **Figure 3B**) in lymphocytes, than the corresponding values in elderly controls. In relation to the different stages of AD, it is important to note that AD patients had in their neutrophils markedly increased GSSG/GSH ratios and MDA levels ($P < 0.05$; **Figures 3E,G**) as well as decreased GSH contents in their lymphocytes ($P < 0.05$; **Figure 3B**) in relation to mAD patients.

Oxidative Stress and Damage Parameters in Human Peripheral Blood Cells of mAD and AD Patients as well as of Healthy Controls

It is known that there is a significant difference between the use of WB and purified and isolated PMNs and mononuclear leukocytes (62). Moreover, various peripheral blood cell types and their proportions can affect the immune response, as well as the inflammatory and oxidative stress conditions (54, 62). For this reason, the same parameters of oxidative stress and damage analyzed in isolated neutrophils and lymphocytes from mAD and AD patients, and adult and elderly healthy subjects, were also evaluated using samples of WB cells, containing both total RBC and leukocyte populations. This kind of sample better reproduces the *in vivo* conditions. The results are shown in **Figure 4**. There were higher values of oxidative stress and damage in blood cells of elderly subjects than in those of adults. Thus, GSH values were lower ($P < 0.001$; **Figure 4A**) and GSSG/GSH ratios higher ($P < 0.05$; **Figure 4C**), as well as both GSSG ($P < 0.01$; **Figure 4B** and MDA ($P < 0.001$; **Figure 4D**) levels, than those in adult subjects.

Regarding AD pathology, AD patients exhibited, in their blood cells, significantly lower GSH contents ($P < 0.05$; **Figure 4A**), higher GSSG and MDA contents ($P < 0.05$ and $P < 0.001$, respectively; **Figures 4B,D**), and GSSG/GSH ratios ($P < 0.05$; **Figure 4C**) than elderly subjects. Similar results were also observed in mAD patients in relation to elderly subjects, which also showed lower GSH levels ($P < 0.01$; **Figure 4A**) and higher GSSG/GSH ratios ($P < 0.05$; **Figure 4C**) and MDA contents ($P < 0.01$; **Figure 4D**) than elderly controls. However, no differences were observed in GSSG contents between mAD and elderly subjects. Finally, regarding the differences between early and advanced stages of AD, it is important to note that AD patients showed higher levels of GSSG and MDA ($P < 0.05$ and $P < 0.01$, respectively; **Figures 4B,D**) than mAD patients.

Basal Cytokine Production in Blood Leukocytes of mAD and AD Patients as well as of Healthy Controls

Since an age-related increase in release of pro-inflammatory cytokines in resting cells leads to a sterile inflammation (30), and this alteration of the inflammatory status in aging ("inflamm-aging") could lead to a chronic situation causing neuronal impairment and loss associated with AD (63), we also assessed extracellular release of IL-6, TNF-α, and IL-10 secreted *ex vivo* by blood cells cultured (4 h) under resting conditions, from mAD and AD patients, as well as from adult and elderly healthy controls.

As shown in **Table 3**, under basal conditions, in elderly subjects the values of IL-6 and TNF-α released were higher ($P < 0.01$ and $P < 0.05$, respectively) than in adults. Regarding AD pathology, a similar cytokine profile was also observed in AD patients in relation to both adults and elderly subjects. Thus, AD patients showed higher levels of IL-6 and TNF-α ($P < 0.05$) than elderly controls, whereas mAD patients only showed statistically significant higher values ($P < 0.05$) in the TNF-α levels. Basal IL-10 release was not detectable by the multiplex, so the IL-10/TNF-α ratio could not be calculated. Interestingly, regarding differences between early and advanced stages of AD, AD patients showed higher basal IL-6 release ($P < 0.05$) than the individuals with mAD.

DISCUSSION

To our knowledge, this is the first study that analyzed the changes in several parameters of function and oxidative-inflammatory stress state in different types of peripheral blood immune cells, such as neutrophils and mononuclear cells, from patients with mild and severe AD, compared with a healthy age-matched control group of subjects without cognitive impairment. Additionally, all these parameters were also studied in healthy adult subjects to evaluate

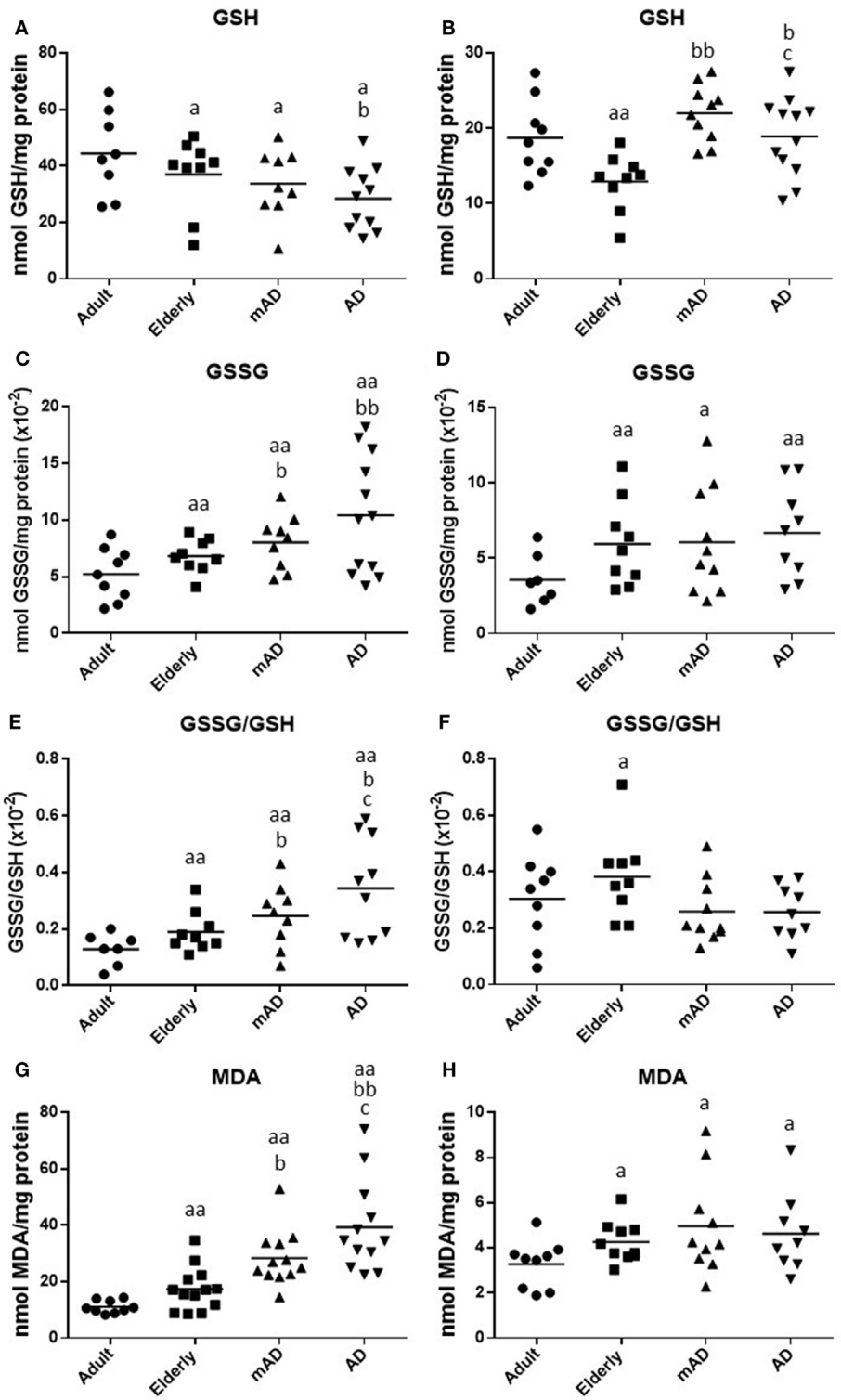

FIGURE 3 | Oxidative stress parameters and lipid peroxidation in isolated peripheral blood neutrophils and mononuclear cells of mild Alzheimer's disease (mAD) and Alzheimer's disease (AD) patients, as well as of adult and elderly healthy subjects. Intracellular reduced glutathione (GSH) contents (nmol/mg protein) in neutrophils **(A)** and lymphocytes **(B)**; intracellular oxidized glutathione (GSSG) contents (nmol/mg protein) in neutrophils **(C)** and lymphocytes **(D)**; GSSG/GSH ratios in neutrophils **(E)** and lymphocytes **(F)**; and intracellular malondialdehyde (MDA) contents (nmol/mg protein) in neutrophils **(G)** and lymphocytes **(H)**. Data are shown as the mean (horizontal bar) of 9–12 values corresponding to the number of subjects analyzed in each group (9 adult, 10 elderly, 11 mAD, and 12 AD). Each value is the mean of duplicate assays. a: $P < 0.05$ and aa: $P < 0.01$ with respect to the value in adult subjects; b: $P < 0.05$ and bb: $P < 0.01$ with respect to the value in elderly subjects; and c: $P < 0.05$ with respect to the value in mAD patients.

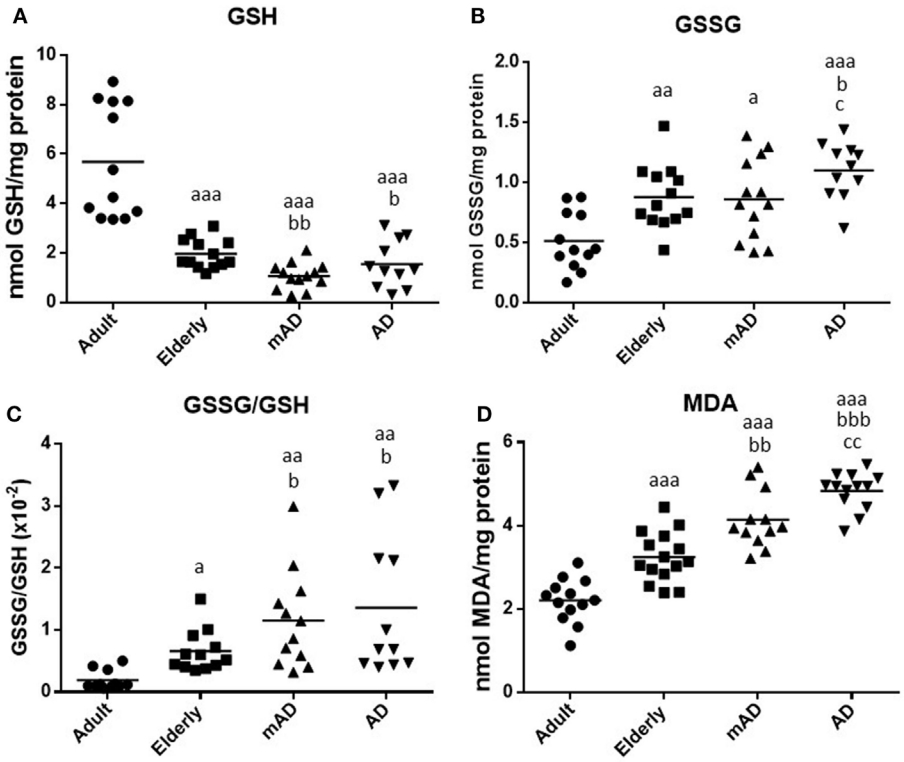

FIGURE 4 | Oxidative stress parameters and lipid peroxidation in whole blood cells (containing both total red blood cells and leukocyte populations) of mild Alzheimer's disease (mAD) and Alzheimer's disease (AD) patients, as well as of adult and elderly healthy subjects. Intracellular reduced glutathione (GSH) contents (nmol/mg protein) **(A)**; intracellular oxidized glutathione (GSSG) contents (nmol/mg protein) **(B)**; GSSG/GSH ratios **(C)**; and intracellular malondialdehyde (MDA) contents (nmol/mg protein) **(D)**. Data are shown as the mean (horizontal bar) of 11–15 values corresponding to the number of subjects analyzed in each group (13 adult, 15 elderly, 13 mAD, and 13 AD). Each value is the mean of duplicate assays. a: $P < 0.05$, aa: $P < 0.01$, and aaa: $P < 0.001$ with respect to the value in adult subjects; b: $P < 0.05$, bb: $P < 0.01$, and bbb: $P < 0.001$ with respect to the value in elderly subjects; and c: $P < 0.05$ and cc: $P < 0.01$ with respect to the value in mAD patients.

TABLE 3 | Levels of interleukin (IL)-6 and tumor necrosis factor (TNF)-α (pg/mL) released by peripheral whole blood cells of adult and elderly healthy subjects, as well as of mild Alzheimer's disease (mAD) and severe Alzheimer's disease (AD) patients, after 4 h of culture in basal conditions.

Basal cytokines	Adult	Elderly	mAD	AD
IL-6 (pg/mL)	8.89 ± 1.93	16.23 ± 4.72[a]	16.21 ± 3.84[a]	21.60 ± 5.17[b, c,d]
TNF-α (pg/mL)	24.76 ± 6.14	40.30 ± 14.89[e]	90.76 ± 26[a,c]	105.8 ± 22.5[a,c]

Data are expressed as mean ± SD of the values corresponding to 10 adult, 10 elderly, 8 mAD, and 8 AD subjects. Each value is the mean of duplicate assays.
[a]P < 0.01 with respect to the value in adult subjects.
[b]P < 0.001 with respect to the value in adult subjects.
[c]P < 0.05 with respect to the value in elderly subjects.
[d]P < 0.05 with respect to the value in mAD patients.
[e]P < 0.05 with respect to the value in adult subjects.

the differences due to the aging process. Our results revealed an impairment of the immune functions of both neutrophils and mononuclear cells at different stages of AD. Several alterations were only observed in severe AD patients and some only in mAD patients. Furthermore, our results also demonstrated that immune blood cells from mAD and AD patients, and especially neutrophils, showed an increased oxidative stress and oxidative damage in relation to elderly subjects. This altered redox balance could be mediated by the higher production of pro-inflammatory cytokines, which has been also observed in AD patients.

The immune system has a profound implication in the pathology of AD, not only at the central level but also at the peripheral level (8–10, 17, 19). Although several studies have reported alteration of both innate and acquired immunity at different stages of AD (12), there are many contradictory results (11–15, 17). Moreover, most of these studies in human and animal models have focused on the alteration of the adaptive immune response. However, little is known about the changes in innate immunity, especially those carried out by phagocytes (e.g., neutrophils). Our findings revealed that severe AD patients show lower neutrophil and lymphocyte chemotaxis, PHA- and LPS-lymphoproliferation, as well as higher basal lymphoproliferation in comparison to elderly healthy subjects. These AD-related changes are similar to those with aging (28, 30), which was also observed in the present study, although more exacerbated. This shows the presence of a

lesser competent immune system in severe AD patients than in elderly subjects, which could contribute to the high mortality of these individuals. Our results agree with some of those observed in humans and experimental animals with AD (11, 16, 17, 26, 33, 52). Thus, leukocytes of 3xTg-AD mice presented an impairment of chemotaxis capacity (16, 26, 52). In relation to lymphoproliferative response in stimulated conditions some studies also report a decrease in this function in cells obtained from patients with severe AD (11, 17, 33, 64).

Surprisingly, mAD patients, but not severe AD patients, showed higher values in neutrophil and lymphocyte adherence than elderly subjects. This increase in adherence is a characteristic of aging (24, 28). Although at this moment we cannot explain the possible regulation mechanisms that severe AD patients use to present similar values to age-matched controls, this parameter could be an early marker of the appearance of AD. In mAD patients, a lower proliferative response of lymphocytes to the mitogen PHA than in elderly subjects also occurred. Thus, this function of lymphoproliferative response to mitogens, which typically decreases with aging (24, 28, 30) appeared even lower in cells of mAD and AD.

Alzheimer's disease patients in comparison to mAD showed lower lymphocyte chemotaxis and antitumoral NK activity. In this regard, although limited in number, several studies in human and 3xTg-AD mice revealed that these immune function parameters were altered in AD (16, 26, 32, 52). Thus, NK cell activity, which has been proposed as one biomarker of immune alterations in the progression of AD (32) and which was decreased in cells of 3xTg-AD mice in advanced stages of AD (16, 26), was lower in severe AD than in mAD patients. In the case of chemotaxis of lymphocytes, a function that decreases with aging (28, 30), our results reflect a clear deterioration of this activity in AD patients, even higher than shown by cells of elderly subjects and of mAD. Thus, this parameter could be considered as a possible marker of the progression of the disease.

Interestingly, it should be noted that although the decline of the immune functions showed with aging were also observed in mAD and AD patients, several of these immunological alterations were greatly exacerbated in individuals with severe AD in comparison to healthy elderly subjects of the same chronological age. This suggests that AD patients suffer an accelerated immunosenescence. Thus, since these immune function parameters are good markers of health and predictors of longevity (28, 30), these could be used as peripheral biomarkers of the progression of AD.

A chronic oxidative-inflammatory stress, a condition in which oxidant and pro-inflammatory compounds overwhelm antioxidant and anti-inflammatory defenses, is associated with aging and several age-related pathologies (26, 28). Indeed, several studies in human and animal models, including some by our group, have demonstrated that increased peripheral oxidative stress markers are associated with aging and, more specifically, with AD (16, 26, 30, 47, 52, 54). In AD, oxidative stress has been recognized as an essential contributor to the pathogenesis and progression of the disease (38, 40, 52, 65). As occurs in the brain (7, 40), an enhanced oxidative stress during AD is also present in peripheral blood cells (27, 41–43). Most studies have focused on mononuclear cells, mainly in lymphocytes, which reflect the pathological oxidative stress conditions observed in the brains of AD patients. These studies found an elevated production of oxidative compounds, lipid oxidation, DNA damage, mitochondrial susceptibility, basal apoptosis as well as altered levels of antioxidant enzymes in lymphocytes of AD patients, as well as even in those of MCI subjects, in comparison with these cells of healthy subjects (27, 66–68). These results suggest that some of these parameters could be prodromal markers of AD. However, little is known about the changes in the redox balance of neutrophils during the progression of AD. Therefore, since phagocytes may be the immune cells that contribute most to the oxidative stress and damage associated with immunosenescence (28, 54), we also analyzed different redox state and oxidative damage parameters in both isolated peripheral blood neutrophils and mononuclear cells, in order to elucidate possible differences between them. Interestingly, our results showed that neutrophils suffer a marked increased oxidative stress and damage during the progression of AD. In particular, the neutrophils of both mAD and AD patients had lower antioxidant GSH contents and higher oxidant markers, such as GSSG and MDA contents, as well as GSSG/GSH ratios, than elderly subjects. Nevertheless, this redox state alteration was not observed in mononuclear cells, which paradoxically showed higher GSH levels in individuals with mild and severe AD than those observed in age-matched controls. In this context, it has been suggested that in early states of the disease, individuals developing AD can suffer a situation of "reductive stress" as a result of the activation of a compensatory response to cope with a high exposure of oxidant compounds (40). Therefore, targeting reductive stress may be a strategy to delay and prevent the onset and progression of AD (69). Thus, it is plausible that an overexpression and high levels of GSH could be induced by an increased oxidant production in lymphocytes of mAD patients. Nevertheless, these levels were lower in severe AD patients in comparison to mAD subjects. The glutathione cycle is one of the main intracellular mechanisms to preserve a competent intracellular redox state (70). The role of this has been extensively studied in AD, in both humans and animal models, where a depletion in GSH contents and an imbalance in GSSG/GSH ratios in favor of GSSG have been noted at central (39) and peripheral levels, such as in plasma, peritoneal leukocytes, and blood cells (47, 52, 71). Moreover, it has been described that the altered levels of these circulating compounds are also directly related to the severity of cognitive impairment (47, 67). The imbalance in GSSG/GSH ratios could be the result of a defective respiratory chain caused by the activation of Aβ peptide on its complexes, which leads to overproduction of oxidant compounds, which has also been observed in peripheral leukocytes (72). In addition, an adequate immune response will require optimal levels of GSH (70). Therefore, the lower GSH observed in neutrophils of AD could contribute to the impairment of the functions of these cells.

Since the brain is very susceptible to oxidative damage (36), lipid peroxidation is one of the most promising procedures in AD diagnosis (47). Thus, higher MDA content has been reported in the brain and the peripheral levels (e.g., plasma/serum, erythrocytes, and peripheral leukocytes) of not only AD patients but also MCI subjects (42, 46, 47). Some authors suggested that lipid peroxidation could be one of the main factors responsible for

cognitive deterioration and that there was a negative correlation between MDA and MMSE scores (43). Others reported that the differences depended on the stage of AD (42, 43, 47). However, although no difference between moderate and advanced AD was detected (46), our results showed that MDA levels in neutrophils were higher in patients at the advanced stage of the disease than in elderly controls and even in mAD. Thus, this parameter could be a useful marker of early stage of AD as well as progression of the disease.

Given the different pattern observed in the oxidative stress and damage parameters between neutrophils and mononuclear cells in the progression of AD, we have also analyzed these parameters in WB cells. This kind of sample better reproduces the *in vivo* conditions of immune response. Interestingly, a similarly altered redox state and oxidative damage pattern were observed in WB cells to that in neutrophils. Thus, mAD and AD patients, in general, showed lower GSH and higher GSSG contents and GSSG/GSH ratios, together with a marked increase in MDA levels, than cells from elderly subjects. Moreover, it is important to highlight that AD patients had a higher GSSG/GSH ratios, as well as GSSG and MDA contents, in their neutrophils and WB cells than mAD patients. The results suggest that the estimation of these parameters in WB cells would be a useful biomarker in the assessment of AD progression. Moreover, WB samples are clinically more feasible, reproducible, cost effective, easy to implement and apply, compared to purified and isolated neutrophils and mononuclear blood leukocytes.

In addition, since oxidation and inflammation processes are able to induce and exacerbate one another (36), the higher oxidative stress and damage observed in WB cells from mAD and AD patients could also be triggered by an increase in basal inflammation. Given that previous works supported the importance of cytokines in mediating the activity of peripheral immune cells in AD (73, 74), we also assessed the release of IL-6, TNF-α, and IL-10 *ex vivo* by blood cells cultured under resting conditions. Our results showed that mAD and AD patients had a higher basal TNF-α than elderly subjects, whereas basal IL-6 levels only were significantly higher in severe AD. Basal IL-10 release was not detectable. Even though cytokine levels are hard to detect at basal conditions, due to their short half-life, several studies also investigated these inflammatory markers in CSF, serum and plasma at different stages of the AD patients (73–76). Although the results are controversial, a strong upregulation of pro-inflammatory cytokines has been observed in these patients, but also in MCI (73, 74, 76, 77). Furthermore, these patients showed higher plasma levels of IL-6 than those at an early stage of disease and healthy controls (77). This agrees with our results, in which severe AD patients also showed higher IL-6 release than mAD. Interestingly, given that increased basal IL-6 levels in plasma have been shown to be a risk for decline in cognitive functions (73), as well as being described as one the most powerful predictors of morbidity and mortality in the elderly (30), these results suggest the possibility of using peripheral IL-6 production as a helpful tool to characterize immune dysfunction during AD progression.

The chronic oxidative-inflammatory stress situation observed in blood cells of AD patients could be involved in the impairment of their functions. Thus, the higher oxidative stress in severe AD patients could be the underlying mechanism for the decrease in PHA- and LPS-induced lymphoproliferation, as well as for the increase in the basal proliferation. This basal proliferation, which has scarcely been studied, was also increased with aging (30), as well as in the leukocytes of 3xTg-AD mice (52). The higher levels of basal proliferation in AD lymphocytes show an overactivation of these cells. This could be due to the increase age-related accumulation of damage-associated molecular patterns, which, through the production of pro-inflammatory cytokines, may be responsible for the activation of the immune system and the establishment of "sterile inflammation" (78). In addition, after a mitogenic LPS-stimulus, immune blood cells from AD patients also produced a higher release of IL-6 and TNF-α, as well as lower IL-10 levels and, consequently, a lower IL-10/TNF-α ratio, compared to age-matched controls. This reaffirms the existence of an altered immune response in advanced stages of AD. Although data on peripheral cytokines in AD patients are controversial (73, 74), several authors also found an upregulation of these cytokines in LPS-stimulated WB in AD patients in relation to elderly controls (75), as also occurred in samples of brain, serum, and other cell cultures (79). A possible explanation would be that βA peptide, which can activate the overproduction of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, etc.) in peripheral blood monocytes (80, 81), could lead to an abnormal inflammatory response. This could be attributed either to an adaptive control on innate immunity and/or a compensatory mechanism for the lack of effectiveness of adaptive immunity (81). Our results also demonstrated an altered balance in the IL-10/TNF-α ratios, which showed similar values in mAD patients and elderly subjects, whereas the values were lower in AD patients. Since this IL-10/TNF-α ratio has been proposed as an indicator of successful aging and longevity (30), its determination also may be a helpful parameter for evaluating the progression of AD. Since the decline in PHA-lymphoproliferation and the high release of IL-6 after LPS stimulation not only occur in severe AD patients but also begin to decline in mAD patients, this suggests that both parameters could be used as possible early peripheral biomarkers of AD.

In conclusion, the present study shows the impairment of several immune functions of human peripheral blood neutrophils and mononuclear cells at different stages of AD. However, a different pattern of altered immune response was observed between mild and severe AD patients. Thus, several alterations were only observed in severe AD patients (e.g., chemotaxis, basal, and LPS lymphoproliferation) and others (e.g., adherence) only in individuals with mAD. Other alterations detected in the mild stage of the disease increased in the late stage (e.g., PHA lymphoproliferation and IL-6 release). As occurs in aging, this impairment of immune cell functions could have as their basis an oxidative-inflammatory stress situation. Thus, our findings also demonstrated that several peripheral oxidative stress and damage markers were increased in immune blood cells from mAD and AD patients, especially in neutrophils (e.g., low GSH levels, high GSSG/GSH ratios, and GSSG and MDA contents), in relation to elderly subjects. However, this increased oxidative stress and damage were higher in severe AD patients than in mAD and could be mediated by the higher production of peripheral

pro-inflammatory cytokines in these patients. Furthermore, the fact that neutrophils showed higher oxidative stress and damage than mononuclear leukocytes supports the idea that phagocytes are the immune cells that most contribute to the peripheral chronic oxidative stress and damage associated with AD, especially in the advance stages of the disease. Therefore, our results support the idea that severe AD patients show an accelerated immunosenescence due to the parameters of function and redox state studied, these being greatly deteriorated in these patients in comparison to elderly subjects of the same chronological age. Therefore, since WB cells are very easy to obtain and reproduce the results observed in neutrophils, the assessment of oxidative stress and damage parameters, as well as peripheral cytokine release, together with the analysis of several functions in isolated neutrophils and mononuclear cells, would be useful markers of AD progression. Nevertheless, additional studies are needed to identify function and oxidative-inflammatory stress alterations in peripheral blood phagocytes and lymphocytes, especially in subjects with mild cognitive deterioration in order to identify potential prodromal and preclinical biomarkers of AD.

ETHICS STATEMENT

All procedures were carried out according to the Declaration of Helsinki, and approval was obtained from the corresponding Research Ethic Committees.

AUTHOR CONTRIBUTIONS

MF—formulated the original problem, designed the work, provided direction and guidance, wrote the manuscript and critically reviewed the final version of the manuscript. CV—provided input for experimental design, carried out experiments with patient samples, analyzed data results, and wrote the manuscript; IT—provided input for experimental design and carried out experiments with patient samples; AG—carried out experiments with patient samples; EC and JM provided the samples for the experimental study; and JM made the clinical diagnosis of Alzheimer's disease patients and control's selection.

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Negative Effect of Age, but Not of Latent Cytomegalovirus Infection on the Antibody Response to a Novel Influenza Vaccine Strain in Healthy Adults

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Older adults are more vulnerable to influenza virus infection and at higher risk for severe complications and influenza-related death compared to younger adults. Unfortunately, influenza vaccine responses tend to be impaired in older adults due to aging of the immune system (immunosenescence). Latent infection with cytomegalovirus (CMV) is assumed to enhance age-associated deleterious changes of the immune system. Although lower responses to influenza vaccination were reported in CMV-seropositive compared to CMV-seronegative adults and elderly, beneficial effects of CMV infection were observed as well. The lack of consensus in literature on the effect of latent CMV infection on influenza vaccination may be due to the presence of pre-existing immunity to influenza in these studies influencing the subsequent influenza vaccine response. We had the unique opportunity to evaluate the effect of age and latent CMV infection on the antibody response to the novel influenza H1N1pdm vaccine strain during the pandemic of 2009, thereby reducing the effect of pre-existing immunity on the vaccine-induced antibody response. This analysis was performed in a large study population ($n = 263$) in adults (18–52 years old). As a control, memory responses to the seasonal vaccination, including the same H1N1pdm and an H3N2 strain, were investigated in the subsequent season 2010–2011. With higher age, we found decreased antibody responses to the pandemic vaccination even within this age range, indicating signs of immunosenescence to this novel antigen in the study population. Using a generalized estimation equation regression model, adjusted for age, sex, and previous influenza vaccinations, we observed that CMV infection in contrast did not influence the influenza virus-specific antibody titer after H1N1pdm vaccination. Yet, we found higher residual protection rates (antibody level ≥40 hemagglutinin units (HAU)) in CMV-seropositive individuals than in CMV-seronegative individuals 6 months and 1 year after pandemic vaccination. In the subsequent season, no effect of age or CMV infection on seasonal influenza vaccine

response was observed. In conclusion, we observed no evidence for CMV-induced impairment of antibody responses to a novel influenza strain vaccine in adults. If anything, our data suggest that there might be a beneficial effect of latent CMV infection on the protection rate after novel influenza vaccination.

Keywords: cytomegalovirus, influenza vaccine, aging, immunosenescence, pandemic, antibody response, *de novo* immune response

INTRODUCTION

Aging of the population poses an important public health problem. With age, the function of the human immune system declines, a phenomenon also referred to as immunosenescence (1). Profound changes of the immune system include the gradual loss of naïve cells, increase of memory cell numbers, and decreased diversity of the T cell and B cell repertoire (1–3). These changes contribute to reduced protection against infectious diseases and reduced vaccine responses in older adults. Indeed, the incidence of influenza virus infections is increased and accompanied with more complications and higher mortality in older adults (4, 5). Most developed countries recommend yearly influenza vaccination in individuals above 60 or 65 years of age (6), in order to prevent influenza virus infection by the induction of protective antibodies (4, 7). However, the antibody response to influenza vaccination in older adults is impaired, causing a suboptimal protection in this vulnerable group (7–9).

Accumulating evidence indicates that latent cytomegalovirus (CMV) infection is associated with age-related changes of the immune system, and might enhance immunosenescence (2, 10, 11). CMV is a common β-herpesvirus with a prevalence of 45–100% worldwide, which increases with advancing age (12). CMV infection causes morbidity and mortality in severely immunocompromised patients, while the virus rarely causes clinical symptoms in healthy individuals. Despite the ability of the immune system to control primary infection, the virus establishes a latent infection, with episodes of viral reactivation during lifetime (13). The frequent reactivation of CMV causes continuous antigenic stress for the immune system (3). Anti-CMV IgG levels increase with age (14–16) and are thought to increase after viral reactivation episodes, thereby reflecting the amount of experienced CMV antigenic stress during lifetime (12, 14, 17). The profound effect of CMV infection on the immune system is especially shown by the progressive large expansion of oligoclonal CMV-specific CD8 T cells and, to a lesser extent, CD4 T cells. Furthermore, CMV-seropositivity is strongly associated with an inverted CD4/8 ratio (18), bias of the TCR repertoire (19), and an increase of highly differentiated T cells (20).

It has been suggested that CMV-enhanced immunosenescence could impair the immune response to influenza vaccination (21, 22). Indeed, in several studies, CMV-seropositivity or a high anti-CMV IgG titer was associated with lower antibody responses to influenza vaccination in both adults (23–25) and older adults (25–28). However, others did not find an effect of CMV infection (29, 30), or reported even an enhanced antibody response to influenza vaccination in both young (31, 32) and older CMV-seropositive individuals (33).

The overall impact of latent CMV infection on the antibody induction by influenza vaccines remains controversial and depends, among other factors, on pre-existing immunity to influenza virus (34). Most studies investigated the antibody response to seasonal influenza vaccination; a yearly recommended trivalent influenza vaccine that often contains overlapping influenza vaccine strains in consecutive years. Natural exposure to influenza virus and previous vaccination causes pre-existing immunity, which influences the consecutive vaccine response. Higher pre-vaccination antibody titers (pre-titers) indeed were shown to result in lower post-vaccination antibody titers to subsequent vaccination (7, 35). Furthermore, one could expect a larger effect of immunosenescence on *de novo* immune responses (36, 37). A seasonal influenza vaccination is, therefore, a suboptimal study setting to investigate the effect of latent CMV infection on influenza vaccine antibody response.

We hypothesize that the effect of latent CMV infection on the antibody response to influenza vaccination can best be studied when a novel influenza virus strain is introduced into a naïve population. In this study, we had the unique opportunity to investigate the effect of latent CMV infection on the antibody response during the pandemic season of 2009 to the novel H1N1pdm vaccine strain in a large study population and at multiple time points after vaccination. This allowed a sophisticated study design to test the effect of latent CMV infection on a *de novo* influenza vaccine response by minimizing pre-existing immunity due to previous exposure by vaccination or natural infection. As a control, the influence of latent CMV infection on the memory antibody response to the vaccination in the subsequent year was also investigated, which included both the same H1N1pdm vaccine strain and an H3N2 vaccine strain.

MATERIALS AND METHODS

Study Population and Design

The current study is embedded in a trial that evaluated the immune responses to pandemic and seasonal influenza vaccination that was conducted in 2009–2011 (the Pandemic influenza vaccination trial, Netherlands Trial Register NTR2070). This study was carried out in accordance with the recommendations of Good Clinical Practice 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 Central Committee on Research Involving Human Subjects of the Netherlands. Healthy individuals, between 18 and 52 years of age, were recruited among health care workers in the Utrecht area in the Netherlands. Individuals

over 52 years of age were not included because of potential pre-existing immunity due to exposure to the influenza A/H1N1 strain that circulated until 1957 (38). Serum samples and questionnaires were used from the vaccine group of the Pandemic influenza vaccination cohort.

Vaccines

In the pandemic season, individuals received two doses of the monovalent MF59-adjuvanted influenza vaccine containing influenza A/California/7/2009(H1N1pdm09) with a 3-week interval (Focetria, Novartis, Italy). Blood samples were collected before vaccination (T1), 3 weeks after vaccination at which also the second pandemic vaccine dose was given (T2), 6 weeks after the first vaccination (T3), 26 weeks after the first vaccination (T4), and if participants continued with the study during the 2010–2011 season, also 52 weeks after the first vaccination (T5) (**Figure 1**). Self-reported vaccine history (2006–2009) was extracted from the questionnaires. If study subjects received seasonal trivalent vaccination in 2009–2010 (Solvay, the Netherlands), it took place at least 3 weeks prior to the study or at the end of visit at time point 3 of the study. In season 2010–2011, individuals received the seasonal trivalent subunit vaccine Influvac 2010–2011, containing the influenza A vaccine strains A/California/7/2009(H1N1pdm09) and A/Perth/16/2009(H3N2) (Solvay, the Netherlands). Blood was collected before vaccination (T1), 3 weeks after vaccination (T2), and 20 weeks after vaccination (T3).

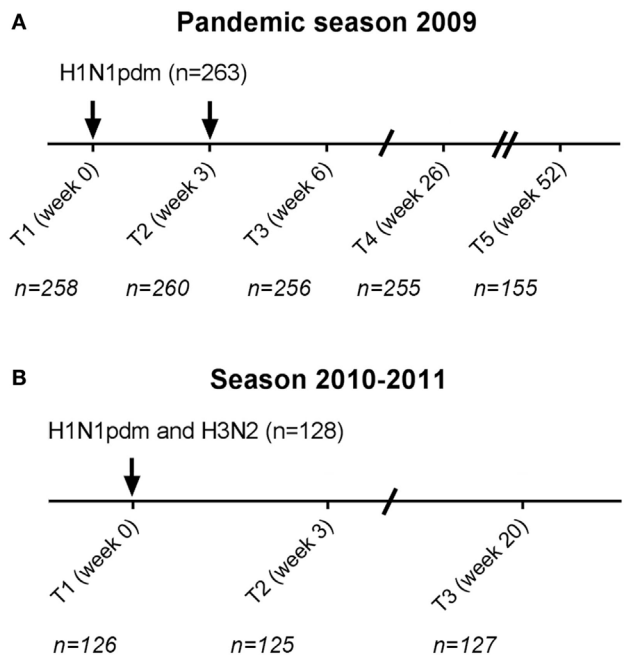

FIGURE 1 | Study schedule. Participants received in the pandemic season two monovalent influenza H1N1pdm vaccinations with a 3-week interval **(A)**. In total, 263 cytomegalovirus (CMV)-seropositive and CMV-seronegative individuals were vaccinated. 155 participants continued for the subsequent year in the study (T5 season 1). In the season 2010–2011, 128 individuals were vaccinated (T1 season 2) with the seasonal trivalent influenza vaccination which contained among others the same H1N1pdm vaccine strain and an H3N2 vaccine strain **(B)**. Arrows (↓) indicate the moment of vaccination. Time points (T) indicate the moment of blood withdrawal. For each time point, the number (*N*) of individuals with data of influenza antibody levels is indicated.

Assessment of Serum Anti-CMV Antibody Titers

Anti-CMV IgG antibody concentrations were measured using a commercial ELISA (IBL international GMBH, Hamburg, Germany) according to manufacturer's instructions. Participants with a CMV antibody level of ≥12 U/ml or higher were considered CMV-seropositive, a level of ≤8 U/ml were considered CMV-seronegative, and a level between 8 and 12 U/ml was considered equivocal and these participants were excluded for further analysis. CMV-seropositive individuals were divided into low anti-CMV levels (≤30 U/ml), medium anti-CMV levels (>30 U/ml, ≤90 U/ml), or high anti-CMV levels (>90 U/ml) according to the standards in the CMV ELISA kit.

Hemagglutination-Inhibition (HI) Assay

Hemagglutination-inhibition assays were performed in the pandemic season for A/California/7/2009(H1N1pdm09) and in season 2010–2011 for A/California/7/2009(H1N1pdm09) and A/Perth/16/2009(H3N2) to determine influenza virus-specific antibody titers before and after vaccination. Briefly, a dilution series of cholera filtrate-treated serum samples was incubated with four hemagglutinin units (HAU) of influenza virus for 20 min, 0.25% turkey erythrocytes for 45 min and scored for agglutination (39). The influenza antibody titer is the inverse of the last dilution of the serum that completely inhibited hemagglutination. A detectable influenza antibody body titer is defined as >5 HAU.

Statistical Analysis

Antibody responses to H1N1pdm influenza vaccination in the pandemic season were expressed in two different ways: (1) influenza antibody titer and (2) protection rate (antibody titer ≥40 HAU). For all statistical analyses, influenza antibody titers were log (base 2) transformed, and presented as geometric mean titer (GMT) with 95% confidence interval (CI) in the figures.

First, a two-tailed Student's *t*-test (for two groups) or one-way ANOVA (for three or more groups) was used to explore group differences in influenza antibody titers (e.g., between low, medium, and high CMV IgG groups). For the two-tailed *T*-test, equality of variances was tested with Levene's test for equality. Group differences in categorical variables were compared with the Fisher exact test.

Second, we investigated the effect of latent CMV infection in a multivariate context; the effect of CMV infection on influenza antibody titers was adjusted for potential confounders using a generalized estimation equation (GEE) regression model (Table S1 in Supplementary Material) (40). This model takes repeated measurements for the same individuals into account. For the continues variable outcome (influenza antibody titer) the normal distribution and for the categorical variable (influenza protection rate) the binomial distribution of the model

was used. The effect of CMV infection was investigated in two ways: (a) CMV-serostatus: CMV-seropositive individuals were compared to CMV-seronegative individuals and (b) anti-CMV IgG groups: low, medium, and high anti-CMV IgG levels were compared within CMV-seropositive individuals. Confounders included were age, sex, time, and various variables concerning vaccination history (see Table S1 in Supplementary Material). The model yielded a beta regression coefficient for each variable, which reflects how a category (e.g., highest age group) compares to the reference category (e.g., lowest age group). Regression coefficients of the GEE models are given in Tables S2–S7 in Supplementary Material. The model also yielded adjusted results (i.e., influenza antibody titers or protection rates) for each time point at which comparisons between CMV-serostatus or anti-CMV IgG group were performed, by including an interaction term between time and CMV-serostatus or anti-CMV IgG group in the GEE models. The adjusted outcomes of the models and pairwise comparisons are presented in the figures. These analyses were also performed for the influenza vaccine response in season 2010–2011 for H1N1pdm and H3N2. *P* values of ≤0.10 were considered a trend and of ≤0.05 were considered significant. Data were analyzed using SPSS statistics 22 for Windows (SPSS Inc., Chicago, IL, USA) and R 3.4.0 (https://www.r-project.org/).

RESULTS

Characteristics of the Study Population

In total, 288 individuals were vaccinated with the pandemic influenza vaccine in the pandemic season (**Figure 1**). CMV-serostatus was determined and 25 individuals with an equivocal CMV status were excluded from further analysis. Of the remaining 263 individuals, 171 were CMV-seropositive (65%). Groups of CMV-seropositive and CMV-seronegative individuals were comparable for sex, age, and previous influenza vaccinations (**Table 1**). In season 2010–2011, 128 of the 263 participants were vaccinated with the seasonal vaccination of which 76 (59.4%) were CMV-seropositive. Also, in the subsequent season, no differences in sex, age, and previous influenza vaccinations between CMV-seropositive and CMV-seronegative individuals were observed (**Table 1**).

Negative Effect of Age on Influenza Titers after *De Novo* Pandemic Influenza Vaccination

We investigated if there was an effect of age on the induction of antibodies to the pandemic influenza vaccination in our study population. After pandemic vaccination, H1N1pdm influenza virus-specific antibody titers were negatively correlated with age at all time points post-vaccination except T5 (see Table S8 in Supplementary Material). Representative data are depicted for T2 in **Figure 2A** (T2, $p = 0.0013$, $R = -0.198$). Individuals are divided into three age groups for further analysis by approximately 10-year intervals. Significant differences were also observed between age groups in the H1N1pdm titers (e.g., T2, $p = 0.016$), with lower responses in the oldest age group compared to the youngest two age groups (e.g., T2, 19–30 versus 40–52 year $p = 0.007$) (**Figure 2B**) (see Table S8 in Supplementary Material). Similar results were observed for the different age groups when analyzing the data by protection level, defined by reaching a titer of ≥40 HAU (data not shown). These data indicate that there are already signs of immunosenescence-driven impaired vaccine responses to a novel antigen challenge in middle-aged individuals.

No Effect of CMV-Seropositivity on Antibody Titers after Pandemic Influenza Vaccination

Next, the effect of latent CMV infection on the influenza virus-specific antibody response to the vaccine with the newly introduced H1N1pdm influenza vaccine strain was investigated. CMV-seropositive individuals were compared to CMV-seronegative individuals for influenza titers before and after vaccination. No differences between CMV-seropositive and CMV-seronegative individuals in influenza titer at any time point in both seasons were found (Figure S1A in Supplementary Material). Some individuals did already show a detectable pandemic titer before vaccination, although on average the pre-titer was very low (GMT 9.4 HAU). To correct for this and other potential confounders, influenza titers of CMV-seropositive and CMV-seronegative individuals were analyzed adjusted for pre-titer, sex, age, and previous influenza vaccinations with a GEE model (Table S2 in Supplementary Material). No significant

TABLE 1 | Characteristics of study population for pandemic season and season 2010–2011.

	Pandemic season				Season 2010–2011			
	Total (n = 263)[a]	CMV+ (n = 171)	CMV− (n = 92)	Significance	Total (n = 128)	CMV+ (n = 76)	CMV− (n = 52)	Significance
Age (mean and SD)	39.9 (7.8)	39.3 (8.6)	39.5 (8.5)	$P = 0.88$	41.3 (8.1)	41.7 (7.7)	40.63 (8.6)	$P = 0.43$
Sex (% men)	45.2%	51.1%	42.1%	$P = 0.19$	48.4%	46.1%	51.9%	$P = 0.59$
Previous influenza vaccination before pandemic season	49.0%	49.7%	47.8%	$P = 0.80$	65.6%	63.2%	69.2%	$P = 0.57$
Seasonal vaccination 2009–2010 before study	23.6%	23.4%	23.9%	$P = 1.00$	87.1%[b]	77.6%	78.8%	$P = 1.00$
Seasonal vaccination 2009–2010 during study	37.3%	38.0%	35.9%	$P = 0.79$				

Cytomegalovirus (CMV)-seropositive and CMV-seronegative group are compared with Student's t-test for age and with the Fischer exact test for categorical variables.
[a]*Time point 5, 52 weeks after pandemic influenza vaccination, blood was collected of 155 participants who continued in the study for season 2010–2011.*
[b]*Seasonal vaccination in 2009 before study or during study combined.*

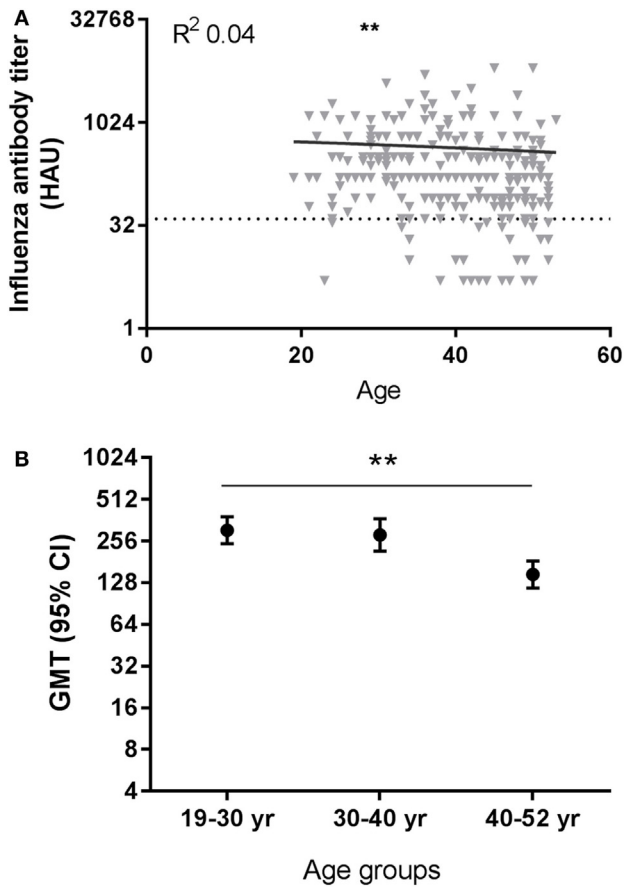

FIGURE 2 | Effect of age on influenza virus-specific antibody titers after influenza vaccination. A representative figure of influenza H1N1pdm antibody titers after pandemic vaccination plotted against age (T2) **(A)**. Dotted horizontal line represents a protective influenza titer of 40 hemagglutinin units (HAU). The geometric mean of the influenza antibody titer **(B)** after vaccination is given for different age groups after vaccination with H1N1pdm for T2 ($p = 0.016$ ANOVA). Correlations are tested with Pearson correlation. Differences between two age groups are tested with Student's *t*-test for log-transformed influenza antibody titers. $^{**}p < 0.010$. GMT, geometric mean titers.

differences were found between CMV-seropositive and CMV-seronegative individuals in antibody titers at each individual time point (**Figure 3A**). So although age shows a negative effect on the novel pandemic H1N1pdm antibody response indicative of immunosenescence to *de novo* response (**Figure 2**), no effect of CMV-serostatus on the influenza virus titer is observed after pandemic vaccination in adults (**Figure 3A**).

Higher Residual Protection Rates after Pandemic Influenza Vaccination in CMV-Seropositive Individuals than in CMV-Seronegative Individuals

Subsequently, we investigated whether there was an effect of CMV-serostatus on the protection rate, as defined by antibody titer ≥40 HAU, against influenza virus after influenza vaccination (41). Shortly after vaccination, no effect of CMV-serostatus on the protection rate was observed. However, CMV-seropositivity was associated with enhanced 6 months and 1 year protection rates after pandemic vaccination. The percentage influenza protected individuals is significantly higher for CMV-seropositive individuals than for CMV-seronegative individuals, both 26 weeks ($p = 0.047$) and 52 weeks ($p = 0.044$) after pandemic vaccination (**Figure 3B**) (unadjusted data in Figure S1B in Supplementary Material). Together, this suggests that latent CMV infection did not impair the protection rate after influenza vaccination, but if anything, might be beneficial for persistence of protection after the *de novo* influenza vaccination.

High Anti-CMV IgG Levels As Surrogate Marker of CMV Reactivation Are Not Associated with Impaired Pandemic Influenza Vaccine Response in CMV-Seropositive Individuals

To study in the CMV-seropositive individuals whether the frequency of CMV reactivation has a negative effect on the influenza antibody responses, anti-CMV IgG levels were used as a surrogate marker of CMV reactivation (25, 42) and associated with the influenza antibody response to vaccination. To this end, CMV-seropositive individuals with low anti-CMV IgG levels (≤30 U/ml), medium anti-CMV IgG levels (>30 U/ml, ≤90 U/ml) or high anti-CMV IgG levels (>90 U/ml) were compared for their influenza antibody titer and protection rate both unadjusted (Figures S1C,D in Supplementary Material) and with the GEE model (Table S3 in Supplementary Material). No differences were observed between anti-CMV IgG groups in the H1N1pdm influenza titers or protection rate after the pandemic vaccination (**Figures 3C,D**). This indicates that despite a negative effect of age on the antibody response to the pandemic vaccination (**Figure 2**), no signs of impairment by CMV reactivation were observed. Also this shows that the positive effect of CMV status on long-term protection after pandemic influenza vaccination (**Figure 3B**) could not be explained by differences in anti-CMV IgG groups within CMV-seropositive individuals.

No Effect of Age or CMV-Serostatus on Seasonal Influenza Vaccination with H1N1pdm and H3N2

The same analyses for the effect of age and latent CMV infection on influenza vaccination were performed for the 128 individuals that continued with the study and were vaccinated in season 2010–2011 with the seasonal influenza vaccination containing the same H1N1pdm strain and an H3N2 strain. A trend of a negative effect of age on the H1N1pdm memory response was observed, but no significant differences in antibody titers for H1N1pdm or H3N2 were found between age groups at any time point after vaccination in season 2010–2011 (e.g., T2, respectively, $p = 0.101$ and $p = 0.434$) (**Figure 4A**). Both the influenza antibody titer and the protection rate did not differ between CMV-seropositive and CMV-seronegative individuals (**Figures 4B–D**; Tables S4 and S6 in Supplementary Material). Surprisingly, influenza antibody titers and protection rate after the

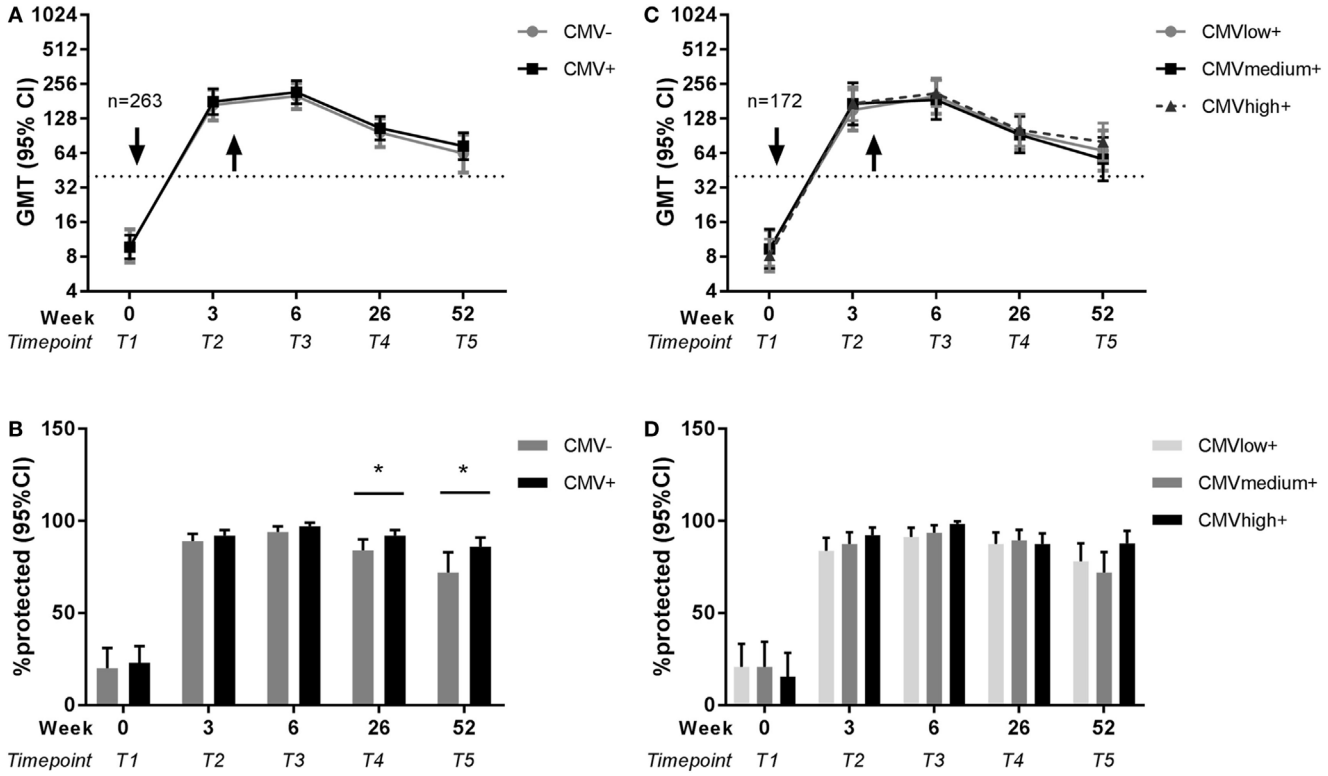

FIGURE 3 | Effect of latent cytomegalovirus (CMV) infection on influenza virus-specific antibody titer and protection rate to pandemic influenza infection. Geometric mean and 95% confidence interval (CI) of influenza antibody titers and the percentage protected (defined as a titer ≥40 HAU) are shown for CMV-seropositive and CMV-seronegative individuals **(A,B)** and for CMV-seropositive individuals with low, medium, and high anti-CMV IgG levels **(C,D)** before and after pandemic vaccination with H1N1pdm in 2009. Arrows (↓) indicate the moment of vaccination. Dotted horizontal line represents a protective influenza titer of 40. Results are adjusted for sex, age group, and previous influenza vaccinations by a generalized estimation equation (GEE) regression model. Significant differences are tested by pairwise comparison between CMV-seropositive and CMV-seronegative individuals or anti-CMV IgG group high and low per separate time point. *$p < 0.05$.

seasonal vaccination were higher for both H1N1pdm and H3N2 in the high anti-CMV IgG levels group compared to low anti-CMV IgG levels group within the CMV-seropositive individuals at most time points after vaccination (**Figures 4E–G**; Tables S5 and S7 in Supplementary Material). In summary, although no clear effect of age or CMV-serostatus, high anti-CMV IgG levels seem to be associated with high influenza antibody titers and protection rate in CMV-seropositive individuals.

DISCUSSION

In this study, we investigated the effect of age and latent CMV infection on the antibody response to a novel influenza vaccine strain in healthy adults. We found evidence of immunosenescence in these adults from the age of 40. However, latent CMV infection did not impair the antibody responses to a *de novo* influenza vaccine response. Interestingly, indications for the contrary were observed: CMV-seropositive individuals even showed a higher long-term influenza protection rate after pandemic influenza vaccination. These results suggest that latent CMV infection does not always further weaken age-related impaired immunity, but if anything, might be beneficial.

Our study showed no negative association between latent CMV infection and the antibody response to influenza vaccination. Other studies did report negative effects in adults (23, 25, 32) and older adults (25, 26, 28, 43). However, most of these studies investigated the effect of latent CMV infection on the influenza vaccine response in the presence of pre-existing immunity. In one study, all subjects were even seroprotected (influenza antibody titer >40 HAU) before influenza vaccination (26). It is known that individuals with high pre-titers show a lower increase in influenza antibody response after influenza vaccination (7, 19, 35, 44). Therefore, high pre-titers are associated with lower seroconversion (antibody titer ≥40 HAU and ≥4-fold increase) and higher protection rate (>40 HAU). Furthermore, in all but two studies (25, 43), vaccine history was not taken into account, while previous vaccination is associated with lower seroconversion independently of pre-titers (7). Not accounting for pre-existing immunity in influenza vaccine responses, therefore, may obscure findings and lead to different findings on the effect of latent CMV infection. Here, we controlled for pre-existing immunity by investigating the effect of latent CMV infection on pandemic vaccination for which pre-existing immunity was low, and by performing analysis adjusted for pre-titers and vaccine history. By doing so, we found that influenza vaccine responses are not

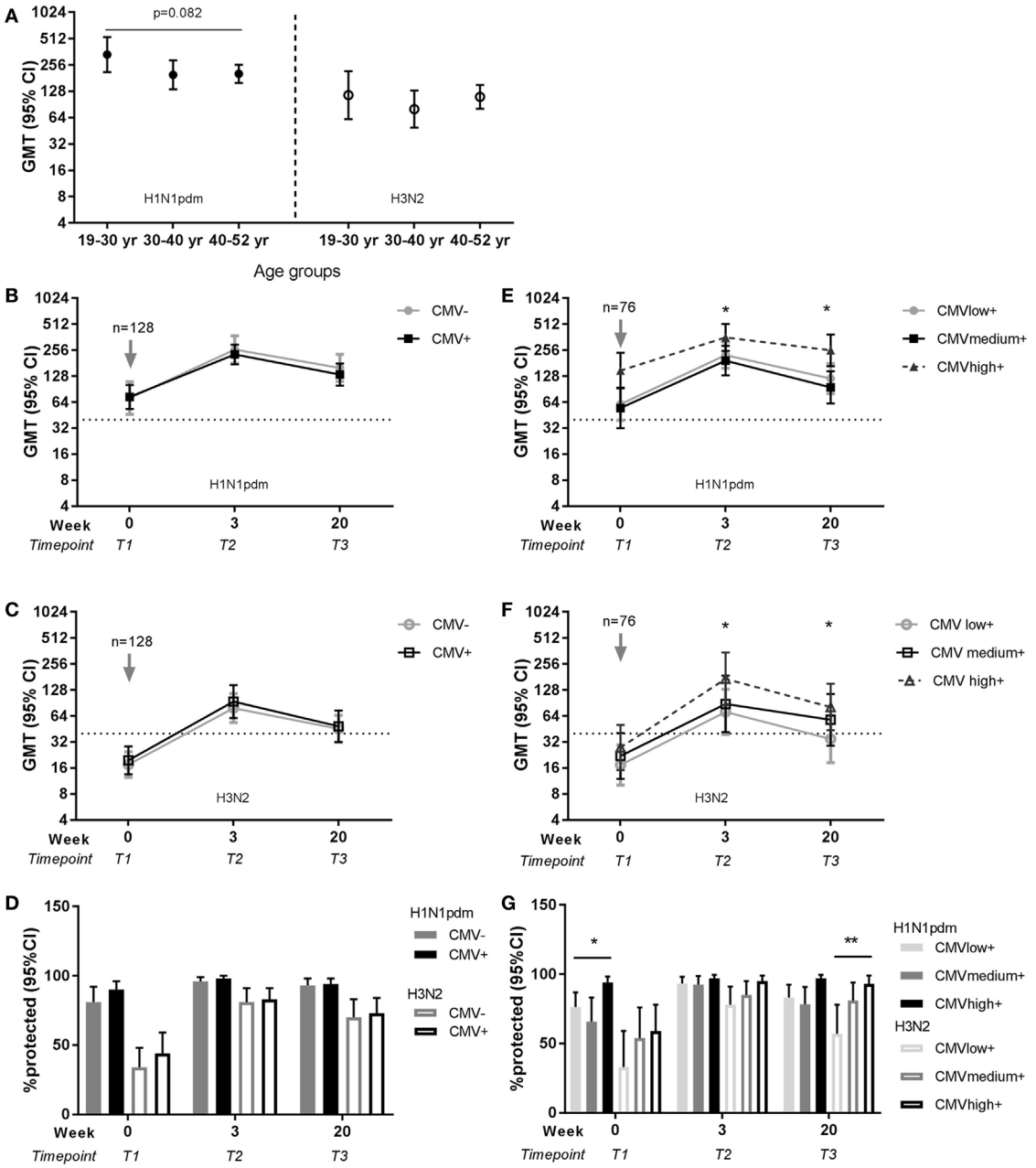

FIGURE 4 | Effect of age and latent cytomegalovirus (CMV) infection on influenza virus-specific antibody titer and protection rate to seasonal influenza infection. Geometric mean and 95% confidence interval (CI) of influenza antibody titers are shown per age group for H1N1pdm (left panel) and H3N2 (right panel) for the representative time point T2 (3 weeks) after seasonal influenza vaccination 2010–2011 **(A)**. Geometric mean and 95% CI of influenza antibody titers **(B,C)** and the percentage protected [defined as a titer ≥40 hemagglutinin units (HAU)] **(D)** are shown for CMV-seropositive and CMV-seronegative individuals for H1N1pdm and H3N2 strain before and after seasonal vaccination 2010–2011. For CMV-seropositive individuals with low, medium, and high anti-CMV IgG levels geometric mean and 95% CI of influenza antibody titers **(E,F)** and the percentage protected (defined as a titer ≥40 HAU) **(G)** are shown for H1N1pdm and H3N2 strain before and after seasonal vaccination 2010–2011. Arrows (↓) indicate the moment of vaccination. Dotted horizontal line represents a protective influenza titer of 40 HAU. Results for the effect of latent CMV infection are adjusted for sex, age group, and previous influenza vaccinations by a generalized estimation equation (GEE) regression model. Significant differences are tested by pairwise comparison between CMV-seropositive and CMV-seronegative individuals or anti-CMV IgG group high and low per separate time point. Significant differences between age groups were tested with ANOVA and differences between two age groups are tested with Student's *t*-test for (log transformed) antibody titers. *$p < 0.05$.

impaired by latent CMV infection. If anything, signs of enhanced persistence of protection after influenza vaccination were observed in CMV-seropositive individuals. We observed similar results when we analyzed the effect of latent CMV infection on the seroconversion rate. No impairment by CMV-latent infection on the vaccine response was found, but CMV-seropositive

individuals showed a higher seroconversion rate 6 months and 1 year after vaccination (T4, $p = 0.044$; T5, $p = 0.02$) (data not shown).

A beneficial effect of latent CMV infection on the immune system has been indicated (10) and is suggested to reflect higher activation status of innate cells after primary CMV infection or reactivation. Accordingly, an increased antibody titer short term after influenza vaccination in young CMV-seropositive compared to young CMV-seronegative individuals was observed (31–33) and suggested to depend on boosting by low-grade inflammation and high levels of circulating IFNγ in CMV-seropositive young individuals (31, 33). A beneficial effect of latent CMV infection on the long-term persistence of protection after vaccination in adults has to our knowledge not been reported. Waning of protection is thought to be most significant in individuals above 65 years of age (45) and accelerated by latent CMV infection (46). Our results might suggest a positive effect of CMV infection in adults on the protection rate. Thereby our data fit in a scenario in which latent CMV infection has a beneficial effect in adults and may become detrimental with aging.

Two studies that reported a short-term negative effect of latent CMV infection in adults did take the factor pre-existing immunity into account by either correcting for antibody titers pre-vaccination (24) or by investigating the effect of latent CMV infection on the novel pandemic vaccine (23). However, these studies differ from our study in terms of vaccine type and analysis of the antibody response. Turner et al. studied the fold increase of influenza antibody titers to seasonal vaccination, corrected for pre-titers before vaccination (24). They reported a negative effect on the influenza antibody fold increase in one strain of the trivalent vaccine in CMV-seropositive adults with high anti-CMV IgG levels compared to CMV-seronegative adults. Wald et al. (23) also reported a negative effect of CMV-seropositivity in adults, by investigating the same pandemic H1N1pdm vaccine response in 2009 as we did. However, they did not adjust for confounders in the analysis (23). These differences in findings of the effect of latent CMV infection on the influenza vaccine response without pre-existing immunity are unexplained. We speculate that the vaccine dose and adjuvant use may be a reason for these differences. In Turner et al., half the recommended dose was used (24). Likewise, an unadjuvanted monovalent vaccine (47) was used in Wald et al., while in our study the vaccine was adjuvanted. The use of MF59 adjuvant is expected to activate the CD4+ T cells and further enhance antibody production, thereby eliciting a stronger immune response compared to an unadjuvanted vaccine. Taken together, it may be possible that only with less potent influenza vaccines, a short-term negative effect of latent CMV infection is present.

The correlation of lower antibody response to the novel pandemic influenza vaccination with age points to an immunosenescence-driven weakened immune response. Typically, lower antibody responses to influenza vaccination are associated with high age (>60 years old). Interestingly, we observed already an effect of age in this group of non-elderly (18–52 years of age), although small. This effect of age was due to a lower influenza antibody response from the age of 40 years onward. It is suggested that differences between age groups to influenza vaccination responses might also explained by HA imprinting (48). HA imprinting implicates that the immune response is skewed to the group of HA antigens of the influenza strain that is first encountered during childhood. However, this was not the case and HA imprinting could be excluded as an explanation for the age differences.

Similar analyzes were performed for the effect of age and latent CMV on the seasonal influenza vaccine response in season 2010–2011. Seasonal vaccination in 2010–2011 contained the same H1N1pdm strain of the pandemic season and the antigen-drifted H3N2 strain that overlaps in serological response to great extent with previous H3N2 strains (49). Thus, both seasonal strains elicit an immunological memory response. Immunosenescence mainly affects the *de novo* immune responses (36, 37). In line with this, effects of age on an influenza vaccine response diminish after further vaccination with the same strain (34), explaining the different findings for the effect of age between the pandemic season and season 2010–2011. It was surprising to find that individuals with high anti-CMV IgG levels showed a higher influenza titer and protection rate to seasonal vaccination. We cannot exclude that these individuals might be high-antibody producers in general, as previously shown for respiratory syncytial virus and the response to other respiratory viruses (50). Also, the total group that continued to season 2010–2011 with the study was smaller ($n = 128$) and had a higher number of previous vaccinations than the group of the pandemic season ($n = 263$), complicating the adjusted analysis. Different results were obtained for using seroconversion rate instead of protection rate as definition of responder on the seasonal vaccination. A positive effect of high anti-CMV levels group was not observed on the seroconversion rate (data not shown). This shows the importance for correcting in our statistical model for these factors and strongly implies caution with interpretations of CMV-induced effects in small study groups or non-adjusted studies as reported in literature.

Important strengths of our study compared to others are the use of a novel influenza vaccine strain, the relatively large groups of study subjects in the pandemic season and the adjusted analysis with the GEE model. Since aging and latent CMV infection are thought to affect the immune system both independently and by interacting with each other, separation of these factors in analysis is crucial (51). A limitation of the study is that the study population consists of health care workers who received repeated previous influenza vaccinations. Individuals with repeated previous seasonal influenza vaccinations show in general higher pre-vaccination titers than first-time vaccinated individuals (44). Even in the pandemic season, cross reactivity was reported for the H1N1pdm strain (52, 53). Together with potential natural exposure to the H1N1pdm strain just before the study, this may explain the detectable titers before pandemic vaccination in this study. The seasonal 2009 vaccination 3 weeks before the study in the pandemic season indeed increased the pandemic pre-titer (data not shown). However, vaccine history of the past years preceding the vaccine trial of the study subjects was reported and was adjusted for in the analysis. Importantly, pre-titers did not affect the study results, since individuals in our study without detectable pre-titers

($n = 203$) for pandemic influenza vaccination showed comparable results for the effect of CMV infection for the pandemic season (data not shown).

The influenza response in humans is complex and raises the question if influenza vaccination is the best model to investigate the effect of latent CMV infection on vaccine responses A less complicated model, in which a vaccine for people that are truly naïve is used, might be a better study design for this question. However, we consider that influenza vaccination represents the most relevant because of its high societal importance. Therefore, knowledge on the effect of CMV infection on the influenza antibody response is of great importance.

In conclusion, we used a novel influenza vaccine strain to investigate the effect of age and latent CMV infection on the *de novo* immune response to influenza. We found indeed already impaired antibody responses to vaccination in adults with increasing age, but latent CMV infection did not impair the influenza virus-specific antibody response. Thereby, we show that CMV infection does not *per se* enhance the age-related impaired immunity as assumed, but if anything might give opposite effects. A model in which CMV infection boosts the immune system during adulthood, while in older adults CMV infection enhances the aging of the immune system, might be appropriate. These results are important in the decision to invest in preventing latent CMV infection in healthy individuals through strategies such as CMV vaccination.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Good Clinical Practice 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 Central Committee on Research Involving Human Subjects of the Netherlands.

AUTHOR CONTRIBUTIONS

SB, DB, and JB conceptualized the study. SB, MH, and RJ executed the laboratory experiments. SB and AW performed the statistical analysis. SB, AW, DB, and JB interpreted the data and wrote the manuscript. All authors critically revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00082/full#supplementary-material.

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Parallels in Immunometabolic Adipose Tissue Dysfunction with Ageing and Obesity

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Ageing, like obesity, is often associated with alterations in metabolic and inflammatory processes resulting in morbidity from diseases characterised by poor metabolic control, insulin insensitivity, and inflammation. Ageing populations also exhibit a decline in immune competence referred to as immunosenescence, which contributes to, or might be driven by chronic, low-grade inflammation termed "inflammageing". In recent years, animal and human studies have started to uncover a role for immune cells within the stromal fraction of adipose tissue in driving the health complications that come with obesity, but relatively little work has been conducted in the context of immunometabolic adipose function in ageing. It is now clear that aberrant immune function within adipose tissue in obesity—including an accumulation of pro-inflammatory immune cell populations—plays a major role in the development of systemic chronic, low-grade inflammation, and limiting the function of adipocytes leading to an impaired fat handling capacity. As a consequence, these changes increase the chance of multiorgan dysfunction and disease onset. Considering the important role of the immune system in obesity-associated metabolic and inflammatory diseases, it is critically important to further understand the interplay between immunological processes and adipose tissue function, establishing whether this interaction contributes to age-associated immunometabolic dysfunction and inflammation. Therefore, the aim of this article is to summarise how the interaction between adipose tissue and the immune system changes with ageing, likely contributing to the age-associated increase in inflammatory activity and loss of metabolic control. To understand the potential mechanisms involved, parallels will be drawn to the current knowledge derived from investigations in obesity. We also highlight gaps in research and propose potential future directions based on the current evidence.

Keywords: adipose, ageing, immunometabolism, obesity, inflammageing, immunosenescence

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INTRODUCTION

Metabolically driven inflammation is a hallmark of cardiovascular disease and type-II diabetes mellitus (1). Several organs including the liver, the skeletal muscle, and the gut have a role in the generation and progression of these diseases. However, it has become clear that adipose tissue accumulation, sometimes within and around these organs, is a major driving force for metabolic and inflammatory dysfunction (1–3). Obesity, defined herein as a body mass index (BMI; $\geq$30 kg/m^2), leads to extensive adipose tissue deposition and negative health consequences, due to chronic caloric overconsumption and an excessively sedentary lifestyle (4, 5). Globally, over 2.1 billion adults were overweight (BMI $\geq$ 25 to <30 kg/m^2) or obese in 2014 (6). This number has been steadily increasing

with global age-standardised mean BMI scores increasing by 0.4 and 0.5 kg/m^2 each decade in men and women, respectively (7).

Obesity is characterised by poor metabolic control, oxidative stress, mitochondrial dysfunction, impaired immune function, and chronic, low-grade inflammation (8–13). In particular, it is well established that adipose tissue in obesity is a potent contributor to chronic, low-grade systemic inflammation, which can result in multiorgan dysfunction. One of the main factors associated with obesity-induced adipose tissue inflammation is dysregulation of the immune cell populations within the tissue stromal fraction, which contribute to the propagation of a pro-inflammatory microenvironment that spills over into the circulation and other organs (1, 14).

In contrast to obesity-centric research, the role of immunometabolic adipose tissue dysfunction in human ageing has been largely unexplored. In older people, many biological systems and processes become dysregulated (15). Indeed, age-associated immunological dysregulation potently modulates systemic inflammation, multiorgan dysfunction, and longevity (16–18). Moreover, molecular modulation of adipose tissue inflammatory and metabolic processes influences longevity in animal models, suggesting that adipose tissue may play an important role in the ageing process (19, 20). Further, in humans, an age-associated redistribution of adiposity towards the abdominal cavity occurs independently of obesity (21, 22). However, the role of adipose-resident immune cells and the interplay between metabolic and inflammatory processes in ageing adipose tissue is poorly characterised.

The aim of this review is to summarise the immunological alterations that take place in adipose tissue with obesity and ageing, with the aim to improve understanding of the role for adipose tissue immunometabolic dysfunction in age- and obesity-associated disease. This review first evaluates evidence showing obesity-specific immunological alterations in adipose tissue, which will then be used as a basis for understanding the potential role of adipose tissue immunological alterations in the pathophysiology of age-associated diseases.

THE IMMUNE SYSTEM AND INFLAMMATION

The immune system protects the host against viruses, bacteria, fungi, parasites, and tumours through a complex integration of innate and adaptive defences. The innate immune system provides a first line of defence, comprising physical barriers such as the skin and chemical barriers on surfaces exposed to the environment, preventing pathogens entering the body. In addition, the innate immune system has cellular defences, including monocytes, macrophages, natural killer (NK) cells, mast cells, neutrophils, eosinophils, basophils, and dendritic cells. Some cells possess characteristics of both the innate and adaptive immune system. For example, the small population of innate lymphocytes that are cluster of differentiation (CD) 1d restricted referred to as invariant natural killer T cell (iNKT) and other T-cells that express NK cell-associated surface proteins (NKT-like cells). Professional antigen-presenting cells link the two arms of the immune system. For example, dendritic cells act as tissue sentinels and, upon antigen encounter, travel from peripheral tissues (e.g., places in contact with the external environment; like the digestive system and dermis) to the lymph nodes where they activate cells of the adaptive immune system: B-cells and T-cells (23, 24). T-cells comprise 60–75% of all lymphocytes and originate from the bone marrow, maturing in the thymus, and circulate in blood as either antigen-naive or antigen-experienced cells (25). B-cells comprise 5–15% of all lymphocytes and elicit their effector functions, including their memory response, *via* soluble immunoglobulins (Igs), which can neutralise toxins or flag pathogens and target cells for elimination by other cells of the immune system such as macrophages and NK-cells (24).

In response to injury or infection, a local immune response is initiated, characterised by swelling, heat, and pain. One of the first local changes is an increase in blood flow facilitating an influx of acute-phase reactants, such as C-reactive protein, and an accumulation of innate and then adaptive immune cells for pathogen elimination and tissue repair. However, alterations to the tissue microenvironment and local stimuli can result in uncontrolled inflammation. Such alterations to the anti-inflammatory or pro-inflammatory milieu can disrupt systemic homeostasis and metabolic demand, perpetuating the inflammatory response that has profound health implications. A degree of inflammation within adipose tissue is central to tissue remodelling, as many of the cells, cytokines, and pro-oxidants produced at normal levels, regulate tissue homeostasis (26). However, prolongation of this normally transient and well-controlled process drives chronic, low-grade systemic inflammation that is central to the impaired health with obesity and ageing.

ADIPOSE TISSUE INFLAMMATION AND METABOLIC DISEASE

Impairments in adipose tissue function associated with structural and functional changes to the tissue results in the propagation of abnormal and often pro-inflammatory secretory profiles from adipocytes and cells of the stromal fraction. This association was first understood when murine obesity was linked with increased production of the inflammatory, insulin desensitising cytokine: tumour necrosis factor-α (TNF-α) (27). In the context of obesity, adipose tissue dysfunction is promoted by a chronic positive energy imbalance. Similar metabolic impairments are also observed in other conditions characterised by adipose tissue dysfunction, including ageing and lipodystrophy. Consequently, the similarities between these conditions allow for comparisons to be made to better understand the processes involved (28–30).

To date, a variety of stimuli for immunometabolic deterioration within adipose tissue have been proposed. These include increased gut-derived antigens (e.g., lipopolysaccharide), stimulation of immune cells by dietary or endogenously derived lipids, adipocyte hypertrophy—leading to apoptosis, necrosis, fibrosis, and hypoxia—and adipocyte dysfunction from mechanical stress (31). Collectively, these alterations impact various aspects of adipose tissue function, including changes to local blood flow, which impairs the endocrine potential of the tissue; changes to the extracellular matrix, which instigates monocyte infiltration to manage tissue remodelling; and adoption of a pro-inflammatory and

pro-oxidative microenvironment, which act to recruit immune cells driving their pro-inflammatory polarisation (32–35). Moreover, the dysfunction of preadipocytes (adipocyte stem cell precursors) induced by a pro-inflammatory and pro-oxidative microenvironment inhibits the healthy turnover of adipose tissue, potentiated by, and impacting upon, impaired endothelial function, which exacerbates local hypoxia (34–36). The net result of these disturbances is the aberrant secretion of adipokines, which, *via* paracrine and endocrine means, impact appetite, bone health, metabolic health, and systemic inflammation through the activation of pro-inflammatory signal cascades [i.e., nuclear factor κB (NFκB), NLR family pyrin domain containing 3 (NLRP-3), and *c*-Jun *N*-terminal kinases (JNKs) signalling] (1, 37). Adipose tissue immunometabolic dysfunction also impacts the ability of adipocytes to buffer lipids. This dysfunction leads to the shunting of lipids towards non-adipose tissues, including the liver, the skeletal muscle, and the heart, promoting tissue-specific insulin resistance and inflammation, triggering β-cell, and metabolic dysfunction (1, 38, 39).

METHODOLOGICAL CONSIDERATIONS IN ADIPOSE TISSUE IMMUNOLOGICAL RESEARCH

Adipose tissue is an immunometabolically active organ in a constant state of flux (40). Therefore, assessment of adipose tissue function is a difficult task, and the nature of sampling tissue means the available data tend to represent a snap-shot of adipose physiology and function. In the context of the current review, this means that the assessment of adipose cellular composition at a given point in time will not necessarily provide a full and complete picture of the events that have led to the characterised phenotype. In human research, adipose tissue samples are often collected under fasting conditions, and very little information is available in the post-prandial state where much of the day is spent, which potently alters adipose tissue immunometabolic status (41). Furthermore, in human studies, the adipose tissue sampling methodology (e.g., surgical versus aspiration biopsies) may influence some outcomes (42). In mice, both the breed and inconsistency in whether animals are fasted or fed has the potential to influence their metabolic profile (43). Some investigations have adopted arteriovenous difference blood sampling techniques to sample the blood flowing across the subcutaneous adipose depots in an attempt to understand the dynamic behaviour of the tissue (44). This methodology allows for a greater understanding of how adipose tissue responds, in real-time, potentially giving a more complete picture of adipose tissue functionality.

Adipose tissue is distributed throughout the body with site-specific physiological properties. In humans, abdominal subcutaneous and visceral adipose tissue differ substantially in function and cellularity, and considerable depot differences are also apparent between gluteofemoral and abdominal adipose tissues, which is particularly relevant when comparing males and females due to sex-specific adipose tissue distribution (45, 46). In mice, different adipose tissue depots also possess unique physiological properties both between and within specific depots. Although beyond the scope of this review, differences include adipocyte size and expandability in response to high-fat overfeeding, control of lipolysis, and fatty acid and leukocyte composition (47, 48). Most investigations of adipose tissue immunology have been conducted with animal models, with considerably less work with humans. Although differences in adipose tissue biology between humans and animals is beyond the scope of this review, it is important to recognise that human adipose tissue is, indeed, very different to that of animals such as mice in terms of functionality, physiology, distribution, and make-up (i.e., quantities of brown and white adipose tissue, respectively) (49).

To assess the constituents of the adipose tissue stromal fraction, many approaches have been employed, including immunohistochemistry, analysis of gene expression and protein level, and flow cytometry. Each method has benefits and drawbacks, which are summarised in **Table 1**. For example, it has been shown that immunohistochemistry yields reproducible results, but with flow cytometry, the recovery of macrophages after tissue digestion can be a possible source of variation (50). However, there is good agreement between flow cytometric analysis of adipose tissue cellular composition and the expression mRNA for key cell surface markers across a range of adiposities in humans (**Figure 1**) (35). Moreover, differences between studies in the selection of cell markers, coupled with lack of consensus over cell identification strategies, make direct comparison between studies complex and could feasibly explain conflicting results.

The mode of reporting assessments of cellular composition also requires consideration. Reporting proportional representation of cell types within the stromal fraction versus absolute cell counts can produce different findings. For instance, a proportional decline in certain populations in relation to total stromal cell yields may be a consequence of an expansion of another cell population within the tissue instead of an absolute decline in specific cell numbers. For example, one report showed that, compared to older mice, young mice exhibited around twice as many macrophages in adipose tissue when expressed as a percentage of the total stromal yield. However, these age-related differences were not seen when data were expressed per gram of adipose tissue (51). In summary, when interpreting the results of different studies, it must be considered whether the laboratory methods and data presentation/expression could in-part explain discrepant results, especially when interpreting cell counts and/or proportions within adipose tissue.

TRADITIONAL CONCEPTS IN ADIPOSE TISSUE IMMUNOLOGY: AN INFLAMMATORY ROLE FOR MONOCYTES AND MACROPHAGES

Macrophages were first discovered within adipose tissue forming multinucleated crown-like structures of ~15 cells clustering around dead or dying adipocytes in obese C57/BL6J mice (52). In young obese mice and humans, these structures have been shown to comprise 90% of all adipose tissue macrophages (53). Macrophages are the main leukocyte in the adipose stromal fraction of young, non-obese mice and humans, comprising

TABLE 1 | Appraisal of three commonly utilised methods of assessing the cells of the adipose tissue stromal fraction.

Method	Benefits	Drawbacks
Immunohistochemistry	– Small amount of tissue required – Information on cell locality – Intact undisturbed tissue sample – Long-term storage – Some sample remains available for future analysis	– Manual counting is subject to investigator bias – Time consuming for manual counting – Limited to 2 or 3 antibodies per tissue slice
Flow cytometry	– Commonly 10–12 antibodies can be used in parallel – Thousands of cells analysed from one sample – Absolute cell counts can be determined – Isotype control and fluorescence minus one tubes can be added to an experiment to minimise the influence of subjectivity when gating populations	– Extensive processing required to extract the stromal fraction possibly leading to artefacts – Possibility for selective cell type loss with processing – Cell autofluorescence complicates gating of some populations (i.e., macrophages) – Large (>300 mg) tissue sample required – Small margin for bias when gating cell populations
Gene and protein expression (RT-PCR and Western blot)	– Very little tissue required – Little processing of the tissue required prior to freezing – Long-term storage – Some sample remains available for future analysis	– Requires isolation of a specific marker not expressed on any other cell type – Cannot be multiplexed by examining multiple markers on cell types

RT-PCR, reverse transcription polymerase chain reaction.

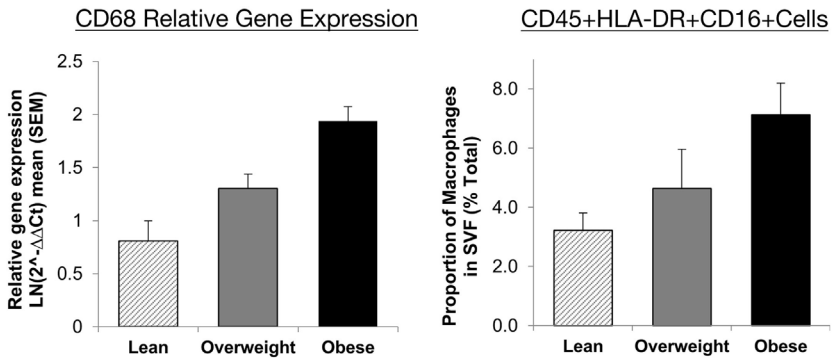

FIGURE 1 | Agreement between RT-PCR and flow cytometric assessment of macrophage abundance in lean, overweight, and obese individuals subcutaneous abdominal adipose tissue. Relative gene expression of CD68 was used to identify macrophages (n = 30) and presented as mean $2^{-\Delta\Delta Ct}$ ± SEM. Proportions of macrophages in the adipose tissue SVF as a percentage of total cells (n = 17). Macrophages were identified as CD45+HLA-DR+CD16+ cells. Presented with permission from Travers et al. (35). Abbreviations: CD, cluster of differentiation; HLA-DR, human leukocyte antigen-antigen D related; RT-PCR, reverse transcription polymerase chain reaction; SVF, stromal vascular fraction.

4–15% of total stromal cell count (54, 55). Macrophage content increases with adiposity, and the accumulation is greatest in visceral depots in humans (55, 56). Adipose tissue macrophages are derived from monocytes, which differentiate in response to growth factors, including macrophage colony-stimulating factor and granulocyte–macrophage colony-stimulating factor (57, 58). Monocytes, defined by their cell surface expression of CD14 and CD16 (see **Table 2**), circulate in peripheral blood until they migrate into adipose tissue primarily in response to chemokine (C-C motif) ligand-2 (CCL-2)—also known as monocyte chemoattractant protein 1 (MCP-1)—released from adipose tissue (57, 59). MCP-1 promotes the local proliferation of adipose-resident macrophages, contributing to the large accumulation of these cells with obesity (60). Monocytes also enter the adipose microcirculation in response to adipocyte-derived cell-stress markers, including CCL-5, also known as regulated on activation, normal T-cell expressed and secreted (RANTES), interleukin (IL)-6, interferon-γ (IFN-γ), and TNF-α (61). Production of these signals, which enhances macrophage accumulation, also polarises macrophages into highly inflammatory, classically activated cells, referred to herein as M1-like macrophages to be consistent with the reporting of data in the studies we summarise. However, we acknowledge that the M1 and M2 classification of macrophages is simplistic and becoming outdated[1] (see **Table 2**).

[1]Traditionally, macrophages have been categorised as M1 (classically activated, often thought to be pro-inflammatory) and M2 (alternatively activated, often thought to be anti-inflammatory). It is now understood that this is an oversimplification of macrophage phenotype. However, much of the available research in adipose tissue, especially in mice, has maintained the M1 and M2 categorisation. In this article, for consistency and ease of comparing studies, the terms "M1-like" and "M2-like" will be used. Within adipose tissue, it is now accepted that tissue-resident macrophages have a tissue-remodelling and pro-inflammatory phenotype, representing an intermediate population in mice and humans (i.e., cells that display an M2-like surface expression profile while demonstrating an M1-like secretory profile and function) (33, 63, 79, 170, 246, 247, 248, 249). Therefore, the role of macrophages in expanded and/or dysfunctional adipose tissue is likely to be far more complex than originally envisaged.

TABLE 2 | Adipose tissue macrophage phenotypic classifications in humans.

Type	Cell surface expression profile	Secretion profile	Function
M1-like *"classically activated"*	CD14+CD16++ CD11c+CD31+CD44+CD45+CD206−HLA-DR+	TNF-α, IL-6, IL-8, IL-1β, NO	Pro-inflammatory, pro-oxidative, antimicrobial, pathogen clearance
Intermediate population	CD14+CD16+ CD11c+CD45+CD206+	TNF-α, IL-6, IL-8	Pro-inflammatory and pro-oxidative, tissue remodelling
M2-like *"alternatively activated"*	CD14++ CD16+ CD45+CD163+CD206+	IL-10, TGF-β	Anti-inflammatory, fibrosis, tissue surveillance, angiogenesis

Text in italics and quotes emphasises other common terms to describe these cells. Intermediate population refers to adipose tissue macrophages expressing M2-like surface markers (e.g., CD206), while demonstrating an M1-like pro-inflammatory function. Expression pattern of CD14 and CD16 is best established on monocytes rather than macrophages. It is important to note that macrophage populations within adipose tissue can express markers of alternatively activated cells (e.g., CD206+) while also imparting a pro-inflammatory, classically activated function within the tissue, highlighting the spectrum of macrophage polarity within adipose tissue.
CD, cluster of differentiation; HLA-DR, human leukocyte antigen-antigen D related; IL, interleukin; NO, nitric oxide; TGF-β, transforming growth factor-beta; TNF-α, tumour necrosis factor-α.

It has been consistently shown that there is a positive relationship between the number of macrophages in adipose tissue and the degree of whole-body insulin resistance in mice and humans (35, 62, 63). Moreover, TNF-α—which is primarily produced by macrophages in adipose tissue—is a potent inhibitor of insulin signalling and an activator of NFκB signalling (27, 64). In obese and inflamed adipose tissue, NFκB is activated by the fatty acid chaperone—Fetuin-A—interacting with the lipid-sensing toll-like receptor 4 (TLR-4) on the macrophage cell surface (65–67). In the commonly studied 3T3-L1 adipocyte cell line, TNF-α has been shown to directly impact insulin signalling by inactivating insulin receptor substrate 1 (IRS-1) through p44/42 mitogen-activated protein kinase (MAPK) activation (68). Chronic activation of pro-inflammatory stress sensors and signalling pathways: p44/42, JNK, p38, and MAPK by TNF-α derived from subcutaneous adipose-resident macrophages indirectly stimulates lipolysis in adipocytes, impairing lipid handling capacity (69–71). It has also been observed, *in vitro*, that monocytes incubated with palmitate—an abundant saturated fatty acid—exhibit upregulated IL-6 and TNF-α mRNA (72). Further, upon macrophage–adipocyte co-culture, there is a substantial increase in macrophage-derived TNF-α and IL-1β (65). As TNF-α, IL-6, and IL-1β stimulate a feed-forward activation of NFκB and JNK, this leads to IRS-1 phosphorylation at serine residues rather than tyrosine, perpetuating insulin signalling impairments (73). Another factor influencing macrophage function in adipose tissue is hypoxia brought about by adipocyte expansion, without concurrent angiogenesis, impairing oxygen supply (74, 75). Hypoxia potentiates the palmitate-induced pro-inflammatory and pro-oxidative polarisation of human macrophages, while hypoxia-inducible factor-1α (HIF-1α) can directly induce pro-inflammatory M1-like macrophage polarisation and pro-inflammatory cytokine productions (76, 77). Macrophage-derived reactive oxygen species produced as a result of hypoxia may also impair lipid and glucose metabolic control through the S-nitrosylation of the peroxisome proliferator-activated receptor-γ (PPAR-γ) nuclear receptor (78).

Macrophages are considered central regulators of fibrosis and may also increase progenitor cell deposition into the extracellular matrix as a means of protecting hypertrophic adipocytes from rupturing (59). M2-like macrophages possess a pro-fibrotic potential and are, therefore, recruited into the adipose tissue in response to "danger signals" (i.e., cytokines, acute-phase proteins, and stress signal cascades) released from hypertrophic adipocytes (79). However, the exposure of macrophages to hormones, cytokines, chemokines, and fatty acids found within dysfunctional adipose tissue encourages them to adopt a pro-inflammatory phenotype potentiating metabolic deteriorations and inflammation (80, 81). In human obesity, perhaps counter intuitively based on the simple macrophage phenotyping, it is the M2-like, and not M1-like, macrophages, with a tumour-associated genetic signature and remodelling phenotype that correlates with obesity in subcutaneous adipose tissue (33). Another stimulus that might influence macrophage infiltration into adipose tissue is endoplasmic reticulum stress, which promotes a pro-inflammatory microenvironment through the initiation of the unfolded protein response. In mice, increased fatty acid-mediated oxidative stress upregulates pro-inflammatory cytokine expression, which, in humans, is also initiated by elevated TNF-α and IL-1β (82, 83). Endoplasmic reticulum stress responses involving the inositol-requiring enzyme 1α and CCAAT-enhancer-binding protein homologous protein direct pro-inflammatory, M1-like macrophage polarisation in adipose tissue (84, 85). Finally, it is unknown whether macrophages initiate inflammatory responses in adipose tissue or whether other cells present stimulate macrophage accumulation and dysfunction. For example, some studies in mice indicate that macrophages infiltrate adipose tissue in the absence of other leukocytes, whereas other studies show that different leukocyte subsets are central to macrophage accumulation (86).

Recent evidence has indicated that a unique adipose-resident macrophage population exists. In mice fed a high-fat diet, a population of sympathetic neuron-associated macrophages accumulate within visceral adipose tissue. This macrophage population regulates adipocyte exposure to norepinephrine by sequestering and degrading norepinephrine released into the adipose tissue interstitium. This process is brought about by the selective expression of the norepinephrine transporter, *SLC6A2*, and genes controlling norepinephrine-degradation such as monoamine oxidase-A by these macrophages (87). However, unlike other adipose-resident macrophage populations, these sympathetic neuron-associated macrophages do not increase within obese adipose tissue *via* proliferative mechanisms, but instead appear to infiltrate the tissue selectively (87). Given that catecholamines increase lipolytic rate in adipocytes *via* adrenergic receptors triggering the downstream hydrolysis of triglycerides, selective

knockout of these sympathetic neuron-associated macrophages protects against high-fat diet-induced obesity, in mice. Moreover, the capacity to buffer regional norepinephrine releases, which in healthy adipose tissue may act as a protective mechanism to avoid the dangerous effects of chronic exposure to norepinephrine, is also sufficient to modulate overall sympathetic tone in murine adipose tissue, influencing whole-body metabolism (88).

In summary, a number of factors including adipose tissue cellular composition and secretions plus broader anatomical and physiological characteristics of the tissue have been found to modulate metabolic and inflammatory processes and influence macrophage phenotype and function. However, due to adipose sampling in humans being a "snap-shot" of the dynamic changes that the tissue undergoes with obesity, further work is required to fully understand these interactions. A summary of the likely interactions between adipocytes and adipose-resident macrophages with obesity is presented in **Figure 2**.

A BROADER PERSPECTIVE ON ADIPOSE TISSUE IMMUNOLOGY

Adipose tissue dysfunction is the result of a complex interaction between all cell types within the tissue. Understanding this interplay has been the subject of intensive investigation, which will first be discussed in the context of obesity as a foundation for understanding the function of adipose tissue with ageing. The following sections will summarise research examining different immune cell populations in adipose tissue in the context of obesity, considering cells of the weight of evidence (most to least) supporting their role within dysfunctional adipose tissue.

Neutrophils in Obesity-Associated Adipose Tissue Dysfunction

In mice fed a high-fat diet, the accumulation of neutrophils (identified in mice as Ly6g+CD11b+) occurs in visceral, but not subcutaneous, adipose tissue after 3 days (89). Neutrophils

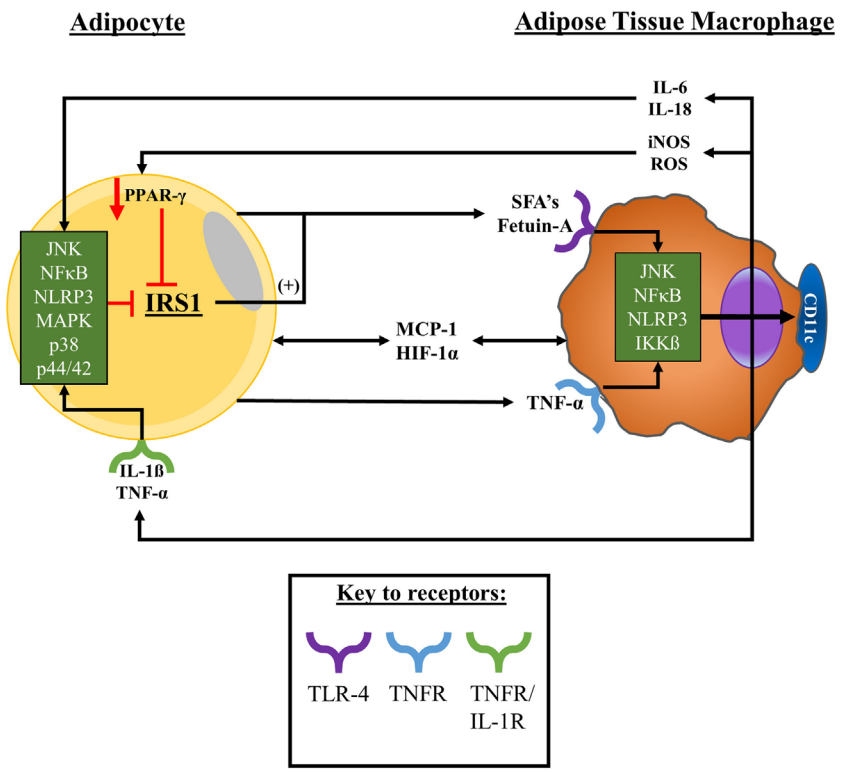

FIGURE 2 | Adipocyte–macrophage interactions with obesity. With obesity, adipocytes expand promoting a state of proinflammation, ER stress, and tissue hypoxia, promoting adipocyte apoptosis/necrosis. Upon these changes in obese adipose tissue macrophages are signalled into the tissue by hypoxic, chemokine, and cytokine signals to surround dysfunctional adipocytes. Macrophages in obese adipose tissue release, and are exposed to, TNF-α and MCP-1—among other factors, including SFAs—which collectively promote the activation of a variety of stress signalling cascades. Consequently, adipose-resident macrophages adopt a pro-inflammatory secretory profile and upregulate their surface expression of CD11c. As a result, adipocytes become exposed to secreted factors such as TNF-α and MCP-1, exacerbating the pro-inflammatory microenvironment of the tissue and potentiating the activation of stress signal cascades within adipocytes. These interactions promote a feed-forward recruitment of monocytes into the tissue and their pro-inflammatory polarisation as well as impairing tissue insulin sensitivity that potentially promotes the release of FFAs into the microenvironment. Abbreviations: CD, cluster of differentiation; ER, endoplasmic reticulum; FFAs, free-fatty acids; HIF-1α, hypoxia-inducible factor-1α; IKKβ, inhibitor of nuclear factor kappa B kinase subunit beta; IL, interleukin; IL-1R, interleukin-1 receptor; iNOS, inducible nitric oxide synthase; IRS-1, insulin receptor substrate 1; JNK, c-Jun *N*-terminal kinases; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein 1; NFκB, nuclear factor kappa B; NLRP-3, NLR family pyrin domain containing 3; PPAR-γ, peroxisome proliferator-activated receptor gamma; ROS, reactive oxygen species; SFAs, saturated fatty acids; TNFR, tumour necrosis factor receptor; TNF-α, tumour necrosis factor-α.

are 20-fold more abundant in adipose tissue from mice fed a high-fat diet compared to chow-fed mice, accounting for ~2% of total stromal cells (89–91). The accumulation of neutrophils in adipose tissue in obese mice leads to elevated elastase production (89). *In vitro* experiments have shown that increased exposure to elastase can contribute to IRS-1 downregulation, resulting in impaired glucose tolerance and insulin resistance (92). Further, systemic and intraperitoneal glucose tolerance has been shown to be impaired in mice injected with elastase compared to those treated with an elastase inhibitor. Neutrophil-derived elastase also imparts pro-inflammatory actions on murine intraperitoneal macrophages *via* TLR-4 signalling, upregulating *TNF-α, IL-1β,* and *IL-6* gene expression, potentially resulting in a feed-forward recruitment of neutrophils and M1-like macrophages into adipose tissue (89).

In humans, immunohistochemical analyses (CD66b+ staining) have shown neutrophils to accumulate in the microvasculature of adipose tissue with increasing adiposity, correlating with elevated NFκB and cyclooxygenase-2 (COX-2) staining (93). Further, it has also been suggested that elevated chemokine (C-X-C motif) ligand (CXCL)-2 release from obese adipose tissue may promote neutrophil infiltration (94). However, in humans at least, several questions remain unanswered. For example, whether neutrophil accumulation is an early step in adipose tissue dysfunction and to what extent neutrophils modulate ongoing inflammatory responses with adipose tissue.

NK-Cells in Obesity-Associated Adipose Tissue Dysfunction

Comprehensive investigations in mice have suggested that a population of NK-cells, uniquely expressing the IL-6 receptor alpha and colony-stimulating factor 1 receptor (IL6Ra+Csf1r+), induced *via* IL-6/STAT-3 (signal transducer and activator of transcription-3) signalling, accumulate with obesity within visceral adipose tissue (95). Ablation of IL6Ra+Csf1r+ NK-cells improves insulin and glucose control and reduces overall adiposity, adipocyte size, macrophage infiltration, and macrophage crown-like structures (95). It has been suggested that IL-15 and aberrant leptin secretion with obesity might instigate an adipose-specific NK-cell expansion and activation in response to high-fat diet overfeeding, stimulating NK-cell IFN-γ, TNF-α, and MCP-1 production, negatively impacting whole-body insulin sensitivity (96–98).

In humans with obesity and type-II diabetes, activated NK-cells accumulate in visceral and subcutaneous abdominal adipose tissue, producing IFN-γ and TNF-α (99–101). Moreover, the increase in IL6Ra+ NK-cells in peripheral blood of people with obesity suggests that obese dysfunctional adipose tissue may cause a pro-inflammatory NK-cell population expansion that spills out into the circulation (95). It has been proposed that adipose-resident macrophages produce CCL-3, CCL-4, and CXCL-10, stimulating the recruitment of NK-cells into adipose tissue, whereupon macrophage-derived IL-15 promotes NK-cell proliferation and activation (102). Once within adipose tissue, NK-cell modulation of MCP-1 expression could potentiate macrophage recruitment and proliferation (60), and finally, through an upregulation of NK-cell-derived TNF-α and IFN-γ, NK-cells may guide M1-like macrophage polarisation. Therefore, NK-cells may be pivotal in the generation of obesity-associated immunometabolic adipose tissue dysfunction.

B-Cells in Obesity-Associated Adipose Tissue Dysfunction

In lean murine visceral adipose tissue, B-cells constitute 10% of the stromal fraction, increasing to 20% in response to high-fat overfeeding, and this accumulation has been reported to occur before macrophage infiltration (103). B-cell localisation around macrophage clusters may be central to their functional relevance within adipose tissue in obesity, suggesting a role in steering macrophage functionality, perhaps *via* LTB4/leukotriene-B4 receptor 1 (LTB4R1) signalling. LTB4/LTB4R1 signalling promotes leukocyte infiltration into tissues, influences cytokine production, and is increased in obese murine visceral adipose tissue B-cells (103). In support, B-cells promote visceral adipose tissue macrophage recruitment and TNF-α production, *in vivo* and *in vitro,* in mice fed a high-fat diet (104). Moreover, B-cells have also been linked with the accumulation and differentiation of IFN-γ-producing CD4+ and CD8+ T-cells within murine visceral adipose tissue (103, 105). Indeed, CD8+ T-cells have been shown to produce 30% less IFN-γ in B-cell-deficient mice fed a high-fat diet suggesting a role for B-cells in T-cell activation (104, 105). B-cells may also promote the expansion of a senescent population of CD4+CD153+PD-1+ T-cells in murine visceral adipose tissue that, in turn, secrete osteopontin that promotes B-cell IgG production and suppresses IL-10 secretion (105, 106).

In obese human subcutaneous adipose tissue, B-cells comprise <4% of all stromal cells, but localise around macrophage crown-like structures and in the perivascular space (107). In obese human adipose tissue, a ~3-fold increase in the expression of the B-cell marker *B220* mRNA has been reported (108), although flow cytometric analysis of human subcutaneous adipose tissue has shown that very few B-cells are present in adipose tissue (109). Contradictory findings could be an artefact of methodology, indicating that further work in humans, employing a combination of methodological approaches, is warranted. In summary, B-cells may play a role in the early stages of obesity-induced adipose tissue dysfunction, modulating the functions of other cells within the stromal fraction, promoting a pro-inflammatory microenvironment.

T-Cells in Obesity-Associated Adipose Tissue Dysfunction

Within lean human adipose tissue, T-cells represent ~10% of the stromal fraction (one-third CD8+ T-cells and two-thirds CD4+ T-cells). More than half of the CD4+ T-cell compartment within murine adipose tissue are regulatory T-cells (110). Studies in humans have shown that T-cells are recruited into adipose tissue in response to adipocyte-derived CCL-20 binding to CCL-6 on the surface of T-cells, as well as CCR-5 to RANTES interactions (109, 111, 112). Moreover, adipose-resident T-cells display a visceral adipose tissue-specific antigen-driven expansion, suggesting a signal within the tissue promotes their clonal expansion. A potential signal has been suggested to derive from B-cells, which

may infiltrate or respond to dysfunctional adipose tissue earlier than T-cells (105, 113, 114). T-cells typically exhibit a differentiated or activated phenotype shown by low-surface expression of CD62L and high expression of CD25 in murine and human adipose tissue, respectively (35, 113).

CD8+ Cytotoxic T-Cells in Obesity-Associated Adipose Tissue Dysfunction

In mice fed a high-fat diet, CD8+ T-cells are the predominant T-cell subpopulation to accumulate in adipose tissue. The early selective recruitment of CD8+ cells into visceral adipose tissue in mice occurs in parallel with a reduction in blood CD8+ T-cells in the first 2 weeks of overfeeding (113). Mouse models also show that adipose-resident CD8+ T-cells have an activated CD62L−CD44+ effector phenotype, which is brought about, *in situ*, by MCP-1 release from adipocytes (113, 115). Co-culturing CD8+ T-cells with obese murine adipocytes has been shown to initiate, potentiate, and maintain the inflammatory response by instigating the infiltration of macrophages towards adipocytes, followed by their activation by CD8+ T-cell-derived MCP-1 (113). Moreover, selective depletion of CD8+ T-cells in obese mice has been shown to improve metabolic health and reduce local tissue and serum levels of IL-6 and TNF-α (113).

In obese humans, CD8A mRNA expression is increased in visceral adipose tissue (113, 116), whilst in subcutaneous adipose tissue CD8+ T-cells adopt an activated phenotype compared to blood (35, 109). As serum MCP-1 is substantially elevated in patients with type-II diabetes and is a potent activator of CD8+ T-cells, MCP-1 may be a cause of elevated adipose tissue CD8+ T-cell activation with obesity or metabolic disease (115, 117). The stimulus for CD8+ T-cell migration into obese adipose tissue is yet to be confirmed *in vivo*, in humans, although significantly elevated CCL-20 release by obese adipose tissue has been observed *in vitro* (109). In the circulation, CD8+ T-cells from obese individuals express elevated levels of TNF-α, IFN-γ, and RANTES. Importantly, IFN-γ has been found to inhibit insulin-mediated upregulation of fatty acid synthase (FAS) and lipoprotein lipase and was associated with a down-regulation of phosphatidylinositol 3-kinase regulatory subunit alpha expression (a key component of insulin signalling) (109). These observations suggest a modulatory capacity for CD8+ T-cells in adipose tissue metabolic health by influencing insulin-mediated triglyceride storage (109).

CD4+ Helper T-Cells (Th Cells) in Obesity-Associated Adipose Tissue Dysfunction

CD4+ T-cells accumulate within human subcutaneous adipose tissue with obesity, exhibiting an activated CD25+ phenotype (35). Recent findings suggest that circulating CD4+ T-cells adopt an effector memory (CXCR-3+CD62L−) phenotype with human obesity in response to a metabolically driven adaptation within T-cells *via* the p110δ subunit of phosphoinositide 3-kinase (PI3K) (118). Indeed, lipid activation of this subunit is also associated with sustained activation of T-cells, indicating that dendritic cell-independent, metabolically driven activation could drive chronic activation within adipose tissue (119, 120). The Th cell balance has the potential to play an important role in adipose tissue inflammatory disorders. For example, in mice, IFN-γ secretion is increased mainly as a consequence of Th cell (Th-1) cell predominance within visceral adipose tissue upon high-fat diet overfeeding (112, 114). The direct effect of IFN-γ in obesity induced by a high-fat diet includes impaired insulin signalling and the promotion of macrophage infiltration and crown-like formations *via* MCP-1 (121). In addition, IFN-γ from Th-1 cells instigates CXCL-10 release from 3T3-L1 adipocytes, providing a positive feedback recruitment loop (122). Moreover, IFN-γ secretion, when combined with TNF-α, can lead to chronic activation of NFκB signalling (123). However, Th-2 cells could serve to promote M2-like macrophage maturation through their production of IL-4 and IL-13 (124). Human and murine research indicates that CD4+ T-cells within both visceral and subcutaneous adipose tissue not only control their own recruitment, activation, and differentiation but are also influenced by other tissue-resident immune cells (i.e., B-cells) and adipocytes. Consequently, CD4+ T-cells have both direct and indirect roles in obesity-associated adipose tissue immunometabolic dysfunction.

Regulatory T-Cells in Obesity-Associated Adipose Tissue Dysfunction

Regulatory T-cells represent more than half of all CD4+ T-cells in lean murine adipose tissue, and in obesity, regulatory T-cells accumulate around macrophages, produce IL-4 and IL-10, and promote Th-2 T-cell polarisation (110). Regulatory T-cells also suppress adipocyte-derived inflammatory markers and restore metabolic health by promoting translocation of glucose transporter-4 to the cell membrane, countering the effects of TNF-α. However, adipose-resident regulatory T-cells appear to decline with increasing duration of obesity in mice (125). It is thought that the decline in regulatory T-cell numbers is a result of IFN-γ-induced impairments in proliferative capacity in visceral adipose (125). These changes occur in parallel with insulin resistance in mice and humans (110, 113, 114). In human obesity—which occurs over a much longer time-span than in mice—it has been shown that there is a substantial increase in regulatory T-cells within subcutaneous adipose tissue, and this could be a compensatory mechanism to counter adipose tissue inflammation (35). However, a proportional decline in regulatory T-cells has also been reported within the adipose tissue stromal fraction in obesity (126). These observations suggest that regulatory T-cells may limit ongoing pro-inflammatory events that occur with obesity-induced adipose tissue dysfunction. However, it is possible that with chronic local inflammation, regulatory T-cell proliferation is impaired and numbers decline.

iNKT Cells in Obesity-Associated Adipose Tissue Dysfunction

Within lean human and murine adipose tissue, iNKT cells typically express low levels of CD4 and NK1.1 and secrete large amounts of IL-4 and IL-10, demonstrating an anti-inflammatory capacity (127). Indeed, with obesity, iNKT cells may initially provide an anti-inflammatory function to limit the ongoing pro-inflammatory response driven by other infiltrating leukocytes.

However, with obesity, iNKT cells decline substantially although the reason for this decline is unclear (127). Further, in mice, iNKT cell accumulation in adipose tissue is limited with adipocyte CD1d knockout and, in turn, these animals exhibit a poor metabolic profile (128). However, it has been suggested that CD1d knockout models may reduce IFN-γ production, independent of iNKT cells (128). In addition, recent investigations have shown that iNKT cells have a role in adipocyte browning and modulating regulatory T-cell responses in mice, promoting the anti-inflammatory actions of regulatory T-cells (129, 130). In non-obese humans, visceral adipose tissue is also enriched with iNKT cells, and these cells exhibit a distinct Th-2 cytokine profile (131, 132). However, with obesity, the proportion of iNKT cells within the adipose tissue stromal fraction declines substantially, in tandem with the development of metabolic syndrome and inflammation, as observed in obese mice (128, 131, 132). In summary, iNKT cells likely assist in the regulation of a healthy adipose tissue microenvironment in a similar way to regulatory T-cells and eosinophils (see Eosinophils in Obesity-Associated Adipose Tissue Dysfunction). Therefore, a reduction in iNKT cells with obesity might contribute to the diminished anti-inflammatory signals within dysfunctional adipose tissue.

Dendritic Cells in Obesity-Associated Adipose Tissue Dysfunction

Dendritic cells accumulate in adipose tissue of mice fed a high-fat diet and likely contribute to the pro-inflammatory microenvironment *via* macrophage recruitment and IL-6 production (133, 134). Myeloid dendritic cells may also promote Th cells to adopt a Th-1 phenotype, which contribute to IFN-γ production (133, 135–137). However, in mice, both the flow cytometric analyses and knockout models commonly utilised are subject to criticism due to their non-specificity for "true" dendritic cells (133, 138). It has recently been shown that type-I IFN signalling is pivotal to the development of obesity-associated metabolic disease in mice, and therefore, plasmacytoid dendritic cells have been implicated in diet-induced obesity (139). Indeed, in mice fed a high-fat diet, plasmacytoid dendritic cells have been shown to increase ~3-fold, possibly as a result of elevated recruitment and activation with obesity by the adipokine, chemerin. In turn, dendritic cell accumulation has been suggested to enhance type-I IFN signalling, leading to IFN-β-induced MCP-1 production and macrophage recruitment/proliferation (60, 133, 139–143). In humans, distinct CD1c+ myeloid dendritic cell populations have been identified within subcutaneous, but not visceral adipose tissue, and the number of these cells correlates positively with BMI (138). Moreover, dietary lipids modulate the abundance of dendritic cells within lymphoid structures in adipose tissue, and as dendritic cells communicate with other lymphoid cells *via* lipid-derived molecules, dendritic cells may modulate adipose tissue immunometabolic responses (144, 145).

Eosinophils in Obesity-Associated Adipose Tissue Dysfunction

Eosinophils in the abdominal adipose tissue of mice have a role in improving insulin sensitivity and stimulating anti-inflammatory responses through the production of IL-4 (146–148). Mice fed a high-fat diet exhibit a decline in eosinophil numbers within adipose tissue (147). In addition, eosinophils have been estimated to produce up to 90% of the IL-4 found within murine adipose tissue, promoting M2-like macrophage polarisation and tyrosine hydroxylase expression. In turn, macrophage tyrosine hydroxylase expression drives the production of catecholamines by macrophages, triggering the expression of uncoupling protein 1 in white adipose tissue (148–150). Moreover, it has been shown that mice fed a high-fat diet and injected with eosinophils intravenously do not accumulate adipose-resident macrophages (147). Further, obese eosinophil knockout mice demonstrate significantly greater degrees of insulin resistance compared to wild-type mice (147).

Mast Cells in Obesity-Associated Adipose Tissue Dysfunction

Obesity in mice is associated with an accumulation of mast cells in adipose tissue and higher levels of tryptase in serum—a granule released by mast cells (151). In mice fed a high-fat diet, mast cells have been reported to accumulate in visceral adipose tissue and may contribute to the development of obesity through the stimulation of adipocyte protease expression, promoting microvessel growth, enabling leukocyte infiltration, and adipose tissue expansion (151, 152). In humans with obesity and type-II diabetes, pharmacological stabilisation of mast cells to prevent degranulation leads to improvements in a number of parameters, including glycosylated haemoglobin, fasting blood glucose, total cholesterol, low-density lipoprotein, triglycerides, and high-density lipoprotein, independent of changes to BMI (153). Therefore, despite contradictory findings in murine research, study on humans indicates a potential role for mast cell dysregulation in the aetiology of metabolic diseases. However, whether this is a result of adipose-resident mast cells specifically is yet to be established.

Summary of the Cellular Changes Associated with Obesity-Induced Adipose Tissue Dysfunction

Adipose tissue dysfunction with obesity is associated with changes to the numbers and/or function of a variety of immune cells. With this ongoing immunological dysregulation, adipose tissue adopts a pro-inflammatory bias, dramatically impacting the metabolic health of the tissue. Consequently, dysfunctional adipose tissue influences whole-body immunometabolic health contributing to the generation of diseases including type-II diabetes and cardiovascular diseases. A summary of obesity-associated changes to adipose tissue-resident immune cell populations is presented in **Figure 3**.

IMMUNOLOGICAL AGEING AND ADIPOSE TISSUE

Most physiological systems become dysfunctional with ageing due to accumulations of cellular changes that contribute to the onset of disease. Nine hallmarks of ageing have been proposed

characterising age-associated whole-body dysfunction, which include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, dysregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intracellular communication (15). The physiological stresses induced by obesity also cause a range of cellular and whole-body deteriorations that underpin the pathophysiology of adipose tissue accumulation and dysfunction. Many of these obesity-associated changes overlap with the hallmarks of ageing; thus, it has been proposed that obesity could be considered an accelerated model of ageing (13, 28, 154, 155).

Ageing is associated with a decline in immune function, referred to as immunosenescence, which is thought to contribute to chronic, low-grade inflammation or "inflammageing" (16, 156–159). The broader consequences of an ageing immune system are increased susceptibility to infections and cancer in combination with weaker responses to novel antigens, including those administered by vaccination (160). Immunosenescence is best characterised and most marked among cells of the adaptive immune system. However, a common general observation is that lymphoid cell numbers decline (along with their proliferative capacity), whereas myeloid cell numbers typically increase, but only some innate immune cells exhibit age-associated dysfunction (156). The most robust hallmarks of immunosenescence are alterations to the T-cell pool in peripheral blood. These changes are, in-part, not only due to thymic involution limiting the output of naive T-cells but also due to a lifetime of antigen exposure, which drives the differentiation of naive T-cells into memory T-cells (160). Thus, ageing is associated with an accumulation of pro-inflammatory effector memory T-cells and a decline in naive T-cells in peripheral blood (160–162).

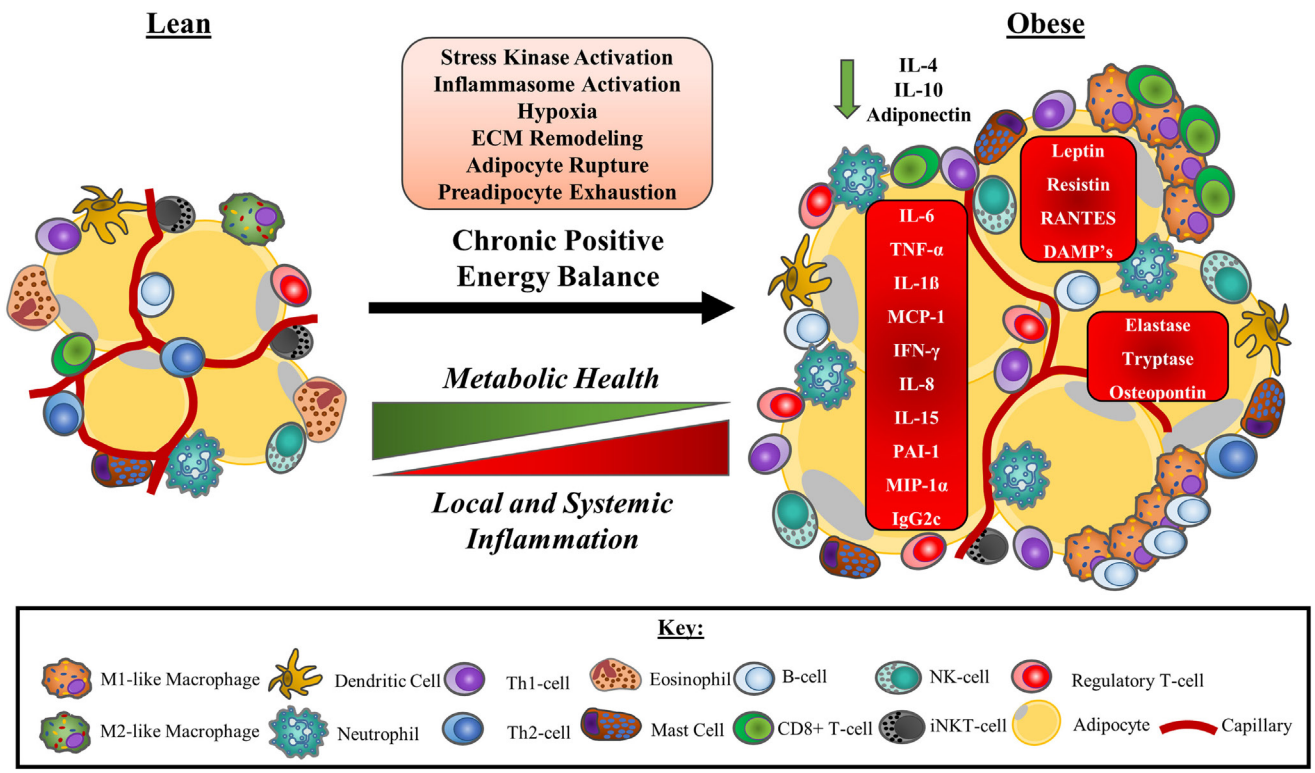

FIGURE 3 | A model of obesity-driven immunological changes within adipose tissue. Under a chronic state of positive energy balance, adipose tissue undergoes a multitude of changes that include the hypertrophic expansion of adipocytes without concurrent angiogenic responses. The consequences of these adaptations include local tissue hypoxia as a result of reduced tissue blood flow and impaired oxygen delivery. The rapid expansion of adipocytes also leads to unstable adipocytes prone to rupturing, releasing their lipid contents. This instability is further exacerbated by an exhaustion of the preadipocytes required to facilitate adipose tissue expansion. The net result of these physiological changes to adipocytes is the release of DAMPs and other signalling molecules that initiate stress kinase activation and promote monocyte infiltration to manage the remodelling of the ECM. Neutrophils are the first cells to accumulate in adipose tissue, followed by a range of other immune cells, most notably, B-cells, CD8+ T-cells, and M1-like/intermediate, or "double-negative" (CD11c−CD206−) macrophages that surround unstable adipocytes in crown-like structures. As a consequence, a Th-1 cell and M1-like macrophage secretion profile predominated and some anti-inflammatory immune cells such as eosinophils and iNKT-cells leave the tissue. Other anti-inflammatory cells, including regulatory T-cells, may increase to compensate. Pro-inflammatory cytokines and adipokines dominate the tissue microenvironment potentiating the activation of stress kinases and inflammasomes. Local inflammation reduces adipose tissue insulin sensitivity, impairing metabolic health and promoting ectopic lipid deposition. Pro-inflammatory adipose tissue contributes to the chronic, low-grade inflammation characteristic of obesity, and metabolic disease. Abbreviations: CD, cluster of differentiation marker; DAMP, damage-associated molecular pattern; ECM, extracellular matrix; IFN-γ, interferon gamma; Ig, immunoglobulin; IL, interleukin; iNKT-cell, invariant natural killer T cell; MCP-1, monocyte chemotactic protein 1 (CCL-2); MIP-1α, macrophage inflammatory protein 1 alpha (CCL-3); PAI-1, plasminogen activator inhibitor 1; RANTES, regulated on activation, normal T-cell expressed and secreted (CCL-5); Th cell, helper T cell; TNF-α, tumour necrosis factor-α.

Obesity, as with ageing, is associated with increased susceptibility to infections and an inability to mount effective immune responses to novel antigens (163–166). Thus, obesity might promote immunological decline through several mechanisms, with dysfunctional adipose tissue as a potential-driving factor. For example, obesity might exacerbate immunosenescence by promoting activation and differentiation of immune cells passing through the adipose tissue microvasculature, which could have implications for the phenotype and function of cells found in peripheral blood and other tissues. In addition, immune cell accumulation in adipose tissue promotes differentiation into pro-inflammatory cytokine-producing cells, which likely drives inflammageing. Exacerbated inflammation influence processes early in the immune response to novel pathogens, such as antigen recognition, processing, and presentation, but might also impair the ability to control latent or chronic infections and undergo processes such as wound healing. Experimental evidence supports some of these ideas, suggesting that adipose tissue dysfunction in obesity promotes immunological ageing (105).

Although there is less research specifically examining adipose tissue in the context ageing compared to obesity, a role for adipose tissue in late-onset disease development has been established. Ageing is associated with increased abdominal and ectopic adipose tissue accumulation, which is linked with the onset of frailty and cardiometabolic disease (167). Moreover, organelle stress and the accumulation of senescent cells (p16INK4a+) within adipose tissue that secrete IL-6, IL-8, and TNF-α further contribute to a pro-inflammatory, dysfunctional adipose tissue with age (168, 169). However, comparatively little is understood about immune cell populations within aged adipose tissue, especially in humans. Thus, the remaining sections will review literature examining the accumulation of different immune cells in adipose tissue with ageing, beginning with cells where there is most evidence, followed by cells that have been examined least.

Macrophages in Aged Adipose Tissue

Very few studies have investigated the effects of ageing on adipose tissue macrophage populations to date; however, the available data support a role for macrophages in age-associated adipose tissue dysfunction. This concept is discussed below and summarised in **Figure 4**. In visceral adipose tissue from old non-obese mice, it has been shown that the absolute numbers of adipose-resident macrophages do not increase substantially but may decline in proportions given the expanded stromal vascular fraction with age. However, with age, visceral adipose tissue macrophages have a predominantly pro-inflammatory, so-called "double-negative" (CD11c−CD206−) phenotype producing IL-6 and TNF-α and down regulating *PPAR-γ* (51, 170). Furthermore, peritoneal macrophages cultured with adipose-derived conditioned media induces M1-like polarisation, indicating that the products of adipose tissue have a direct impact on immune cells (170). Age-associated adipocyte-derived inflammation likely comes about by the activation of the NFκB signalling pathway (51). As a result, NFκB activation may act as a stimulus for the pro-inflammatory polarisation of adipose tissue macrophages, supporting the idea that changes within adipose tissue, independent from total adipose tissue mass, drive age-associated adipose tissue inflammation (171). These observations in mice likely correspond to those made in obese adipose tissue from humans (**Figure 1**) but experimental evidence is limited at present. Moreover, another study in middle-aged (11 months old) mice suggests that the proportions of "double-negative" (CD11c−CD206−) and M2-like macrophages are largely unaltered, whereas M1-like macrophages decline slightly when compared to young murine visceral adipose tissue (172). Note that these 11-month-old mice are representative of humans aged 30–40 years (173).

Sympathetic neuron-associated, catecholamine-degrading macrophages which impair lipolysis have also been reported to accumulate in aged murine visceral adipose tissue (175). With obesity, these sympathetic neuron-associated macrophages appear to infiltrate the tissue selectively (87). However, with ageing, these cells appear to accumulate in adipose tissue *via* proliferative mechanisms, *in situ* (175). The metabolic effects of these cells are due to NLRP-3 activation, which upregulates growth differentiation factor 3 (GDF3) expression within macrophages (175). GDF3 interacts with adipocytes to inhibit lipolysis and stimulates the expression of enzymes that oxidise and remove catecholamines (87, 175). Therefore, in the context of ageing, sympathetic neuron-associated macrophages likely regulate whole-body metabolism, inhibiting lipolytic signalling and free-fatty acid mobilisation as with obesity, contributing to immunometabolic dysfunction both locally and systemically.

In humans, only one study has investigated how ageing influences adipose tissue macrophages. Metabolically healthy Pima Indians, aged 18–44 years, were assessed for insulin sensitivity and subcutaneous adipose tissue macrophage content. Adipose tissue macrophage number increased until ~31–33 years old, plateauing or slightly declining thereafter, but with ageing, adopted an activated phenotype as shown by plasminogen activator inhibitor 1 and *CD11c* mRNA expression (**Figure 4**). The expression of these two macrophage activation markers was also associated with a decline in insulin sensitivity, independent of adiposity. Further, a negative relationship was observed between advancing age and whole-body insulin sensitivity ($r = -0.23$, $P = 0.07$). These findings suggest that although adipose-resident macrophages may decline or plateau in middle age, activation of these cells might impart an insulin-desensitising effect (174). Although this study offers the only direct assessment of adipose-resident macrophage populations with advancing age in humans, there are several factors that should be considered. For example, this study provides data up until middle age only and was conducted in a specific population with unique metabolic, genetic, and lifestyle characteristics. In addition, examining adipose-resident macrophages using gene expression and immunohistochemical analysis has some limitations (see **Table 1**).

The stimulus for age-associated macrophage pro-inflammatory polarisation is unclear, but endoplasmic reticulum stress, as shown in obese adipose tissue and adipose tissue macrophages, has been implicated. For example, it has been shown in aged murine adipose tissue stromal cells that endoplasmic reticulum stress promotes an inflammatory environment, elevating IL-6, MCP-1, and TNF-α production within adipose tissue, potentially in response to altered autophagy (176, 177). Ageing is associated

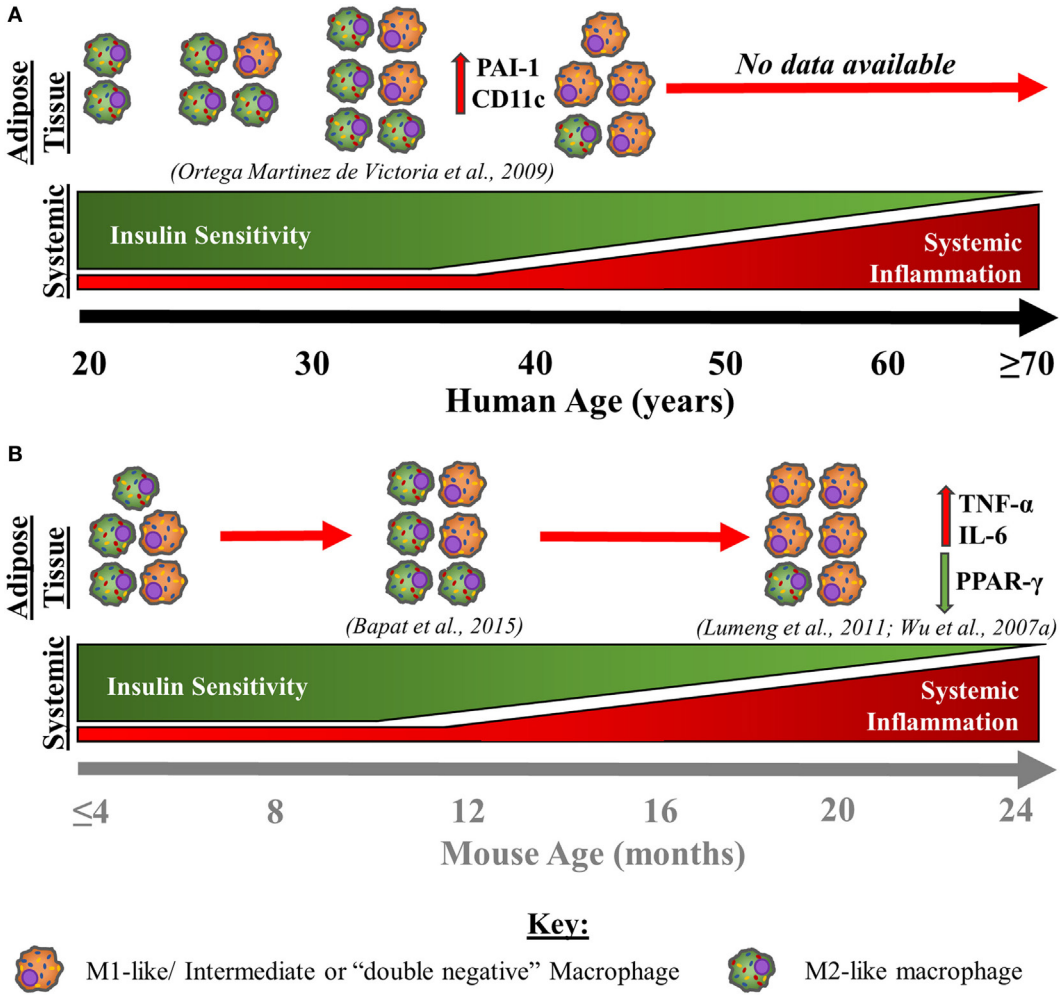

FIGURE 4 | Age-associated changes in adipose tissue macrophages and immunometabolic health in humans **(A)** and mice **(B)**. This schematic is a theoretical representation based on the limited empirical evidence that is available. With advancing age in humans **(A)**, subcutaneous adipose tissue macrophage content increases steadily until 31–33 years whereupon numbers slightly decline, until 45 years old (174). Although absolute adipose tissue macrophage content declines from around the age 31–33 years old until 45 years old, at least, the presence of more pro-inflammatory activated macrophages [M1-like/intermediate, or "double-negative" (CD11c−CD206−) macrophages] increases, which is associated with a decline in insulin sensitivity. With advancing age, insulin sensitivity continues to decline, while low-grade systemic inflammation begins to steadily increase from middle age into old age. With advancing age in mice **(B)**, an increase in pro-inflammatory macrophages within visceral adipose tissue that produce TNF-α and IL-6 and have downregulated PPAR-γ expression is also observed up to 18–24 months, at least (51, 170). Further, in middle-aged mice (44 weeks old), "double-negative" (CD11c−CD206−) and M2-like macrophages are largely unchanged or are modestly elevated, while M1-like macrophages exhibit a slight decline (172). Therefore, it is theorised that there is a continued pro-inflammatory macrophage activation/polarisation with advancing age in humans, independent of changes in absolute counts within the adipose tissue. Further, the same pattern of events appears to occur within murine adipose tissue, although the age at which this shift to a pro-inflammatory bias occurs is unknown. The similarities between data from aged mice and humans indicate that macrophages adopt a predominantly pro-inflammatory M1-like/intermediate, or "double-negative" (CD11c−CD206−) phenotype into old age, contributing to the increased systemic inflammation and impaired metabolic health exhibited by older individuals. Abbreviations: CD, cluster of differentiation marker; IL, interleukin; PAI-1, plasminogen activator inhibitor 1; PPAR-γ, peroxisome proliferator-activated receptor gamma; TNF-α, tumour necrosis factor-α.

with decreased vascularisation as a result of impaired angiogenesis, supporting local hypoxia, which might drive adipose tissue macrophage accumulation (36, 178–180). Ageing is also associated with reductions in the mitochondrial cytochrome *c* oxidase subunit 5B (COX5B) component of complex IV within adipocytes, which represses HIF-1α (181). Therefore, reductions in COX5B in human visceral adipose tissue with age both elevates HIF-1α and intracellular lipid storage as a result of decreased fatty acid oxidation, promoting adipocyte enlargement, *in vitro* (181). If this hypertrophic expansion was to continue, stress signals prompting macrophage infiltration could be released, as is observed in obesity (53, 79, 182).

Regulatory T-Cells in Aged Adipose Tissue

As with obesity, regulatory T-cells increase in the adipose tissue of old compared to young mice (110, 170, 172). The expanded population of these regulatory T-cells is transcriptionally more closely related to adipose-resident conventional T-cells

than splenic regulatory T-cells (i.e., selective enrichment for *PPAR-γ* gene expression, among others), indicating that their characteristics may be driven, in part, by their location within the adipose tissue (172). However, unlike in obesity, an increase in regulatory T-cells in aged murine adipose tissue is linked with a decline in metabolic function independent of macrophage accumulation (172). These observations indicate that, in aged adipose tissue, a reduction in regulatory T-cells may be protective, because the immune profile of adipose tissue regulatory T-cell knockout mice is shifted towards those of young mice (172). Moreover, *ex vivo* adipose tissue basal glucose uptake was nearly twice as high for adipose-resident regulatory T-cell knockout mice compared to control tissues, supporting a causal association between adipose tissue regulatory T-cells and age-associated insulin resistance (172).

Although it is unclear whether the function of regulatory T-cells with ageing in humans is impaired, studies in aged mice indicate that these cells retain their suppressive capacity (172, 183). As a degree of inflammation is essential for the maintenance of healthy adipose tissue, the substantial increase in adipose tissue regulatory T-cells combined with the retention of their suppressive capacity may be an indication of overcompensation, impacting the immunometabolic properties of aged murine adipose tissue (26, 172). Although the observations concerning regulatory T-cells within aged murine adipose tissue provide evidence that age- and obesity-associated insulin insensitivity may be orchestrated by unique immune cell populations, further work in the adipose tissue of older humans is required to better understand this immunometabolic interplay. Additional work in older mice is also warranted as the animals examined by Bapat et al. (172) were substantially younger than those examined in other studies investigating age-associated adipose tissue immunological dysfunction (51, 170, 172) (see **Figure 4**). Nonetheless, recent evidence offers some support for a non-obesity-dependent regulatory T-cell-driven, late-onset metabolic deterioration, called type-IV diabetes (172).

B-Cells in Aged Adipose Tissue

It has been shown that the visceral adipose tissue of obese, old mice is enriched with a specific B-cell population compared to young, obese mice, and these cells differ from splenic B-cells (184). Co-culturing splenic B-cells with visceral adipocytes from old, obese mice caused B-cells to adopt an age-associated phenotype (184). These B-cells, which had been altered by adipose tissue, had higher levels of basal inflammation and NFκB protein within the nuclear extract, as has been shown with peripheral blood B-cells from older humans (185). In addition, the visceral adipose tissue of old mice has also been shown to be enriched with IgG2c (a mouse-specific IgG isoform), an observation that has also been made in obese mice (104, 184). It was proposed by Frasca and Blomberg (184) that increased hypoxia and *CCL-2* expression, along with increased adipocyte-derived TNF-α, IFN-γ, IL-6, and IL-21, prompted an obesity-driven accumulation of pro-inflammatory B-cells, which is exacerbated with ageing (184). Nonetheless, it should be noted that older mice in this study were heavier than younger mice and had more visceral adiposity. Thus, it is difficult to tease apart the effects of ageing or obesity in this work. Other evidence suggests that metabolic secretions released from dysfunctional adipose tissue with age, rather than typical pro-inflammatory factors, contribute to B-cell dysfunction. For example, it has been shown that incubation of B-cells from young mice with leptin induces STAT-3 signalling within B-cells, leading to TNF-α and IL-6 production, possibly contributing to the elevated production of these cytokines in aged murine adipose tissue (51, 171, 186).

CD4+ Th Cells and CD8+ Cytotoxic T-Cells in Aged Adipose Tissue

CD4+ T-cells within the visceral adipose tissue of obese mice display an "aged" phenotype and accumulate 4-fold with obesity (105, 170). High-fat overfeeding in mice causes an accumulation of CD4+ T-cells in the visceral adipose tissue expressing markers associated with senescence such as CD153+PD-1+CD44^{high}CD4+ T-cells in visceral adipose tissue. These cells secrete osteopontin, causing local inflammation (105). Moreover, CD4+ T-cells expressing markers such as CD153 also accumulate in the circulation with age in mice, perhaps linking changes in blood to changes in adipose tissue, or *vice versa* (187). Compared to the 4-fold increase in CD4+ T-cells in aged murine visceral adipose tissue, CD8+ T-cells exhibit a 7-fold increase, in parallel with an increase in *CCR-5* gene expression, which is involved in the migration and homing of effector T-cells (170, 188). However, whether adipose tissue CD8+ cells impart a pro-inflammatory function with age is unclear. CD8+ T-cells within aged adipose tissue have not been studied in isolation; although when adipose tissue stromal cells from aged mice were cultured *ex vivo*, no difference was found in the production of IFN-γ compared to young mice (170). Although there was a greater production of TNF-α from aged murine adipose tissue, this was attributed to macrophages (170). It is, therefore, currently unclear what role CD8+ T-cells play in aged adipose tissue. Furthermore, it is unclear what signal promotes this CD8+ T-cell expansion within aged adipose tissue and whether this contributes to, or merely reflects, the increased CD8:CD4 T-cell ratio in the circulation of older individuals (189).

Under-investigated Cells in Aged Adipose Tissue

Neutrophils, eosinophils, mast cells, dendritic cells, NK-cells, and iNKT cells have a role in obesity-associated adipose tissue dysfunction. Indeed, obesity is associated with alterations in the numbers, proportions and functions of many cell types, either resulting in a pro-inflammatory change (i.e., neutrophils, mast cells, dendritic cells, NK-cells) or an anti-inflammatory change (i.e., eosinophils and iNKT cells). These alterations contribute towards, or occur in response to, the immunometabolic dysfunction within adipose tissue caused by obesity. However, currently, there is no direct evidence for a similar role for these cells within adipose tissue in an ageing context.

Although no research has specifically examined neutrophils in aged adipose tissue in mice or humans, elevated *COX-2* mRNA has been shown in visceral adipose tissue from older mice, which

may indicate neutrophil activation in response to elevated basal prostaglandin E2 (PGE_2) expression (51, 93, 190, 191). Moreover, neutrophils migrate through tissues secreting proteases (e.g., elastase) in response to homing signals including, among others, leptin, and aberrant secretion of this adipokine with age may promote neutrophil entry into adipose tissue (192–195). Neutrophils also demonstrate misdirected chemotaxis, with ageing, which, if neutrophils were signalled into adipose tissue in response to aberrant leptin secretions, would potentially exacerbate damage to the tissue, as membrane-bound proteases would be released over a wider area (196). In obese adipose tissue, increased elastase release by tissue-resident neutrophils impacts upon the metabolic health of adipose tissue by interacting with IRS-1 and TLR-4 signalling, impairing insulin sensitivity (89, 92, 197, 198). Therefore, using obesity as a model, neutrophil degranulation as a result of impaired navigation through the adipose tissue may contribute to age-associated adipose tissue dysfunction.

Mast cell and eosinophil numbers and function in adipose tissue has not been investigated in an ageing context. With eosinophils, considering that the number of these cells do not change with ageing in the circulation, it might be speculated that the same is true in adipose tissue (199). However, in obesity, the decline in adipose tissue eosinophils in mice might promote a pro-inflammatory macrophage predominance due to less eosinophil-derived IL-4 and IL-10. Subsequently, a loss of eosinophil-derived anti-inflammatory signals could contribute to fewer M2-like macrophages and the increase in M1-like or "double-negative" (CD11c−CD206−) macrophages with ageing in visceral adipose tissue. If mast cells are shown to be present in aged adipose tissue, the reported increase in PGE_2 within aged murine adipose tissue may promote mast cell degranulation, considering that PGE_2 inhibits the release of mediators that limit mast cell activation (51, 200).

Although no research has examined dendritic cells in adipose tissue with ageing, some evidence suggests that the pro-inflammatory profile of aged murine adipose tissue, as shown by high gene expression for IL-6, TNF-α, and PGE_2, could influence dendritic cell function (51). PGE_2 augments IL-23 production in aged murine and human dendritic cells, promoting Th-17 responses, while a pro-inflammatory aged adipose tissue microenvironment could promote NFκB activation within dendritic cells, as is seen in obese adipose tissue (138, 201–205). Dendritic cells from the circulation of older individuals may also have the potential to guide pro-on within adipose tissue due to increased intracellular IL-6 and TNF-α and increased expression of co-stimulatory and activation markers, as is observed in the context of obesity (133, 137, 158, 206). Nonetheless, these observations have yet to be investigated within aged adipose tissue and can, therefore, only represent speculative mechanisms at present.

We are unaware of any research that has investigated the role of NK-cells in aged adipose tissue. However, in mice, aberrant leptin secretions have been suggested to drive specifically activated $CD56^{dim}$ NK-cell polarisation, contributing to elevated TNF-α production and metabolic impairments (95, 96, 194). As well as leptin, the elevated TNF-α within aged murine adipose tissue may also serve to recruit NK-cells as TNF-α is essential for NK-cell recruitment into other organs such as the liver (207). It remains unclear whether the aberrant secretory profile of aged adipose tissue influences NK-cell function. However, as circulating $CD56^{dim}$ NK-cells accumulate in the blood of older people, if these cells were recruited into adipose tissue by TNF-α, leptin, or another yet to be established signal, they may contribute towards pro-inflammatory macrophage polarisation (102, 208–212).

Adipose tissue iNKT-cells have not been investigated with ageing, but considering that IL-4- and IL-10-producing NK1.1+iNKT-cells decline with obesity (127), it is possible that ageing has similar effects. However, given endogenous lipid antigens derived from adipocytes influence the secretion profile of iNKT-cells (127, 213, 214), then interactions between adipocytes and iNKT-cells might contribute towards iNKT-cell dysregulation in aged adipose tissue. For example, loading glycolipids into the CD1d receptor on adipocytes is mediated by intracellular ceramides (127), and sphingolipid ceramide content is higher in adipose tissue from old mice compared to young (51). Thus, in principle, enhanced ceramide-mediated glycolipid loading within aged adipocytes might drive overactivation of iNKT-cells with ageing.

SUMMARY, CONSIDERATIONS, AND FUTURE DIRECTIONS

Poor metabolic control, insulin resistance, inflammation, and impaired immune function are hallmarks of ageing and also characteristics of obesity. In addition, as outlined in this review, individuals who are obese, or elderly, exhibit similar immunological profiles in adipose tissue (**Tables 3–6**). Given the overlap between obesity and ageing, it is likely that changes to the numbers and function of some immune cells within obese and aged adipose—especially macrophages—contribute to dysfunction of this tissue. Moreover, it is possible that alterations to the immune profile of obese adipose tissue accelerate local and whole-body ageing. Finally, changes to the numbers and function of some immune cells within obese, and likely aged adipose tissue, whether a cause or consequence of adipose tissue dysfunction, may also underpin the development of metabolic diseases, as shown with obesity. However, there is limited work in an ageing context.

Improving our understanding of the interactions between ageing and adipose tissue dysfunction may help with the development of therapeutic and preventative strategies to improve longevity and quality of life in ageing populations. For example, with obesity, immunological dysregulation in adipose tissue is a well-established contributor to type-II diabetes and has consequently been a target for strategies such as caloric restriction, physical activity, and pharmacological manipulation. Caloric restriction imparts beneficial effects on metabolic control and blunts the production of pro-inflammatory cytokines from obese, murine adipose tissue (232, 233). Caloric restriction in older mice also delays ageing, in part due to beneficial effects on adipose tissue metabolic health and function (234). Moreover, in obese women, short- (1 month) and long-term (6 months) caloric restriction promotes an anti-inflammatory profile in abdominal, subcutaneous adipose tissue and reduces total adipose tissue

TABLE 3 | A comparison of the change in abundance for select immune cell subsets within adipose tissue with age and obesity compared to young and lean, respectively.

	Immune cells within adipose tissue			
	Ageing	**Ageing references**	**Obesity**	**Obesity references**
CD8+ T-cells	↑	(170)	↑	(113)
CD4+ T-cells	↑ ← →	(170)	↑	(35, 105)
Regulatory T-cells	↑(?)	(172)	↑/↓	(35, 125)
B-cells	↑(?)	(184)	↑	(104, 107)
Macrophages				
M1-like/intermediate/DN	↑	(51, 170)	↑	(63, 87)
M2-like	↓	(174, 175)	↓	(35, 65)
Eosinophils	?		↓	(147)
Mast cells	?		↑(?)	(151, 152, 215)
NK cells	?		↑	(95, 97)
iNKT cells	?		↓	(128, 132)
Neutrophils	?		↑	(89, 93)
Dendritic cells	?		↑	(133, 139)

↓, decrease; ↑, increase; ← →, little or no change or equivocal data; ?, unclear or no evidence; CD, cluster of differentiation marker; DN, double-negative macrophage (CD11c−CD206−); iNKT cell, invariant natural killer T cell; NK-cell, natural killer cell.

TABLE 4 | Changes to adipose tissue and adipocytes with age and obesity compared to young and lean, respectively.

	Broader Tissue-Specific Changes			
	Ageing	**Ageing references**	**Obesity**	**Obesity references**
Adipocyte size	↑	(216)	↑	(31)
Endoplasmic reticulum stress	↑	(176, 177)	↑	(82, 85)
Mitochondrial dysfunction	↑	(181)	↑	(217)
Stem cell exhaustion	↑	(13)	↑	(13)
Tissue blood flow	↓	(180)	↓	(36, 178)
Tissue hypoxia	↑		↑	

↓, decrease; ↑, increase.

TABLE 5 | Changes in the soluble factors produced and/or released by adipose tissue (adipocytes and cells of the stromal fraction) with age and obesity compared to young and lean, respectively.

	Soluble and secreted factors within adipose tissue			
	Ageing	**Ageing references**	**Obesity**	**Obesity references**
IL-6	↑	(171)	↑	(218)
MCP-1	↑	(170)	↑	(60)
TNF-α	↑	(51)	↑	(27)
IFN- γ	← →	(170)	↑	(113)
IL-10	← →	(170)	↓	(219)
IL-1β	↑	(51)	↑	(79)
Adiponectin	↓← →	(220)	↓	(221)
Leptin	↑	(192, 193)	↑	(222)

↓, decrease; ↑, increase; ← →, little or no change or equivocal data; IFN-γ, interferon gamma; IL, interleukin; MCP-1, monocyte chemotactic protein 1; TNF-α, tumour necrosis factor-α.

macrophage content (235, 236). Physical activity can reduce systemic inflammation, targeting dysregulated chemokine and adipokine secretory patterns in mice and humans (40, 237–239).

TABLE 6 | Changes in the expression of proteins within adipose tissue (adipocytes and cells of the stromal fraction) with age and obesity compared to young and lean, respectively.

	Proteins expressed within adipose tissue			
	Ageing	**Ageing references**	**Obesity**	**Obesity references**
CCR-2	↑	(170)	↑	(223)
CCR-5	↑	(170)	↑	(111, 112)
CCR-7	↓	(170)	↓	(224)
CX3CR-1	↓	(170)	↑ ← →	(225–228)
CXCR-3	↑	(170)	↑	(229)
PGE_2	↑	(51)	↑	(230)
PPAR-γ	↓	(51)	↑	(231)

↓, decrease; ↑, increase; ← →, little or no change or equivocal data; CCR, (C-C motif) chemokine receptor; CX3CR-1, (C-X3-C motif) chemokine receptor 1; CXCR-3, (C-X-C motif) chemokine receptor 3; PGE_2, prostaglandin E2; PPAR-γ, peroxisome proliferator-activated receptor gamma.

In parallel, physical activity also limits the accumulation of immune cells such as CD8+ T-cells—potentially by attenuating RANTES/CCR-5 signalling within human adipose tissue (237). Moreover, physical activity reduces neutrophil number and elastase expression, as well as shifting macrophages to an M2-like phenotype within the adipose tissue of mice fed a high-fat diet (237, 240, 241). In addition, exercise might limit, or delay immunosenescence by inducing an anti-inflammatory/anti-oxidative microenvironment within aged adipose tissue, limiting non-specific activation of T-cells and propagation of inflammation, by dampening adipose tissue-derived signals (242–244). Finally, pharmacological therapies (e.g., the PPAR-γ agonists rosiglitazone) promote M2-like macrophage repolarisation after high-fat overfeeding in mice (245). Further, PPAR signalling overlaps with the network of longevity genes, and PPAR-γ2 (adipocyte specific)-deficient mice have a considerably reduced lifespan, possibly mediated by the anti-inflammatory effects on adipose tissue macrophages (19, 20, 245). Therefore, targeting immunological dysregulation in adipose tissue might promote healthy ageing.

In conclusion, obesity is a useful model to develop our understanding of age-associated adipose tissue dysfunction. There is now an improved appreciation of how this metabolically and immunologically active tissue influences ageing and immunometabolic disease(s). The available evidence indicates that age-associated adipose tissue dysfunction occurs irrespective of changes in adipose tissue masses, and this dysfunction appears to play an active role in the pathophysiology of ageing. Moreover, the accumulation of predominantly pro-inflammatory immune cells in obese adipose tissue exacerbates the immunometabolic dysfunction of the tissue and might act as a potent stimulus for accelerating ageing in obesity. Importantly, countermeasures that target inflammation within dysfunctional adipose tissue offer promise to reduce the burden of obesity- and age-associated immunometabolic diseases.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this work.

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Abnormal Epigenetic Regulation of Immune System during Aging

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Epigenetics refers to the study of mechanisms controlling the chromatin structure, which has fundamental role in the regulation of gene expression and genome stability. Epigenetic marks, such as DNA methylation and histone modifications, are established during embryonic development and epigenetic profiles are stably inherited during mitosis, ensuring cell differentiation and fate. Under the effect of intrinsic and extrinsic factors, such as metabolic profile, hormones, nutrition, drugs, smoke, and stress, epigenetic marks are actively modulated. In this sense, the lifestyle may affect significantly the epigenome, and as a result, the gene expression profile and cell function. Epigenetic alterations are a hallmark of aging and diseases, such as cancer. Among biological systems compromised with aging is the decline of immune response. Different regulators of immune response have their promoters and enhancers susceptible to the modulation by epigenetic marks, which is fundamental to the differentiation and function of immune cells. Consistent evidence has showed the regulation of innate immune cells, and T and B lymphocytes by epigenetic mechanisms. Therefore, age-dependent alterations in epigenetic marks may result in the decline of immune function and this might contribute to the increased incidence of diseases in old people. In order to maintain health, we need to better understand how to avoid epigenetic alterations related to immune aging. In this review, the contribution of epigenetic mechanisms to the loss of immune function during aging will be discussed, and the promise of new means of disease prevention and management will be pointed.

Keywords: immune aging, epigenetics, DNA methylation, histones, environment, age-related diseases

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EPIGENETICS: HOW THE GENOME TALKS WITH THE ENVIRONMENT

Epigenetics (epi = beyond) refers to the study of the heritable information on the chromatin beyond that given by the DNA sequence. Epigenetic marks, represented by different chemical groups added on both the DNA molecule and histone proteins, play key roles in the control of chromatin structure and function (1, 2). Among the most studied epigenetic mechanisms are DNA methylation

Abbreviations: ncRNA, noncoding RNA; 5mC, 5-methylcytosine; DNMT, DNA methyltransferase; SAM, *S*-adenosylmethionine; TET, ten-eleven translocation; 5hmC, 5-hydroxymethylcytosine; 5fC, 5-methylformylcytosine; 5caC, 5-caboxylcytosine; PTM, posttranslational modification; 8-OHdG, 8-hydroxy-2′-deoxy-guanosine; DC, dendritic cell; NK, natural killer; APC, antigen-presenting cell; IFN-γ, interferon-gamma; IL, interleukin; MIP-1α, macrophage inflammatory protein 1-alpha; LPS, lipopolysaccharide; PRR, pattern recognition receptor; DAMP, damage-associated molecular pattern; PAMP, pathogen-associated molecular pattern; TCR, T cell receptor; NFAT, nuclear factor of activated T cells; MHC, major histocompatibility complex; CIITA, class II transactivator; Treg cell, regulatory T cell; TGFβ, transforming growth factor β; RORC, RAR-related orphan receptor C; FOXP3, forkhead box P3; Pax5, paired box 5; AID, activation-induced cytidine deaminase; Ezh2, enhancer of zeste homolog 2; MOZ, monocytic leukemia zinc finger protein; HPC, hematopoietic progenitor cell; TNF-α, tumor necrosis factor-alpha; TLR-2, toll-like receptor 2; CRAT, carnitine *O*-acetyltransferase; F3, coagulation factor III; CRP, C-reactive protein; EWAS, epigenome wide association studies; HSC, hematopoietic stem cell.

and posttranslational histone modifications. The chromatin remodeling, the presence of structural and functional variants of histones, and the regulation by noncoding RNAs are additional epigenetic mechanisms working together with DNA methylation and histone modifications to maintain genome stability and control gene expression.

DNA Methylation

In mammals, each cytosine in the context of a CpG dinucleotide is a potential site for the covalent addition of a methyl group, yielding 5-methylcytosine (5mC) (3). This reaction is catalyzed by DNA methyltransferases (DNMTs)—DNMT1, 3A, and 3B, which transfer the methyl group from *S*-adenosylmethionine (SAM) to the 5-carbon of the cytosine (5mC) (4). SAM, a component of methionine cycle, is considered the universal donor of methyl groups to various biomolecules, including DNA and histones (5). Different components coming from the diet, such as B6 and B12 vitamin, choline, and betaine act as cofactors in reactions taking part in methionine cycle, and the folate cycle is coupled to the methionine cycle, bringing up how cellular nutrient status may modulate epigenetic marks (6). In normal cells, most CpG-rich (CpG islands) gene promoters are unmethylated, making these genes permissive to transcription. Hypermethylated CpG-rich promoters are typically associated with gene silencing, since methylated CpGs can both impair the binding of transcriptional factors and recruit repressive complexes (7, 8). Regions, such as repetitive DNA sequences and transposons, present in high number in our genome, have CpGs heavily methylated in normal cells. The loss of methylation in these regions favors homologous recombination and the expression of undesirable elements, contributing to chromosomal instability (9). The technological advances in genome-wide chromatin profiling have revealed that in fact the role of DNA methylation in gene regulation depends on its position and context. While in promoters it is associated with transcriptional silencing, in other regions it can modulate enhancer activity and splicing (10). The methylation at gene bodies is frequent in ubiquitously expressed genes and correlated with transcriptional activation (11). The tissue-specific DNA methylation seems to occur not at CpG islands, but at regions of lower CpG density about 2 kb distant from CpG islands, named CpG island shores (12). In addition, other non-CpG sites were more recently described as sites for DNA methylation in humans, such as CHG and CHH sites (where H is A, C, or T) (13), but the mechanisms involved in this process are still unknown. The complexity of the covalent modification of DNA has additionally increased with the recent identification of a new family of enzymes known as ten-eleven translocation 1-3 (TET1-3), which are able to oxidize 5mC to 5-hydroxymethylcytosine (5hmC) in a reaction that generates other intermediates (5-methylformylcytosine and 5-carboxylcytosine) (14, 15). These modified bases may be excised by thymine DNA glycosylases (TDG) and, through the base excision repair (BER) process, yield demethylated cytosines (16), which makes this process a mechanism of active DNA demethylation. The DNA can be also actively demethylated by the deamination of 5mC and 5hmC by the activation-induced deaminase/apolipoprotein B editing complex enzymes, followed by BER/TDG activity (17). Alternatively, 5hmC can be passively demethylated during DNA replication, since it is not recognized by DNMT1, yielding in this position non-methylated cytosine in the newly synthesized DNA strand.

Histone Modifications

In the nucleosomes, not only the DNA molecule but also histone proteins carry chemical modifications, which are fundamental for chromatin-dependent gene regulation (18). Several post-translational histone modifications (PTMs) regulate the chromatin structure, by affecting inter-nucleosomal interactions, and recruit proteins and complexes that influence not only the gene transcription but also mediate processes, such as DNA replication, DNA repair, alternative splicing, and recombination (19). A large number of proteins acting as writers, erasers, and readers have been described as components of histone modifier machinery, targeting all core histones H2A, H2B, H3, and H4, and the linker H1 histone, which have their amino acids subjected to covalent modifications mainly in the *N*-terminal tails. Among these modifications are acetylation, phosphorylation, methylation, ubiquitylation, sumoylation, and ADP ribosylation (19). By decreasing the positive charge of histones, acetylation and phosphorylation weaken interactions between histones and DNA, facilitating transcription machinery to access the DNA. Histone methylation occurs mainly on lysines and arginines, which can be, respectively, mono-, di- or trimethylated, and mono- and dimethylated, resulting in a high level of complexity regarding their effects. For example, high levels of trimethylated H3K4, H3K36, and H3K79 are associated with actively transcribed chromatin, while methylated H3K9, H3K27, and H4K20 are associated with transcriptionally inactive chromatin (20). The covalent attachment of the large ubiquitin molecule changes the nucleosome conformation, affecting both intra-nucleosomal interactions and interactions with effector proteins. Sumoylation involves the addition of small ubitiquin-like molecules to histones, and has been associated with repressive functions. Histone mono- and poly-ADP ribosylation has been correlated with a relaxed chromatin state. The number of possible histone modifications has increased with the continuous identification of novel histone PTMs, such as lysine propionylation (21, 22), butyrylation (21), crotonylation (23), succinylation, and malonylation (24), coupling cell metabolism with chromatin structure and function. It is important to keep in mind that a single histone mark is not responsible for the final effect on chromatin, but rather the combination of all marks in a chromatin region defines the biological outcome (25, 26). Besides that, there is interplay of DNA methylation, histone modifications, and nucleosome positioning, and the outcome is a result of the sum of these interactions.

The Plasticity of the Epigenetic Marks

Although presenting the same genome, each cell type in the same individual has a specific group of epigenetic marks, named epigenome. Epigenetic marks are established during embryonic development and transmitted through mitosis, stabilizing gene expression programs, and defining cell-type identities and function (27). In addition to their role in cell differentiation, these marks are fundamental to X chromosome inactivation in females and genomic imprinting during

development (28, 29). Although being relatively stable over time, epigenetic marks can change dynamically in response to cellular conditions and environmental cues (2, 30, 31). Recent studies have shown that the methylation patterns determined by the binding of factors on DNA motifs are less responsive to environment within the lifetime of an individual, and that these patterns would persist across generations (32, 33). However, other DNA regions would have their epigenetic marks more susceptible to internal and external environment, such as stress, smoke, drugs, hormones, circadian rhythms, and metabolic variations caused by diet. In this way, the environment can modulate the epigenotype, and consequently the phenotype, being decisive to direct to health or disease states. In fact, many studies have shown the relation between epigenetic alterations and a wide variety of diseases, including aging-related diseases (34–36).

CHANGES IN EPIGENETIC MARKS DURING AGING

During the aging of an organism, there is a gradual decline of normal physiological functions. In humans, these include decreased immune function, chronic inflammation, sarcopenia, and most importantly increased susceptibility to diseases, such as cancer, cardiovascular disorders, and metabolic and neurodegenerative diseases. Although systemic, these phenotypes are a result of alterations in different cellular processes, such as DNA damage response, mitochondrial and proteasome function, and cell death regulation (37–40). At the molecular level, transcriptional dysregulation is observed with aging, resulting in gene expression changes (41, 42). Epigenetic alterations are important contributors to these changes in the aged transcriptome, and are known as "epigenetic drift" (43–45).

DNA Methylation

Regarding DNA methylation, a progressive global hypomethylation occurs invariably with advanced age (46). Repetitive DNA sequences normally silenced by epigenetic marks become expressed, being at least partly responsible for the well-characterized loss of heterochromatin observed during aging (47, 48). An age-dependent hypomethylation of specific gene promoters, such as *IL17RC*, occurs and induces their transcription (49). At the same time, some gene promoters become hypermethylated and abnormally silenced (50–52). Regarding 5hmC, it was shown that although the global level of 5hmC in the brain increases with aging both in mice (53) and humans (54), it decreases in other tissues, such as blood (55).

Besides the epigenetic drift, which is stochastic non-site-specific changes in DNA methylation that contributes to variability during aging, DNA methylation signatures in specific CpGs, both tissue-specific and present in several tissue types, were identified as associated with chronological age (56). Although age-related DNA methylation alterations are more frequent in CpG islands, tissue-specific changes occur in other genomic regions (57). In a comprehensive study of DNA methylation, Yuan and colleagues (58) showed that besides hypermethylated CpG islands, a great number of age-related differentially methylated regions fell into open sea (regions of megabase extension characterized by low CG content) or shore/shelf regions, which were found hypomethylated with age. These authors have also identified large age-associated hypomethylated blocks, similar to those described associated with cancer (59). Based on the genome-wide methylation profile of whole blood from 656 individuals spanning a wide age range, a quantitative model was built to determine the rate at which an individual's methylome ages, and was shown to represent a strong and reproducible mean to discriminate relevant factors in aging (60).

Histone Modifications

The global DNA hypomethylation observed during aging was shown to be associated with changes in histone modification patterns (61, 62). Changes in the activity, function, and abundance of enzymes of the epigenetic machinery are present with aging (63, 64). Genes identified as hypermethylated in blood cells during aging were associated with the presence of bivalent chromatin domains in embryonic stem cells and with the repressive histone marks H3K27me3 and H3K9me3 in differentiated cells (65–67). A global loss of histones, as well as an imbalance of activating and repressive histone marks, occurs with age (68, 69). For example, a diminished content of acetylated H3K9 (70) and trimethylated H3K27 (71) was described in aged cells. Reduced levels of H3K9me3, which can be a result of the downregulation of SUV39H1/2 (72), were found with age in human and murine tissues and cells and seem to contribute to the loss of heterochromatin (72, 73). An age-decrease in the expression of HP1 (74) and DNMTs (75) could favor DNA demethylation in the heterochromatin. Another alteration that could contribute to a more opened chromatin state is the increased level of H4K16Ac with replicative age, as described in human fibroblasts in culture (76). H4K16 is among the targets of the NAD^+-dependent histone deacetylase SIRT1, which is associated with aging extent and genome maintenance in different organisms (77).

While the levels of the canonical histones decrease during aging, alterations in the replication-independent incorporation of histone variants occur during aging. The replication-independent histone variant H3.3 becomes more abundant with age in general, not just in non-replicating cells, such as neurons (78). Again, this could favor a chromatin state more accessible to the transcription machinery. Another replication-independent histone variant that seems to be linked to aging is the H2A.Z, since H2A.Z knockdown fibroblasts were shown to develop premature senescence (79). The H2A variant macroH2A is characteristic of senescence-associated heterochromatin foci, heterochromatin regions over proliferation-promoting genes in senescent cells (80). An age-dependent increase in the macroH2A level was described both during replicative senescence in cultured human fibroblasts and in many tissues of aged mammals (81).

Effects of the Environmental and Lifestyle Factors on Epigenetic Changes and Aging

A classical study by Esteller's group (82) showed significant differences in epigenetic marks in old monozygotic twin pairs

compared to very young twin pairs, which presented these marks indistinguishable. More interestingly, those old twin pairs that had spent less of their lifetime together and/or had a more different natural health-medical history were those presenting the greatest differences in epigenetic marks. Studies with human population have shown that genetic factors cause no more than 20–30% of the differences observed in the lifespan of identical twins, the epigenetic drift being the main responsible for variation during the lifetime (83, 84). These and other studies (85–88) illustrate how epigenetic marks change with aging and are under the effect of environment. As a whole, these alterations change the chromatin accessibility, resulting in abnormal gene transcription and genomic instability, and have been proposed to be key regulators of the aging process, contributors to the development of age-related diseases and even predictors of the chronological age (52, 89–93). Age-related changes in multiple CpG sites across the genome were shown to accurately predict the biological age of an individual. This epigenetic clock has been shown a potential biomarker of aging in humans and associated with several aging-related disease phenotypes (60, 90, 94, 95). Epigenetic age assessed in blood was able to predict, independently of chronological age, all-cause mortality in different cohorts, including different racial/ethnic groups (93, 95–97).

It is important to emphasize that the epigenome acts as a molecular interface between the genome and the environment. In this way, the lifestyle, including diet habits, exercises, life stressors, smoke, substance abuse, chemical exposition, among others, could alter the epigenetic landscape, affecting the chromatin structure and function, and, consequently, favoring the development of aging-related disease phenotypes. Exercise and nutritional habits remodel epigenetic marks in human skeletal muscle and adipose tissue (98–100). The effect of exercise on the improved cardiorespiratory fitness and running performance, as well as on the decreased low-density lipoprotein levels, was accompanied by a widespread demethylation of CpG islands, opposed of the methylation changes observed during aging (101, 102). Several studies have demonstrated the adverse effect of smoke associated with changes in epigenetic marks. Prenatal smoke exposure affects DNA methylation of blood cells from children of smoking mothers (103). Epigenetic alterations caused by chronic cigarette smoke sensitize bronchial epithelial cells to malignant transformation (104). Tobacco smoking may induce DNA methylation alterations in cell types of both the innate and adaptive immune system (105). Offspring DNA methylation alterations were associated with maternal alcohol consumption (106). The turnover of histones and histone variants was shown to be affected by the alcohol exposure in rats (107). Many of these effects of the environment on aging involve oxidative stress, both in humans and animal models. Although severe acute or chronic stress exposure accelerates aging by favoring error accumulation due to exhausting defense mechanisms, moderate stress has shown to delay aging process by activating defense mechanisms to prevent and/or eliminate errors (108). In the last years, several studies have demonstrated the relation between cellular stress and epigenetic alterations (104, 109–114). Reactive oxygen species (ROS) lead to oxidized DNA lesions that can contribute to DNA methylation alterations. One of the major DNA oxidative damage products is 8-hydroxy-2′-deoxy-guanosine that impairs binding of DNMTs and methyl-CpG binding proteins to DNA (115). In addition, ROS may interfere with TET-mediated DNA demethylation (116). SAM availability can also be decreased by the depletion of glutathione (GSH) because of redox status, inhibiting all methylation reactions (117). Sirtuins play important role in response to a variety of stresses, such as oxidative or genotoxic stress and are crucial for cell metabolism. ROS can both induce DNA damage and SIRT1 relocation to these damage sites, for where SIRT1 recruits other epigenetic machinery components, such as DNMTs and polycomb proteins in order to silence these regions. O'Hagan and coworkers (110, 112) showed that this process could result in stable aberrant epigenetic and gene transcription changes, similarly to alterations observed in cancer. In murine embryonic mesenchymal fibroblasts, increased levels of hydrogen peroxide induce SIRT1 to relocate from repressed DNA sequences to DNA breaks to promote repair, resulting in transcriptional changes that parallel those in the aging mouse brain (118). By responding to environmental stress, sirtuins promote cell survival and, as a result, increase replicative and chronological lifespan. Although not clearly established in mammals, the association of sirtuins with aging and lifespan is suggested by the overexpression of SIRT1 in murine tissues during caloric restriction (CR) (119), the requirement of Sirt1 to the increased physical activity and extended lifespan during caloric restriction (120), and the improved health and survival of mice submitted to a high-calorie diet after resveratrol treatment, which activates Sirt1 (121). Several studies in different model organisms show the role of sirtuins in lifespan extension by CR (122–125), and evidences indicate that epigenetic mechanisms have crucial roles in this process (126, 127). In this context, new and known compounds have been tested as "CR mimetics," including sirtuin-activating compounds, such as resveratrol (128). Compounds inhibiting histone acetylation, such as spermidine, also extend lifespan (129).

As mentioned before, ROS may modify the TET-mediated DNA demethylation (116). Both the increase in endogenous antioxidants and caloric restriction were shown to impair the increase in 5hmC levels in murine aged brains (130). The demethylase activity of TET enzymes can be stimulated by nutrients, such as ascorbic acid (131, 132). Since the activity of many epigenetic enzymes depend on intracellular levels of essential metabolites (methionine, iron, ketoglutarate, NAD^+, acetyl-CoA, SAM), the cellular metabolism controls epigenetic modifications and may regulate longevity (133, 134).

In another aspect, studies in human cohorts have shown that life stressors, in special during early development, can induce lasting epigenome alterations (135–139). Stress and glucocorticoids may induce long-lasting changes in DNA methylation both at the genome-wide level and within selective gene loci, as observed both in humans and rodent models (140–142).

EPIGENETIC REGULATION OF THE IMMUNE SYSTEM

An important characteristic of the immune system is its adaptive capacity to recognize self from non-self to protect the organism in

response to environmental signals of different types and duration, such as potentially pathogenic agents and substances. Several immune cell populations act against potentially hazard environment by both innate and adaptive mechanisms, and their functions depend on highly controlled regulation of hematopoietic cell differentiation. Increasing number of studies have demonstrated the crucial role of epigenetic mechanisms in the development and differentiation of immune system, as well as in related pathologies (143–145). With age, the immunocompetence becomes compromised and this has been linked to the repression of immune cell differentiation genes along with the activation of autoimmunity genes because of DNA methylation alterations (49, 146–148).

Innate Immune Cells

The innate immune system, consisting of macrophages, neutrophils, dendritic cells (DCs), and natural killer (NK) cells, is the first response to pathogenic agents. Macrophages and DCs are professional antigen-presenting cells (APCs) able to capture antigens for processing and presentation to lymphocytes. When activated, resident macrophages can act directly by destroying their targets or indirectly *via* initiating an acute inflammatory response by producing cytokines, chemoattractants, and inflammatory mediators, and recruiting neutrophils, monocytes, and DCs (149). Activated macrophages release different factors in response to the extracellular environment, being able to acquire functionally distinct phenotypes, classic M1 and alternative M2. Activated M1 macrophages are induced by the cytokine interferon-gamma (IFN-γ) and bacterial products and have a pro-inflammatory profile, playing an important role in host defense. Differently, M2 macrophages are induced by interleukin-4 and -10 (IL-4 and IL-10) and helminthic products and have an anti-inflammatory profile, promoting tissue repair. Since mature cells of the immune system have to rapidly respond to pathogens, the contribution of epigenetic mechanisms to the regulation of genes involved in these responses has been substantially described. In this context, epigenetic mechanisms were shown to be involved in the modulation of macrophage polarization, mainly by histone marks present in enhancers of specific genes (150). The first study showing the epigenetic regulation of inflammation was that by Saccani and Natoli (151). They demonstrated the induction of inflammatory cytokines, such as IL-8 and macrophage inflammatory protein 1-alpha (MIP-1α), by the loss of H3K9 methylation at the promoter regions after exposing cultured human monocyte-derived DCs to bacterial endotoxin lipopolysaccharide (LPS). Innate immune cells have a degree of specificity by presenting pattern recognition receptors (PRRs) to recognize damage- or pathogen-associated molecular patterns in non-infectious substances or microbes, respectively (152). Recent evidences indicate that, different from previously believed, cells of innate immune system may keep a memory of past stimulations, named "trained immunity," changing the response upon new stimuli and becoming able to respond to a larger number of microbes than the initial agent (153, 154). This immunological memory involves changes in transcriptional programs by reprogramming epigenetic marks. For example, metabolic changes in monocytes activated by β-glucan from *Candida* are associated with increased levels of the active histone marks, H3K4 trimethylation, and H3K27 acetylation, leading to increased production of IL-6 and TNF cytokines, inflammation, and "trained immunity" (155). Macrophages restimulated with LPS induce an attenuated inflammatory response, although maintaining an intact antimicrobial response. Foster and colleagues (156) showed that genes involved in LPS-tolerance lose the active histone marks H3K4me3 and H4Ac in their promoters during restimulation with LPS, while non-tolerizeable genes maintain these active marks after a secondary challenge with LPS, correlated with a permissive gene transcription. Epigenetic mechanisms also regulate the differentiation of human monocytes into DCs under specific stimuli. For example, the observed increased expression of CD209 during differentiation was shown to be a result of the acquisition of H3K9Ac and loss of H3K9me3, H4K20me3, and DNA methylation in its promoter (157).

T Lymphocytes

The age-dependent deterioration of the immune system, named immunesenescence, is accompanied by alterations in epigenetic marks. Kuwahara and colleagues (158) showed that CD4 T-cell senescence and cytokine homeostasis is controlled by the maintenance of histone acetylation on the *Bach2* locus promoted by the binding of menin. In addition, the increased genomic instability in the thymus with age is associated with a loss of heterochromatin marks, including H3K9me3 with corresponding reduction in SUV39H1 expression (159). The senescence seems to be also activated by DNA hypomethylation since the hypomethylation is observed in senescing but not in immortalized cells (160), and the DNA methylation inhibition leads immortal cells to cell arrest (161).

Cells from the innate immune system present antigens to both B and T lymphocytes, activating them to proliferate and differentiate into effector cells. APCs activate T cell receptor and costimulatory molecules of naïve T cells, initiating T cell differentiation by the activation of the nuclear factor of activated T cells and production of interleukin-2 (IL-2). IL-2 orchestrates the molecular switch of transcriptional programs of immune-responsive genes in response to T cell activation. Naïve and resting $CD4^+$ T cells do not express IL-2, but this cytokine is expressed in T cells under antigen stimulation. Murayama and colleagues (162) showed that demethylation of a single specific CpG site in an enhancer region is a prerequisite for IL-2 transcription and, more interestingly, that this epigenetic change constitutes a memory that $CD4^+$ T cells encountered the antigen.

Peptide antigens are presented by APCs to T cells in the context of the major histocompatibility complex (MHC) molecules. Cytotoxic T cells, expressing CD8, recognize antigens presented by normal cells in the context of MHC class I molecules, being able to directly destroy the infected cells. Activated $CD8^+$ T cells have increased levels of H3Ac at the *IFN-γ* promoter and enhancer, modification that is maintained through memory $CD8^+$ T cells, and permits a quicker and stronger cytotoxic response to additional antigen stimulation (163). MHC class II are the MHC molecules involved in the antigen presentation to $CD4^+$ helper T cells. The class II transactivator (CIITA) is a key factor controlling the expression of MHC-II, and both CIITA expression and CIITA-dependent MHC-II expression are epigenetically

regulated (164). Analysis of chromatin accessibility in PBMCs identified memory CD8+ T cells as the subpopulation with the most profound chromatin remodeling with aging (165, 166).

After antigen recognition, depending on cytokine environment, naïve T lymphocytes differentiate into effector T "helper" (Th1, Th2, and Th17) or regulatory (Treg) CD4+ T cells, and coordinate specific immune responses by producing distinct sets of cytokines (167). The differentiation toward a Th1 profile is induced by IFN-γ, IL-12, or IL-15, whereas differentiation toward the Th2 profile—by IL-4, IL-10, or IL-13; both pathways involve the regulated expression of multiple effector genes. Transforming growth factor beta and IL-6 are responsible for inducing naïve T cell differentiation into Th17 cells. The CD4+ T cells differentiation into these different profiles is tightly regulated to assure specific cytokine signatures and changes in the epigenetic marks are fundamental to complete this process. The *IFNG* promoter, hypermethylated in human naïve T cells, becomes demethylated during the differentiation into Th1 profile (168). Specific histone marks were identified across the *IFNG* locus, where H4Ac and H3K4me3 are present in Th1 cells and H3K27me2 and H3K27me3 in Th2 cells (169). Naïve and Th1 cells present *IL-4* promoter highly methylated, while Th2 cells have the intron 2 of *IL-4* partly demethylated (170). Th17 cells are characterized by the expression of IL-17 cytokine and RAR-related orphan receptor C (RORC) transcription factor. The demethylation of both *IL-17A* and *RORC* loci correlates with gene expression in human Th17 cells (171), and the active histone marks H3Ac and H3K4me3 were found in the *IL-17* locus (172). The demethylation of *Foxp3* locus, as well the hyperacetylation of histones, was shown to be important to maintain the stable expression of forkhead box P3 (FOXP3) and stabilize the regulatory phenotype in Treg cells (173, 174).

B Lymphocytes

After binding to an antigen and be induced by T helper cells, B cells differentiate into antibody-secreting plasma cells. Antibodies bind to the specific antigen, leading to a better recognition and destruction of the pathogen (such as bacteria, virus, and tumor cells) by activating complement and/or interacting with lytic cells. During B cell differentiation, lineage-specific genes are expressed, whereas genes related to multipotent progenitors and alternative lineages are repressed. Complex epigenetic regulatory mechanisms coordinate B cell differentiation and function, including monoallelic V(D)J rearrangement and antibody diversity (175–178). A key transcriptional factor involved in B cell commitment is paired box 5 (Pax5) that, besides having its expression regulated by epigenetic mechanisms (179, 180), recruits chromatin-modifying proteins to regulate the expression of its targets. For example, *CD79a* gene promoter, hypermethylated in the progenitor stage, becomes demethylated during early stages of B cell differentiation, followed by the action of histone acetyltransferases recruited by Pax5, allowing gene expression (181). Pax5 can also interact with chromatin-modifying enzymes to repress genes specific for other lineages (182). V(D)J rearrangement and antibody diversity are necessary for the production of effective antibodies and require the activation-induced cytidine deaminase (AID), expressed by B cells at specific stages of differentiation. In naïve B cells, *AID* gene promoter is hypermethylated and the gene is not expressed. Upon B cells activation, *AID* gene becomes demethylated and acquires increased levels of the active histone mark H3Ac (183). The acquisition of this histone mark in active promoters and distal enhancers is also crucial for gene expression changes occurring during the differentiation of B cells to plasma cells (184). Blimp-1, a transcriptional repressor that maintains plasma cell identity, has its expression epigenetically induced and epigenetically suppresses the expression of mature B cell genes by recruiting histone modifiers (185, 186). After V(D)J rearrangement and antibody diversity processes, B cells can differentiate into memory B cells, which acquire additional epigenetic marks beyond those acquired during B cell activation (187). Different epigenetic modifications, as well as epigenetic enzymes, such as enhancer of zeste homolog 2 (188), histone acetyltransferase monocytic leukemia zinc finger protein (189), and DNMT3a (190), are observed in resting and activated B cells, and indicate that the memory B cell epigenome could favor a faster and more efficient activation than that of naïve cells.

CONTRIBUTION OF EPIGENETIC ALTERATIONS TO IMMUNE AGING

Age-associated defects are observed in all cells from the immune system, affecting their activation and cytokines production.

Innate Immune Cells

Regarding the innate immune system, many immune responses decrease during aging, but at the same time hyperreactivity of some responses are also observed (191). Epigenetic alterations seem to affect the monocyte differentiation with age, since older hematopoietic progenitor cells (HPCs) present hypomethylation of differentiation-related genes compared to progenitor cells from umbilical cord blood (192). It could be related to the reduced pluripotency and decreased potential of differentiation of HPCs from older donors (193). At the same time, older HPCs presented *de novo* methylation of a subset of genes associated with the Polycomb repressive complex that could contribute to the reduced phenotypic plasticity of aged stem cells (192). Indeed, epigenetic dysfunction could be a precursor to hematologic disease in elderly individuals (194). In macrophages, epigenetic mechanisms contribute to the decreased expression of MHC-II observed with age (195). Although the number of NK cells increases in older individuals, their cytotoxic activity decrease, and DNA methylation regulation of IFN-γ and IL-2 seems to contribute to this defected function of NK cells (196). Aging is well characterized by an imbalance between inflammatory and anti-inflammatory responses, where increased levels of inflammatory mediators, such as IL-6 and tumor necrosis factor-alpha (TNF-α), are observed even in the absence of acute infection or other physiologic stress (process known as "inflammaging") (197). TNF-α has its expression increased during aging linked to its promoter demethylation (198). This epigenetic alteration contributes to the increased levels of TNF-α and also IL-1α (199, 200), which initiate the low-grade inflammation associated

with resting neutrophils from aged donors. A major cause of worldwide morbidity in the elderly is the age-associated inflammatory lung disease (201). In this context, promoter hypomethylation of inflammatory genes, such as toll-like receptor 2, carnitine *O*-acetyltransferase, and coagulation factor III, were associated with decreased lung function (202). Zinc is a micronutrient crucial for the development and function of immune system, and its deficiency, frequently observed during aging, contributes to a wide range of immune defects (203), including an enhanced inflammatory response by inducing *IL-6* promoter demethylation (204). Using the C-reactive protein (CRP) as an inflammatory biomarker, Ligthart and coworkers (205) performed a meta-analysis of epigenome wide association studies of DNA methylation on chronic low-grade inflammation. In this study, the authors demonstrated that several inflammation-related CpG sites were associated with the expression of nearby genes, and that many of these CpGs presented association with cardiometabolic phenotypes and incident coronary heart disease. Among these genes is *AIM2*, important in innate immune response since it takes part of host defense mechanisms against bacterial and viral pathogens, and that was found hypermethylated and expressed at low levels in samples with low levels of CRP.

T Lymphocytes

The involution of thymic structure and function, characterized by a reduced number and functional defects of thymic naïve T cells, is other process contributing to the immune aging (206). By analyzing the methylome of CD4$^+$ T cells from newborn and centenarian individuals, Heyn and coworkers (207) showed these immune cells present the same DNA methylation changes that are observed in other tissues during aging, a global DNA hypomethylation and a higher variability of DNA methylation. Later, by an integrated analysis of transcriptome, methylome, and miRNAome in the same CD4$^+$ T cells, Zhao and colleagues (148) found a potential relationship between gene transcription and DNA methylation for age- or immune-related genes, indicating the involvement of DNA methylation in the transcription regulation related to the development and functions of T cells in aging. Mice with a heterozygous *Dnmt1* null mutation have hypomethylated DNA and showed to be phenotypically normal, but presented immune senescence and developed early autoimmunity compared with normal mice of the same age (208). By analyzing naïve CD4$^+$ T cells from 74 healthy 19- to 66-year-old individuals, Dozmorov and colleagues (209) identified sites hypomethylated with age presenting T cell-specific enrichment in active enhancers marked with H3K27Ac and H3K4me1, suggesting a progressive age-associated shift in T-cell epigenomes toward pro-inflammatory and T cell activating phenotype that could contribute to increased autoimmunity with age. It was also shown that aged individuals, who have higher levels of autoantibodies, have T cells presenting demethylation and overexpression in the same genes demethylated and overexpressed in T cells from lupus patients (146). The progressive loss of the costimulatory molecule CD28 in CD4$^+$ T lymphocytes during aging is associated with impaired immune response. Recently, a unique DNA methylation landscape was described in CD28^{null} T cells, leading to the expression of inflammasome-related genes (210). Other recent study found two CpG sites present in the promoter region of *KLF14*, involved in CD4$^+$ T cell differentiation *via* suppression of FOXP3, that exhibit stable methylation early in life and a rapid increase late in life in peripheral whole blood, monocytes, and isolated CD4$^+$ T cells (211). Dysfunctional Treg cells have been considered to be contributors to immune senescence and increased susceptibility to age-associated diseases by suppressing T cell responses. Garg and colleagues (212) showed that the high number of Treg cells observed in aged mice is associated with hypomethylation of the upstream *FoxP3* enhancer, resulting in its increased expression. They also demonstrated that Treg cells from aged mice release more IL-10, are more efficient in downregulating the costimulatory molecule CD86 on DCs, and modulate the extracellular redox environment, suppressing T cells proliferation.

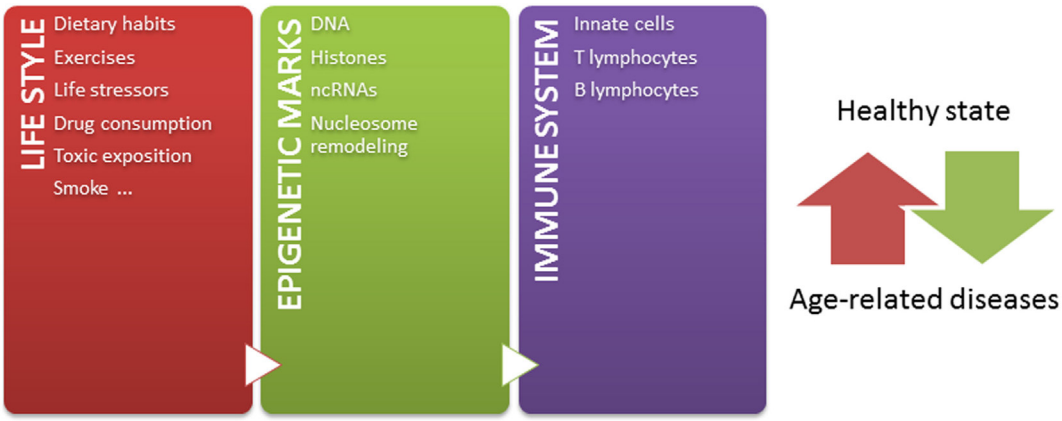

FIGURE 1 | The epigenetic landscape, modulated by intrinsic and extrinsic factors, regulates immune system and contributes to define a healthy or pathological state. Our life style, including nutritional habit, exercises, life stressor, drugs, toxics exposition, and smoke, dynamically sculpts our epigenome along lifetime. The immune system is one of the systems in which functions are importantly regulated by epigenetic mechanisms. In this way, the accumulation of abnormal epigenetic marks in immune cells might contribute to the development of age-related diseases.

Immune senescence is also characterized by a loss of naïve and central memory cells and an expansion of effector memory cells within the $CD8^+$ T cell compartment. A shift toward more differentiated state of chromatin openness was observed in naïve and central memory cells from older individuals, as well a loss of chromatin accessibility at gene promoters mediated in part by the loss of nuclear respiratory factor 1 (NRF1) binding in aged naïve cells (213). By analyzing PBMCs methylation data set in an Italian population, Horvath and colleagues (214) showed that the centenarians are younger than expected based on their chronological age. McEwen and colleagues (215), by examining one of the highest old-age life expectancies populations from Costa Rica (Nicoyans), found this population to possess a significant higher abundance of predicted $CD8^+$ T naïve cells and a lower abundance of estimated $CD8^+$ T memory cells compared with non-Nicoyans, suggesting a younger immune cell profile. In addition, they showed a lower variability in the DNA methylation in Nicoyans compared with non-Nicoyans as an epigenetic characteristic of the longevity in this population.

B Lymphocytes

Considering the role of epigenetic mechanisms in B cell differentiation and function, age-associated epigenetic changes could be the responsible for the decline of humoral immunity in elderly individuals. Loss of function of B cells and their progenitors, reduction in the immunoglobulin diversity and affinity, and shifts in the proportion of naïve and antigen-experienced peripheral B cells subpopulation are characteristics of immune aging (216, 217). Hematopoietic stem cells (HSCs) lose their capacity to differentiate with age, and epigenetic alterations are important contributors to this change. Aged mice present HSCs with an aberrant gene expression profile because of epigenetic deregulation (218). Defects on both B-lineage commitment and transit through early development stage are observed during aging (219).

CONCLUSION AND PERSPECTIVES

Considerable progress has been made in understanding epigenetic alterations involved in aging over the recent years. Most of recent knowledge about epigenetics and aging is almost lack regarding immune aging. Many gaps and questions are still open and should be deeply investigated in this area. However, there are also important challenges and limitations in this study. For example, several analyses in this field use samples contained mixed cell types, instead isolated cells, which may bring confusion in the data interpretation. The dynamic nature of the immune system *per se* becomes challenging to study plastic molecular alterations involved in immune responses. It remains to be determined which epigenetic changes are causally related to aging process, and how they cause immune aging. Future studies are needed to determine the overlapping epigenetic signatures between immune aging and age-related immune diseases.

Independently of these challenges, and taking into account that: (1) epigenetic mechanisms modulate chromatin states, defining gene expression profiles, (2) epigenetic mechanisms play crucial roles in the development and function of immune system, (3) a tightly regulated functioning of immune system is necessary to maintain a healthy state, (4) the environment modifies epigenetic marks throughout lifetime, and (5) epigenetic marks are potentially reversible, the knowledge about how the environment modulates the immune system by epigenetic mechanisms contributing to age-related diseases may lead to the design of novel strategies for prevention and therapeutics. Since some age-related epigenetic alterations are similar across a range of tissues (52, 220), these alterations could be also potentially used as biomarkers for aging-related disease phenotypes in biological samples, such as blood or saliva. But most importantly, considering that both intrinsic and external factors modify epigenetic marks throughout life, it is important to have in mind that healthier lifestyle may be still the most effective way to prevent diseases later in life (**Figure 1**) (221). In conclusion, huge efforts should be undertaken to better understand the relation among epigenetics, immune aging and age-related diseases, in order to define interventions in the lifestyle able to modulate our epigenome for a healthy aging.

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The author confirms being the sole contributor of this work and approved it for publication.

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Proximity of Cytomegalovirus-Specific CD8+ T Cells to Replicative Senescence in Human Immunodeficiency Virus-Infected Individuals

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Antiretroviral therapy (ART) effectively extends the life expectancy of human immunodeficiency virus (HIV)-infected individuals; however, age-related morbidities have emerged as major clinical concerns. In this context, coinfection with cytomegalovirus (CMV) accelerates immune senescence and elevates risk for other age-related morbidities, possibly through increased inflammation. We investigated potential relationships between CMV memory inflation, immune senescence, and inflammation by measuring markers of inflammation and telomere lengths of different lymphocyte subsets in HIV-infected individuals seropositive for anti-CMV antibodies. Our study cohort consists mainly of middle aged men who have sex with men (MSM) and heterosexuals who are stable under long-term ART. Median levels of IL-6, TNF-α, and CRP were significantly higher in those coinfected with CMV. Lymphocyte telomere length in general correlated with age, but for 32/32 subjects tested, there was a consistent hierarchy of telomere lengths with CD8+ T cells' shorter than the general lymphocyte population, CD57+CD8+ T cells' shorter than CD8+ T cells' and CMV-specific CD57+CD8+ T cells' the shortest of all. Telomeres of HIV-specific CD8+ T cells were longer than those of CMV-specific CD8+ T cells in all cases tested and over 10 years, CMV-specific CD8+ T cell telomeres of two HIV-infected individuals eroded faster than those of HIV-specific CD8+ T cells. These data indicate that CMV-specific CD8+ T cells of HIV-infected individuals are the lymphocytes closest to telomere-imposed replicative senescence. Exhaustive proliferation of CMV-specific CD8+ T cells in HIV-infected individuals is a potential source of senescent lymphocytes affecting systemic immune function and inflammation.

Keywords: cytomegalovirus, human immunodeficiency virus, CD8+ T cell, senescence, telomere length, inflammation

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INTRODUCTION

Infection with cytomegalovirus (CMV) is very common and usually problematic only in cases of congenital infection, immune suppression, or immune deficiency (1–5). However, following primary infection, human (H)CMV persists for life in latently infected cells with periodic reactivation contained by the immune system. Immune responses against CMV are generally robust and often

undergo a process termed memory inflation, in which their magnitude increases disproportionately with age, compared to responses against other persistent viruses (6–9). In some old elderly individuals (>80 years), HCMV memory inflation produces an "immune risk profile" (IRP), characterized by pronounced accumulation of terminally differentiated HCMV-specific $CD8^+$ T cells (10). The IRP manifests a $CD4^+/CD8^+$ T cell ratio below 1.0 and is accompanied by phenotypic and functional signs of immune senescence that signify elevated risk for all-cause morbidity and mortality (11–13). Broader population-based studies also associate HCMV infection with development of age-related morbidities, especially cardiovascular disease (14, 15). These findings raise the possibility that either through pro-inflammatory effects of viral reactivation or through long-term influence on immune system character, persistent HCMV infection promotes development of age-related morbidities.

Human immunodeficiency virus (HIV) infection, even when effectively controlled by combination antiretroviral therapy, accelerates HCMV immune memory inflation. This is reflected in earlier, more pronounced expansion of HCMV-specific $CD8^+$ T cells and in greater $NKG2C^+CD57^+$ natural killer (NK) cell accumulation (16–18). Given the relationship between CMV immunity and all-cause mortality in the old elderly, the question arises as to whether the more pronounced immunological influence of HCMV in HIV infection relates to the more frequent and earlier onset of age-related morbidities in HIV infection (19–25). Recent studies indicate that HCMV coinfection is associated with more inflammation, less immune reconstitution, a history of greater $CD8^+$ T cell proliferation, increased vascular intimal-medial thickening, and higher incidence of severe, non-AIDS neurovascular events in HIV infection (17, 26–32). However, any role for CMV-specific immunity as either marker or determinant of this association has not been directly examined.

We previously measured the frequency of T cell receptor excision circles (TREC) in peripheral blood T cell subsets of HIV-infected individuals and observed a lower median TREC frequency in $CD8^+$ T cells of those coinfected with HCMV (32). There was also a significant inverse correlation between the size of CMV-specific $CD8^+$ T cell responses and TREC frequency, suggesting a link between the exaggerated immune response against CMV and exhaustive T cell proliferation in HIV infection (32). In this follow-up study, we investigated the possibility of a direct link between CMV-specific immunity, $CD8^+$ T cell proliferation, lymphocyte senescence, and inflammation. We first measured and compared plasma markers of inflammation in CMV-seropositive and seronegative HIV-infected individuals to assess any association between CMV seropositivity and apparently sterile systemic inflammation. We then selectively examined the telomeres of CMV-specific $CD8^+$ T cells and other lymphocytes from a subset of these individuals for progress toward replicative senescence and potential acquisition of a senescence-associated secretory phenotype (SASP) (33). Our results affirm that CMV infection is associated with inflammation in HIV-infected individuals and position CMV-specific T cells at the leading edge of lymphocyte progression toward exhaustive proliferation and cellular senescence.

MATERIALS AND METHODS

Study Subjects and Sample Collection

Subjects infected with HIV were recruited through the Newfoundland and Labrador Provincial HIV Clinic. All but several were MSM or heterosexual caucasians of western European descent with very few intravenous drug users. All subjects provided informed consent for whole blood collection, immunological studies, and researcher access to medical laboratory records. The Newfoundland and Labrador Health Research Ethics Authority approved this study. Routine clinical assessment with lymphocyte subsets including $CD4^+$ and $CD8^+$ T cell counts and viral load was performed at least once every 6 months. Prior to each clinic visit, whole blood was collected by forearm venipuncture into vacutainer tubes containing acid-citrate-dextrose anticoagulant. Plasma was collected following room temperature centrifugation of whole blood for 10 min at 400 × *g*, aliquoted and stored at −80°C until testing. Packed cells were diluted to two times the original blood volume with phosphate-buffered saline (PBS) and then peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) density gradient centrifugation, washed once in PBS with 1% fetal calf serum (FCS), and resuspended in lymphocyte medium comprising RPMI 1640 with 10% FCS, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer solution, and 2.0×10^{-5} M 2-mercaptoethanol (all from Invitrogen, Carlsbad, CA, USA). Freshly isolated PBMC were resuspended at a minimum of 1.0×10^7 cells/mL in freezing medium composed of lymphocyte medium with 10% dimethyl sulfoxide supplemented to 20% FCS and cooled at 1°C/min overnight to −80°C. Frozen PBMC were then maintained in liquid nitrogen until analysis. To thaw cells, cryopreserved PBMC were immediately immersed in 37°C water bath, gently agitated until almost thawed, then immediately transferred into, and washed three times in, 10 mL lymphocyte medium. Cells were then resuspended at 2.0×10^6 cells/mL in lymphocyte medium and allowed to recover overnight at 37°C, 5% CO_2. Cells were counted after recovery and used only when >70% were viable by trypan blue exclusion.

Measurement of Anti-HCMV Antibodies

Subjects were separated into CMV-seropositive and CMV-seronegative groups based on the presence or absence of CMV-specific antibodies in plasma. The presence of CMV-specific IgG antibodies was assessed as previously described (18). Briefly, antibodies against HCMV were measured in plasma samples by ELISA against CMV AD169-infected MRC-5 cell lysate. To generate lysate, 1×10^7 MRC-5 cells were infected with CMV AD169 at a multiplicity of infection of 0.5 and after 3 days were harvested by scraping, pelleted by centrifugation, and lysed in 1 mL lysis buffer. Lysate diluted 1/1,000 in carbonate/bicarbonate coating buffer was added in 100 μL overnight at 4°C to wells of Immunlon-2 ELISA plates (VWR Scientific, Mississauga, ON, Canada). Lysate prepared as above from uninfected MRC-5 cells was used as control. Plasma samples diluted 1/500 were

incubated on the plates for 90 min, washed and developed with goat-anti-human IgG-horseradish peroxidase conjugate (Jackson ImmunoResearch Labs, West Grove, PA, USA) followed by tetramethylbenzidine substrate (Sigma-Aldrich). Color development ran for 30 min at room temperature, after which the reaction was stopped with 1 N H_2SO_4 and optical density read at 450 nm. CMV AD169 was obtained through the NIH AIDS Reagent Program, AIDS Program, NIAID, NIH from Dr Karen Biron (34). MRC-5 cells were a kind gift of Dr. K. Hirasawa, Division of BioMedical Sciences, Faculty of Medicine, Memorial University of Newfoundland.

Measurement of Inflammatory Markers in Plasma

Fresh plasma was aliquoted to avoid repeat freeze/thaw cycles. Commercial ELISA kits were used to measure the following analytes in plasma as per the manufacturers' instructions. Kits for measuring interleukin (IL)-1β (range = 2.00–200.00 pg/mL), IL-6 (range = 2.00–200.00 pg/mL), and tumor necrosis factor (TNF)-α (range = 4.00–500.00 pg/mL) were from eBioscience, San Diego, CA, USA. Kits for measuring C-reactive protein CRP (range = 15.60–1,000.00 pg/mL) were from R&D Systems, Minneapolis, MN, USA. The sensitivity ranges covered physiologically appropriate levels such that plasma was added neat to the assay, except for CRP where plasma was diluted 1:10,000 with PBS. All measurements were carried out in duplicate with control wells containing only PBS included in each assay. The OD value in control wells was subtracted from all test values to adjust for background. Absorbance was measured at 450 nm on a Biotek synergy HT ELISA reader and standard curves generated as specified.

Peptide Stimulation of PBMC and Surface Marker Staining

All study participants had previously been tested for $CD8^+$ T cell responses against CMV pp65 and immediate early-1 (IE-1) proteins separately using overlapping peptide sets (Peptivator, Miltenyi Biotec, San Diego, CA, USA) (18, 32). Most had also been tested for responses against individual HIV proteins with overlapping peptide sets (NIH AIDS Reagent Program, Germantown, MD, USA). Subjects with sufficiently strong responses for visualization by intracellular flow cytometry were selected to identify CMV-specific or HIV-specific $CD8^+$ T cells for telomere measurement. If responses against both CMV IE-1 and pp65 were present, then PBMC were stimulated with the protein pools in aggregate and responses against the two proteins were not distinguished at this point. Aliquots of 2×10^6 PBMC in 1 mL lymphocyte medium were stimulated with pooled CMV-pp65 (0.5 μg/mL) and IE-1 (0.5 μg/mL) peptide sets or overlapping HIV-1 Nef (1.0 μg/mL) and Gag (1.0 μg/mL) peptides (NIH AIDS Reagent Program, Germantown, MD, USA), for 60 min at 37°C (5% CO_2). Brefeldin A (Sigma-Aldrich, St. Louis, MO, USA), was then added for a final concentration of 10.0 μg/mL and the PBMC left for an additional 4 h before being stained for surface markers and intracellular interferon-gamma (IFN-γ). Cells were washed twice in flow cytometry buffer consisting of PBS with 0.2% NaN3, 5 mM EDTA (Sigma-Aldrich), and 0.5% FCS, then incubated with fluorescein isothiocyanate-conjugated anti-human CD57 (TBO3, Miltenyi Biotec) and Quantum Dot 705-conjugated anti-human CD8 (3B5; Invitrogen) for 20 min at 4°C. Samples were kept in the dark from this point on. After another wash with flow buffer, cells were fixed and permeabilized with InsideStain (Miltenyi Biotec) according to manufacturer's instructions and intracellular staining with allophycocyanin-conjugated anti-human IFN-γ (4S.83, eBioscience) was carried out.

PBMC Subset Telomere Length Measurement by Flow Cytometry

Measurement of telomere length by flow cytometry was carried out with minor adaptation of a previously described protocol (35). Following surface and intracellular staining, samples were resuspended in 250 μL 1% FCS-PBS on ice. To increase the stability of antigen–antibody–conjugate complexes, 7.5 mM bissulfosuccinimidyl suberate (BS_3) crosslinking solution (ThermoFisher Scientific, Waltham, MA, USA) in PBS was added to a final concentration of 5 mM and incubated on ice for 30 min. Residual BS3 was quenched with 3 mL quenching solution (100 mM Tris–HCl, 50 mM NaCl in PBS). Samples were incubated on ice for a further 20 min and standard 1301 T leukemia cells (Sigma-Aldrich) were added at a ratio of 1:4 to sample PBMC. Samples were then washed in flow buffer, split into two aliquots, and transferred to fresh 1.5 mL microcentrifuge tubes, for a probed and unprobed control sample. Samples were pelleted and all but 100 μL supernatant removed *via* pipette to ensure pellet stability. Then, 250 μL of hybridization solution (70% formamide, 30 mM Tris–HCl, 0.2 M NaCl, 1.5% BSA) was added and samples were resuspended with a wide bore 1 mL pipettor and incubated for 10 min at RT. All subsequent resuspensions were done in this manner to avoid unnecessary shear force on fragile samples. Samples were centrifuged at 1,600 × *g* to ensure optimal pellet formation in formamide without compromising cellular integrity. All but 100 μL of supernatant was again removed *via* pipette. Samples were then resuspended in 250 μL hybridization solution with or without the addition of 0.75 μg/mL TelC-Cy3 $(AATCCC)_3$ (Panagene Inc., Daejeon, Korea). An unprobed control to allow correction for formamide-related auto-fluorescence that may artificially increase Cy3 fluorescence was run with every sample. All aqueous reagents were verified pH 7.2 and sterile filtered through a 0.45 μm nylon filter prior to formamide addition.

Samples were then incubated at 84°C for 10 min, placed on ice for 5 min and left to hybridize in a dark chamber for 2 h at RT. Samples were then diluted 3:1 with a post-hybridization solution (70% formamide, 15 mM Tris–HCl, 0.2 M NaCl, 0.15% BSA, 0.15% Tween-20) and centrifuged at 1,600 × *g*. All but 100 μL of supernatant was removed *via* pipette and samples were washed twice with 1% BSA, 0.5 mM EDTA in PBS, centrifuging first at 900 × *g* and then at 500 × *g* to ensure maximal removal of formamide. Samples are then resuspended in the same wash solution and analyzed immediately with a FACSCalibur Cell Analyzer (BD

Biosciences, San Jose, CA, USA). A minimum of 1×10^5 events were acquired per sample.

Calculation of Telomere Length

Although the standard cells were always run together with test samples, representing an internal standard in each telomere length assay, a probed and unprobed sample of 1301 standard cells was also run separately to calculate intra-assay variation in the mean fluorescence intensity (MFI) measured for the standard cells. The SD in geometric MFI for the 1301 cells across 32 assays was 16%. Using Cy3 MFI of the 1301 control cell line and of the sample subsets, the relative and absolute telomere length of the subset is calculated using the known 1301 telomere length [23,480 base pairs (bp)] and the following formula.

$$\text{Relative Telomere Length (RTL)} = \frac{((\text{MFI}_{\text{Cy3}}(\text{Sample}_{\text{probe}}) - \text{MFI}_{\text{Cy3}}(\text{Sample}_{\text{unprobed}})) \times \text{DNA Index of} 1301)}{((\text{MFI}_{\text{Cy3}}(1301_{\text{probe}}) - \text{MFI}_{\text{Cy3}}(1301_{\text{unprobed}})) \times \text{DNA Index of}_{\text{Sample}})} = \frac{\text{SampleTL}_{\text{MFI}}}{1301\text{TL}_{\text{MFI}}}$$

$$\text{Exact Telomere Length(TL)} = \frac{\text{SampleTL}_{\text{MFI}}}{1301\text{TL}_{\text{MFI}}} \times 1301\text{TL}_{\text{bp}}$$

Statistical Analysis

Statistical analyses were carried out using Prism version 6 (GraphPad Software, Inc., La Jolla, CA, USA). Normal distribution of data was assessed by the Shapiro–Wilk test. If deviation from normal distribution was indicated (all TL results, IL-1β, IL-6, TNF-α), data were represented with median ± interquartile range (IQR) and group medians compared by Mann–Whitney test or Wilcoxon-signed rank test. Sex distribution in CMV-seropositive compared to CMV-seronegative groups was compared by Fisher's exact test. Spearman correlation was used to assess relationships between variables. If data were normally distributed (age, $CD8^+$ T cell response), mean ± SD was calculated and Student's *t*-test was used to compare means. Relationships were assessed using Spearman correlation matrices.

RESULTS

Markers of Inflammation in HIV-Infected Groups Distinguished by CMV Infection Status

After excluding individuals coinfected with hepatitis C virus (HCV), we measured levels of IL-1, IL-6, TNF-α, and CRP in plasma samples from 153 HIV-infected individuals characterized for CMV infection status, age, gender, duration of infection, antiretroviral therapy (ART), lymphocyte subset counts, plasma HIV load, CMV-specific T cell immunity, and T cell subset TREC frequency. Of the 153 HIV-infected subjects included, 134 were seropositive for anti-CMV antibodies and 20 of these 134 had at least 1 detectable HIV plasma virus load in the 12 months immediately preceding testing. Three of the 19 individuals seronegative for CMV had at least 1 detectable HIV plasma virus load within the 12 months immediately preceding testing. General demographic characteristics of the subjects with comparisons between the CMV-seropositive and seronegative groups are shown in **Table 1**. Detectable HIV replication was considered any level above the detection limit of the commercial assays used, usually 50 copies HIV RNA/mL plasma, at any one time point within 12 months of the plasma collection for inflammatory marker measurement. Whether individuals with detectable HIV replication within the last 12 months of clinical plasma viral load testing were excluded or not, median IL-6, TNF-α, and CRP levels were significantly higher in the subset coinfected with CMV (**Figures 1C–H**). Median levels of IL-1β were only significantly higher in the CMV-infected group when those individuals with detectable HIV replication within the last 12 months were included in the analysis (**Figures 1A,B**). There were significant correlations between IL-6 levels and age ($r = 0.484$, $p = 0.007$) and between CRP levels and age ($r = 0.403$, $p = 0.027$), but there was no significant difference between median ages of the CMV-seropositive [48 years, interquartile range (IQR) 44–54] and seronegative groups (44 years, IQR 42–50). These data show a broad range in levels of common markers of inflammation in HIV-infected individuals with increased median levels of pro-inflammatory cytokines IL-6 and TNF-α and of the acute-phase protein CRP in the group coinfected with CMV. These differences in inflammatory markers were independent of detectable HIV replication within the 12 months immediately preceding testing.

TABLE 1 | General characteristics of study cohort and comparison of cytomegalovirus (CMV)-infected and uninfected.

	CMV-seronegative		CMV-seropositive		*p*
n (% undetectable human immunodeficiency virus at time of testing)	19	(84%)	134	(77%)	0.5459
n (% male)	11	(61%)	103	(76%)	
n (% female)	8	(39%)	31	(24%)	0.0929
Age (years), median interquartile range (IQR)	44	(42–50)	48	(44–54)	0.1165
β-2 microglobulin (μg/mL), median (IQR)	2.63	(2.06–3.24)	2.64	(2.08–3.33)	0.9082
% CMV-specific $CD8^+$ T cells, mean (SD)	0.01	(0.03)	3.94	(4.15)	<0.0001
Duration of antiretroviral therapy (years), median (IQR)	13	(8–19)	15	(10–19)	0.3753
Nadir $CD4^+$ T cells/μL blood, median (IQR)	190	(86–325)	234	(121–404)	0.3655
$CD4^+$ T cells/μL blood, median (IQR)	742	(522–780)	648	(419–777)	0.7689
$CD8^+$ T cells/μL blood, median (IQR)	648	(442–770)	869	(643–1,217)	0.0093
$CD4^+$:$CD8^+$ T cell ratio, median (IQR)	1.05	(0.86–1.64)	0.68	(0.44–0.92)	0.1273

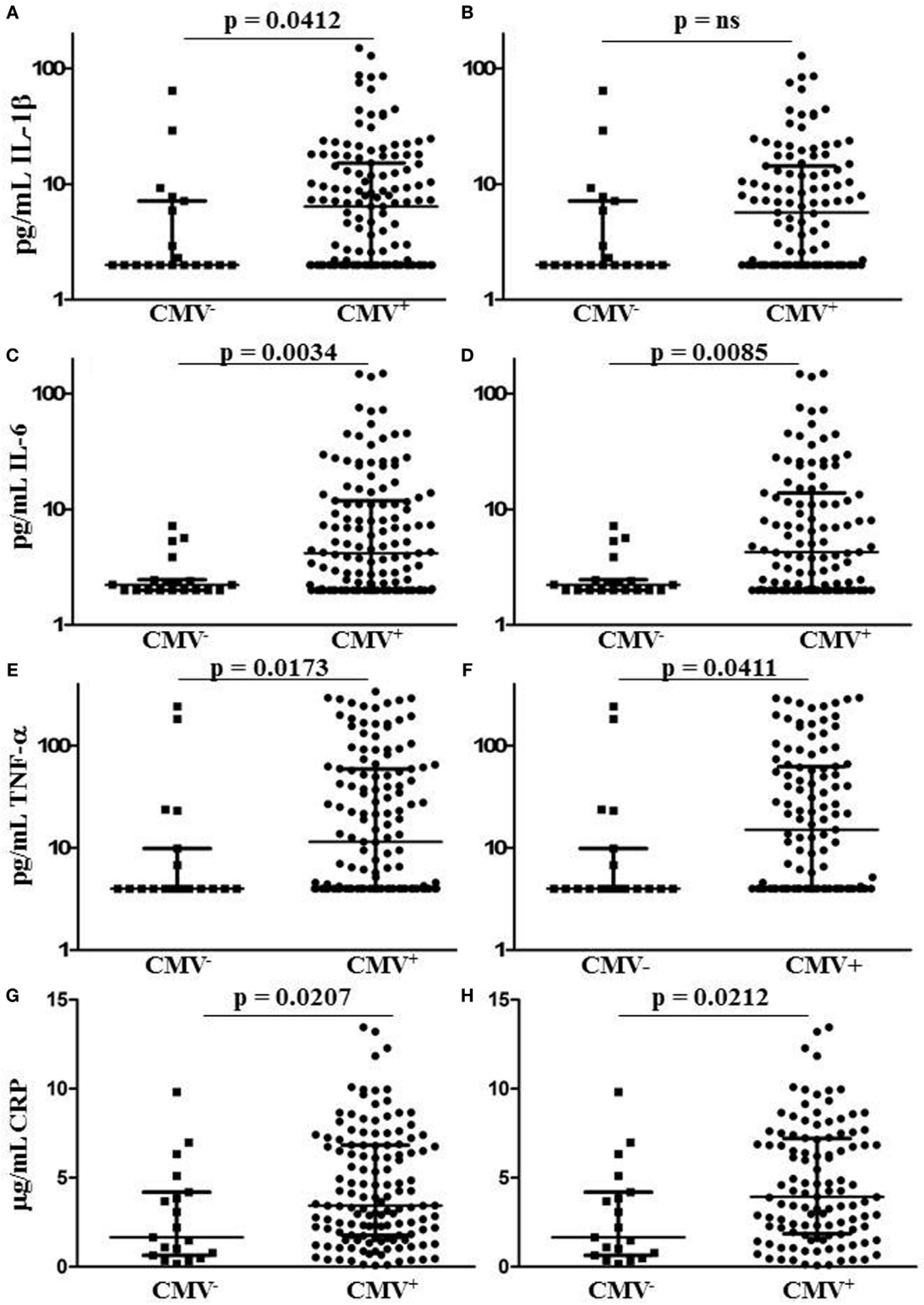

FIGURE 1 | Plasma levels of CRP and pro-inflammatory cytokines IL-1, IL-6, and tumor necrosis factor (TNF)-α in human immunodeficiency virus (HIV)-infected individuals grouped by cytomegalovirus (CMV) seropositivity status. Plots **(A,C,E,G)** show results for all HIV-infected individuals tested, while plots **(B,D,F,H)** show only those individuals with no detectable HIV plasma virus load within the 12 months immediately preceding testing. Horizontal lines bisecting the groups show median values for each measure with interquartile range shown above and below. Significant differences between medians (Mann–Whitney test) are shown above lines spanning the groups compared.

In our HIV-infected study cohort, coinfection with CMV is associated with greater inflammation, unrelated to clinically apparent effects on HIV replication.

Lymphocyte Subset Telomere Lengths in HIV-Infected Individuals

To compare the impact of proliferative history on different lymphocyte subsets in relation to CMV immunity and inflammation, we measured telomere length by fluorescence *in situ* hybridization (FISH) flow cytometry in lymphocytes of a representative set of HIV-infected individuals seropositive for CMV-specific antibodies (**Table 2**). These individuals had cellular immune responses against CMV ranging from 0.2 to 32% of their CD8$^+$ T cells. Sequentially more exclusive analysis gates were applied starting with lymphocytes and proceeding through CD8$^+$ lymphocytes, CD8$^+$CD57$^+$ lymphocytes, and antigen-specific CD8$^+$CD57$^+$ lymphocytes identified by intracellular IFN-γ production (**Figure 2**). Telomere length in nucleotide bp was estimated by the ratio of subset telomere probe geometric MFI to the geometric MFI of the telomere probe hybridized to the 1301 standard control (27.5 kb telomeres) cell line analyzed concurrently (**Figure 3**). There was a broad range of telomere lengths in subjects tested with a significant correlation between lymphocyte telomere length and age ($r = -0.424$, $p = 0.017$, **Figure 4A**). The CD8$^+$ T cells had shorter telomeres than the rest of the lymphocyte population ($p = 0.0018$, **Figure 4B**) and CD57$^+$CD8$^+$ T cells had shorter telomeres than CD8$^+$CD57$^-$ T cells ($p < 0.0001$, **Figure 4C**). These data affirm results of previous lymphocyte subset telomere length assessment studies in HIV infection and validate our application of the FISH flow cytometry assay for telomere length in this setting (36–38).

Telomere Lengths of CMV-Specific CD8$^+$ T Cells

To position CMV-specific CD8$^+$ T cells along the lymphocyte telomere length continuum for each individual and address the potential relationship between CMV-specific immunity and exhaustive CD8$^+$ T cell proliferation, we used CMV peptides to stimulate PBMC from HIV-infected individuals seropositive for CMV prior to FISH. We then compared telomere lengths of CMV-specific CD8$^+$CD57$^+$ T cells producing IFN-γ and the remaining CD8$^+$CD57$^+$ T cells. In 32/32 cases tested, the telomeres of CMV-specific CD8$^+$CD57$^+$ T cells were shorter, by several hundred to several thousand bp (**Figure 4D**, $p < 0.0001$).

TABLE 2 | General characteristics of cytomegalovirus (CMV)-seropositive subjects selected for telomere length analysis.

n (% undetectable human immunodeficiency virus at time of testing)	32	(74)
n (% male)	24	(75)
n (% female)	8	(25)
Age (years), median [interquartile range (IQR)]	50	(47–59)
% CMV-specific CD8$^+$ T cells, mean (±SD)	4.35	4.00
Duration of antiretroviral therapy (years), median (IQR)	15	(8–20)
Nadir CD4$^+$ T cells/μL blood, median (IQR)	218	(60–304)
CD4$^+$ T cells/μL blood, median (IQR)	651	(492–763)
CD8$^+$ T cells/μL blood, median (IQR)	888	(620–1,226)
CD4$^+$:CD8$^+$ T cell ratio, median (IQR)	0.67	(0.45–0.96)

To compare telomere lengths of CMV-specific T cells with another antigen-specific T cell subset, we stimulated the PBMC of a group of individuals with strong HIV-specific CD8$^+$ T cell responses with CMV peptides or appropriate HIV peptides prior to FISH and compared telomere lengths of HIV-specific and CMV-specific CD8$^+$ T cells. In all cases studied, CMV-specific T cells had shorter telomeres than HIV-specific T cells of the same individual ($p < 0.05$, **Figure 4E**). These data indicate by telomere length analysis that CMV-specific T cells of HIV-infected individuals are the T cells most proximal to exhaustive proliferation and replicative senescence.

Since there was a broad range of absolute telomere lengths related to subjects' ages and genetic variation, for broader comparison, we normalized the telomere length of different lymphocyte subsets by expressing it as a fraction of the median length of the general lymphocyte population telomeres for each individual. This representation clearly shows successive relative shortening of telomeres through CD8$^+$, CD8$^+$CD57$^+$, and CD8$^+$CD57$^+$ CMV-specific T lymphocyte subsets (**Figure 5A**). It also clearly illustrates relative preservation of telomere length within the CD8$^+$ T cell subset and within the CD57$^+$ NK subset compared to CD8$^+$CD57$^+$ T cells and CMV-specific CD8$^+$CD57$^+$ T cells (**Figure 5A**). Longitudinal sampling of telomere lengths over 10 years within CMV and HIV-specific CD8$^+$ T cells of two individuals for whom cryopreserved PBMC were available (subjects 178 and 182) showed accelerated erosion of CMV-specific CD8$^+$ T cell telomeres compared to the overall lymphocyte population, CD8$^+$ T cells, CD8$^+$CD57$^+$ T cells, and HIV-specific CD8$^+$ T cells (**Figure 5B**). Both of these individuals were male caucasians infected with HIV for >20 years. For subject 182, the period of telomere measurement spanned age 42–51. He has been receiving ART for 16 years with no recorded detectable HIV replication over the study period. For subject 178, the period of telomere measurement spanned age 59–68. He has been receiving ART for 13 years with two recorded minor transient blips of HIV replication over the study period.

To investigate a direct relationship between CMV immunity-related telomere erosion and inflammation, we assessed correlation between the relative telomere lengths (fraction of the median length of the general lymphocyte population) of different lymphocyte subsets and plasma levels of pro-inflammatory cytokines and CRP. We observed a significant inverse correlation between relative telomere length of CMV-specific CD57$^+$CD8$^+$ T cells and plasma levels of CRP ($r = -0.4756$, $p = 0.0122$). This suggests that telomere erosion of CMV-specific CD8$^+$ T cells in HIV-infected individuals is related to systemic inflammation.

DISCUSSION

Premature functional deterioration of multiple physiological systems, including the immune system, is a primary concern for the extended health of HIV-infected individuals (20, 22, 23, 39). Factors related to immune dysfunction and persistent inflammation may contribute to the earlier and more frequent incidence of age-related morbidities in the HIV-infected population (21, 40). Based on notable associations between CMV infection and cardiovascular disease in the general population and

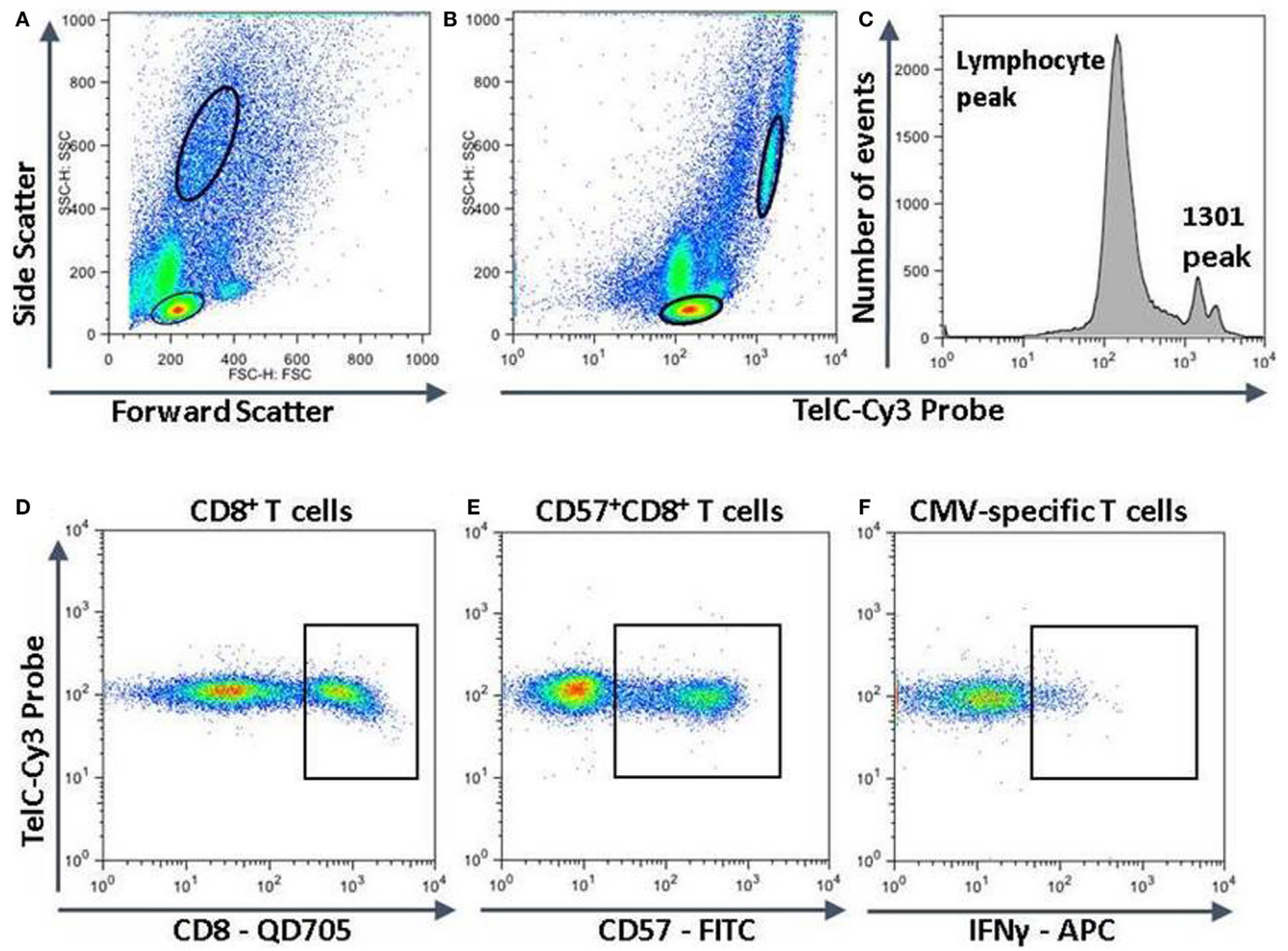

FIGURE 2 | Measurement of telomere length in different lymphocyte subsets. Peripheral blood mononuclear cells were surface stained with fluorescent labeled antibodies against CD8 and CD57, then together with 1301 standard cells were fixed, permeabilized, and subjected to hybridization with a fluorescent Cy3-labeled telomere-specific DNA probe. If stimulated with human immunodeficiency virus or cytomegalovirus (CMV) peptides, they were stained for intracellular interferon-gamma (IFN-γ) before hybridization. The strategy for subset analysis was as shown with successive gating to identify lymphocytes and 1301 cells **(A,B)**, $CD8^+$ T cells **(D)**, $CD57^+CD8^+$ T cells **(E)**, and CMV-specific $CD57^+CD8^+$ T cells producing IFN-γ **(F)**. **(C)** The fluorescence intensities of the entire lymphocyte population and 1301 standard cells. The major 1301 peak represents cells in the G1 phase of the cell cycle while the smaller peak to the right represents cells in S or later phases of the cell cycle.

between CMV infection and immune senescence in old elderly, we and others have investigated the relationship between coinfection with CMV, systemic inflammation, and age-related morbidity in HIV infection (27, 28, 30–32, 41). In this study, we specifically investigated the relationship between $CD8^+$ T cell immunity against CMV and lymphocyte progression toward replicative senescence imposed by telomere loss. If replicative senescence of lymphocytes introduces a SASP, similar to that of other senescent cells, this could promote persistent systemic inflammation and provide a mechanistic link between CMV infection and an increased risk for certain age-related morbidities. After excluding subjects infected with HCV, we compared plasma levels of CRP and of several pro-inflammatory cytokines between groups of HIV-infected infected individuals distinguished by their CMV seropositivity status. Since we did not test for CMV DNA or conduct more sensitive ELISPOT assays to detect T cell responses against CMV, it is possible that some of the 19 CMV-seronegative subjects were not completely CMV-negative. However, we feel this is unlikely based on the consistency with which seropositivity accompanied T cell responses detectable by flow cytometry. Although there was a broad range in the levels of these inflammatory markers, median IL-6, TNF-α, and CRP levels were all significantly higher in the group seropositive for CMV. Levels of IL-1 were significantly higher only when individuals with detectable HIV replication within 12 months of testing were included in the analysis. Thus, elevated IL-1 levels in the coinfected group may be associated with factors favoring HIV replication more so than with CMV coinfection itself. However, the elevated levels of IL-6, TNF-α, and CRP that were independent of detectable HIV replication

support an association between CMV seropositivity and inflammation that could be relevant to the pathogenesis of accelerated aging and of particular age-related morbidities.

The relationship between CMV infection and immune senescence in the old elderly arises through a memory inflation process wherein accumulation of CMV-specific CD8+ T cells lowers the circulating CD4+/CD8+ T cell ratio below 1 (10, 11). Oligoclonal T cell proliferation underlies this accumulation; therefore, CD8+ CMV-specific T cells with the most extensive history of cell division may be approaching replicative senescence imposed by telomere erosion (7, 8). Previous research with non-HIV-infected individuals indicated that CMV-specific T cells did not suffer senescence imposed by telomere shortening (35). Accelerated CMV memory inflation in HIV infection coincides with increases in inflammation and in age-related morbidities, but no direct links have been investigated (16, 18). We speculated that acquisition of a SASP by CMV-specific, or other senescent T cells accumulating through extensive cell division, could link CMV memory inflation to systemic inflammation and its downstream sequelae. Callender et al. recently showed that senescent T cells could be identified by expression of a disintegrin and metalloprotease domain (ADAM) 28 and that they produce high levels of pro-inflammatory cytokines, especially TNF-α (42). In our study, telomere length analysis demonstrated that CD8+ CMV-specific T cells are the circulating lymphocytes nearest to telomere-imposed replicative senescence in HIV-infected individuals, but replicative senescence was not directly addressed. Senescent cells have been proposed as a source of the chronic low-grade inflammation related to immune senescence and development of age-related morbidity (43, 44). In this regard, we found significant correlations of relative telomere length of CD57+CD8+ CMV-specific T cells with age and with plasma levels of CRP, but no significant correlation

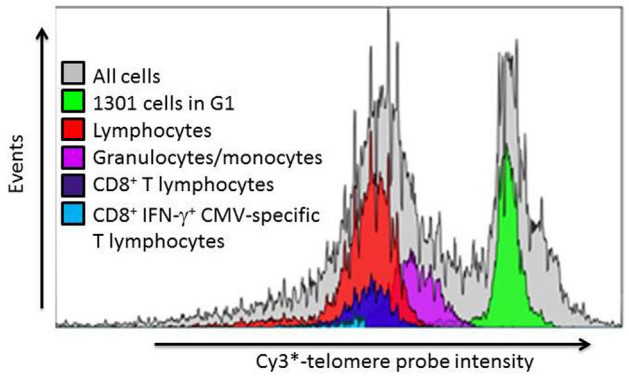

FIGURE 3 | Representative example of leukocyte subset telomere probe fluorescence intensity distribution. After gating on lymphocyte subsets as indicated in **Figure 3**, the telomere probe fluorescence intensity distribution of each individual subset is expressed as a histogram from which the geometric mean fluorescence intensity (MFI) is derived. The telomere length of each subset is then estimated as described in Section "Materials and Methods" from the ratio of subset MFI to that of the internal standard 1301 cells in G1 phase of the cell cycle.

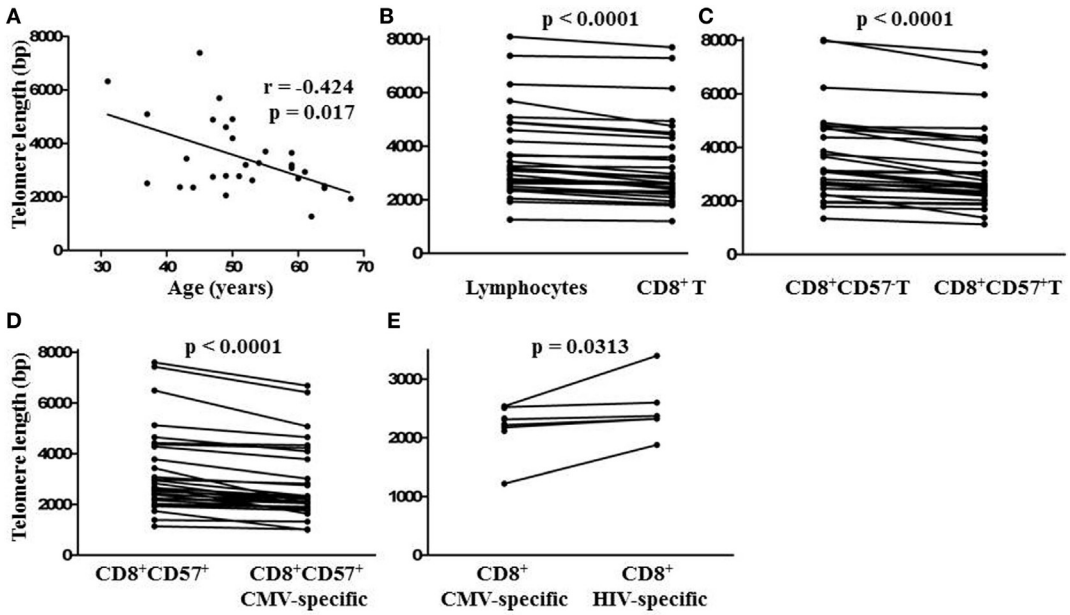

FIGURE 4 | Relationship between lymphocyte telomere length and age and comparison of lymphocyte subset telomere lengths within individuals. **(A)** Spearman non-parametric correlation indicated significant negative correlation between lymphocyte telomere length and age. The correlation coefficient (*r*) and probability of significant correlation (*p*) are shown in the plot frame. Linear regression was done to generate the line of best fit. **(B)** Points representing median telomere lengths for lymphocyte and CD8+ T cell subsets, **(C)** CD8+ and CD8+ CD57+ T cell subsets, **(D)** CD8+CD57+ and CD8+CD57+ cytomegalovirus (CMV)-specific T cell subsets, and **(E)** CD8+ CMV and human immunodeficiency virus (HIV)-specific T cell subsets joined by horizontal lines showing differences between subsets within each individual. Significant differences between subset telomere lengths are shown above the plot frames (Wilcoxon-signed rank test).

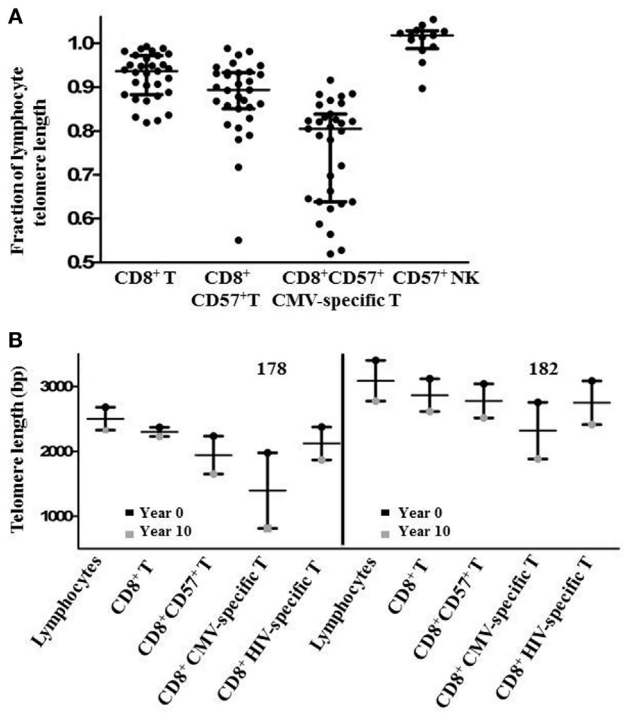

FIGURE 5 | Subset telomere length relative to the lymphocyte population. **(A)** Lymphocyte subset median telomere lengths are shown as a fraction of the total lymphocyte population median telomere length for each individual to normalize values across different ages and genetic backgrounds. Horizontal lines bisecting the groups show median values with interquartile range shown above and below. **(B)** Lymphocyte subset telomere length measurements for two subjects were carried out on peripheral blood mononuclear cells samples collected approximately 10 years apart. Vertical lines represent the loss of telomere length for each subset.

with any of the other markers of inflammation we measured. There was also no significant correlation between relative telomere length of CD57+CD8+ CMV-specific T cells and either concurrent or nadir CD4+ T cell counts. We focused on CD8+ CMV-specific T cells in gauging perisenescence as previous research showed that CD8+ but not CD4+ T cells of HIV-infected individuals have shorter telomeres than those of their HIV-discordant twins (37). We also previously showed that the CD8+ but not CD4+ T cells of CMV-seropositive HIV-infected subjects have lower T cell receptor excision circle frequencies than those of age-matched CMV-seronegative HIV-infected subjects (32). Both findings indicate more extensive proliferation and closer proximity to replicative senescence of CD8+ T cells in HIV-infected individuals.

While this snapshot of peripheral blood lymphocyte telomere length could suggest a direct relationship between CMV-related exhaustive lymphocyte proliferation and systemic inflammation in HIV infection, other factors may complicate or obscure the relationship and also limit the power of our study to link immune senescence, inflammation, and age-related morbidity in HIV infection to CMV immunity. Senescent lymphocytes may undergo rapid clearance, may home from the bloodstream to different tissues such as the liver, or may simply not acquire a SASP that notably impacts levels of the pro-inflammatory cytokines we measured. Accumulation of perisenescent CMV-specific CD8+ T cells may itself have prognostic significance. Further characterization of CMV-specific CD8+ T cells for expression of the senescence-associated proteins p16Ink4a or *FOXO4a* may shed light on their status and allow for finer discrimination of any relationship to markers of systemic inflammation (45, 46). Other inherent limitations to our study involve the primarily cross-sectional nature of our study which does not specifically control for such aspects as previous levels of disease progression, duration of HIV infection, duration of CMV infection, duration of ART, history of CMV reactivation, age, sex, and lifestyle. Thus, these results may apply selectively to the particular HIV-infected population we studied. However, as comparisons of CMV-specific CD8+ T cell telomere length with that of other lymphocytes were always carried out within individuals, thus, serving as their own controls, and the results were consistent across 32/32 subjects, we are confident in the strength of our conclusion that the CMV-specific cells have undergone the most extensive proliferation and are most proximal to telomere-imposed senescence in this setting.

As CMV-specific T lymphocytes are the largest antigen-specific T cell subset and have the shortest telomeres of all lymphocytes, they represent a highly accessible contemporary record of chronic or recurrent immune activation events that have driven clonal proliferation. The extent of telomere attrition relative to that of HIV-specific T cells is somewhat surprising, given the almost universal chronicity of HIV replication in the absence of effective treatment. Overall, antigen-driven proliferation might be expected to be higher in the HIV-specific T cell subset, but CMV could have been acquired much earlier in life than HIV infection for most individuals and the T cell response may be more oligoclonal than that mounted against HIV. We have also speculated that CMV-specific T cells may have a selective advantage over other T cells in homeostatic T cell proliferation, driven by overall lymphocyte attrition and the cytokine environment established in chronic HIV infection. The rate of telomere attrition over 10 years was higher among CMV-specific CD8+ T cells than either HIV-specific CD8+ T cells or the general CD8+ T cell population in both individuals that we studied and neither had experienced any clinically apparent CMV reactivation. Both a higher rate of CMV reactivation and increased homeostatic T cell proliferation could contribute to the accelerated CMV memory inflation occurring in HIV-infected individuals.

While the shortened telomeres of CMV-specific CD8+ T cells directly reflect immunological history as it pertains to CMV, it will be important to determine whether lingering senescent or perisenescent cells portray or influence aspects of immune senescence beyond their own status. Accumulation of CMV-specific lymphocytes in old elderly signifies a global decline in immune function and reduced survival expectancy. It remains unclear whether the accumulation of CMV-specific lymphocytes is simply a symptom associated with generalized immune decline or is itself a driver of immune decline. While there is no direct evidence that CMV-specific lymphocytes contribute to systemic inflammation, their association with morbidity and mortality and positioning at the leading edge of lymphocyte

progress toward cellular senescence is consistent with a negative influence on the function of other immune cells they interact with. We and others have already shown that immune resilience and immune reconstitution is superior in HIV-infected subjects seronegative for CMV (17, 28). The global decline in T cell receptor excision circle (TREC) frequency in CD8+ T cells is significantly correlated with magnitude of the CD8+ CMV-specific T cell response in HIV-infected individuals and higher anti-CMV antibody levels are associated with frailty (14, 32, 47). Although it remains only an association, stronger CMV-specific immune responses clearly have negative implications for the health of aging individuals.

Nonetheless, it is possible to alternatively interpret our findings as the increased inflammation in HIV infection due to bacterial product translocation from the gut or other causes promoting CMV replication, immune memory inflation, and progression of CMV-specific CD8+ T cells toward replicative senescence. To complicate matters further, a recent study demonstrated CMV replication in the intestinal epithelium of HIV-infected individuals, showed that intestinal epithelial cells are fully permissive to CMV infection and found that transepithelial permeability *in vitro* increased with CMV infection (48). Thus, it is also possible that CMV replication in the gut is itself a contributor to the loss of gastrointestinal barrier integrity that is linked with systemic inflammation in HIV-infected individuals (49). In this scenario, the accelerated CMV immune memory inflation seen in HIV infection would be a collateral effect of systemic inflammation, rather than a cause, as we propose.

In order to distinguish effects related to the progress of CMV-specific T cells toward senescence from other effects of aging, age-matched CMV-seronegative HIV-infected control subjects are a critical control group. Many other aspects of immune function beyond immune reconstitution remain to be compared between CMV-infected and CMV-seronegative groups. To investigate a direct role for CMV-specific T cells, the effect of their deletion on the function of other immune cells should be studied *in vitro* as several previous studies reported CMV-specific T cells with a T-regulatory cell phenotype (50–52). In addition, it could be informative to compare the replicative histories of CMV-specific T cells with differing fine specificity for pp65 or IE-1 peptides, rather than group all CMV IE-1 and pp65-specific CD8+ T cells together as we did. Identifying CMV-specific T cells at the leading edge of progress toward senescence also provides an opportunity to observe sequential changes in T lymphocytes as they reach replicative senescence and to define the SASP, if such exists, associated with senescent lymphocytes arising *in vivo* in a setting of chronic infection. Observations on immune senescence and its systemic impact made through the intensifying lens of HIV/CMV coinfection may be broadly applicable to survivors of chemotherapy, graft *versus* host disease, advanced aging, and other chronic conditions.

ETHICS STATEMENT

All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Health Research Ethics Authority of Newfoundland and Labrador.

AUTHOR CONTRIBUTIONS

MDG conceived the study and drafted the manuscript together with JH. JH performed the telomere length measurements, pro-inflammatory marker ELISAs, and some CMV-specific peptide stimulations. NF performed most of the CMV-specific peptide stimulations and all serologic testing for CMV-specific antibodies. NF and MEG processed and archived all samples for this study.

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Checks and Balances in Autoimmune Vasculitis

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Age-associated changes in the immune system including alterations in surface protein expression are thought to contribute to an increased susceptibility for autoimmune diseases. The balance between the expression of coinhibitory and costimulatory surface protein molecules, also known as immune checkpoint molecules, is crucial in fine-tuning the immune response and preventing autoimmunity. The activation of specific inhibitory signaling pathways allows cancer cells to evade recognition and destruction by the host immune system. The use of immune checkpoint inhibitors (ICIs) to treat cancer has proven to be effective producing durable antitumor responses in multiple cancer types. However, one of the disadvantages derived from the use of these agents is the appearance of inflammatory manifestations termed immune-related adverse events (irAEs). These irAEs are often relatively mild, but more severe irAEs have been reported as well including several forms of vasculitis. In this article, we argue that age-related changes in expression and function of immune checkpoint molecules lead to an unstable immune system, which is prone to tolerance failure and autoimmune vasculitis development. The topic is introduced by a case report from our hospital describing a melanoma patient treated with ICIs and who subsequently developed biopsy-proven giant cell arteritis. Following this case report, we present an in-depth review on the role of immune checkpoint pathways in the development and progression of autoimmune vasculitis and its relation with an aging immune system.

Keywords: immune checkpoints, immune checkpoint inhibitors, immune-related adverse events, vasculitis, giant cell arteritis

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INTRODUCTION

Age-associated changes in the immune system are thought to contribute to an increased susceptibility for autoimmune diseases. These changes include shifts in immune cell numbers, distribution, and function in conjunction with alterations in cell surface protein expression. One important class of surface proteins expressed on immune cells is immune checkpoint molecules, which regulate T cell activation by relaying positive (costimulatory) and negative (coinhibitory) signals. The balance between the expression of coinhibitory and costimulatory molecules is crucial in fine-tuning the immune response and preventing autoimmunity.

By exploiting the activation of specific inhibitory signaling pathways, cancer cells are able to evade recognition and destruction by the host immune system. Currently, several coinhibitory molecules are targeted by antibody-based antagonist biologicals in cancer immunotherapy. The rationale for this approach is that blockade of inhibitory checkpoints causes an unrestrained immune response allowing the host's tumor-specific T cells to attack the tumor cells. This immune checkpoint blockade strategy has proven to be very effective, producing long-lasting antitumor responses in multiple cancer types (1–3).

Nevertheless, immune checkpoint therapy has its disadvantages. Blocking the inhibitory signaling pathways may unleash reactivity to healthy tissues, which consequently may result in inflammatory manifestations in patients receiving these agents, termed immune-related adverse events (irAEs) (3–6). These irAEs are often relatively mild, but more severe irAEs have been reported as well including several forms of vasculitis such as granulomatosis with polyangiitis (GPA) (7), lymphocytic vasculitis (8), and polymyalgia rheumatica/giant cell arteritis (9–11) (**Table 1**).

However, little is known about the role of immune checkpoints in vasculitis. In this article, we discuss the evidence that age-associated changes in expression and function of immune checkpoint molecules leads to an imbalance of the immune system. An immune system out of balance is prone to tolerance failure and the development of autoimmune vasculitis. The topic is introduced by a case report from our hospital describing a melanoma patient treated with immune checkpoint inhibitors (ICIs) and who subsequently developed biopsy-proven giant cell arteritis. This case study sets the stage for a more in-depth review on the role of immune checkpoint pathways in the development and progression of autoimmune vasculitis and its relation with the aging immune system.

CASE VIGNETTE

A 70-year-old man with a history of hepatitis A and who had a myocardial infarction in 2001 developed a melanoma of the skin of the left temple in 2015. He was diagnosed with stage IIIB BRAF mutated melanoma and was treated with modified radical dissections including a parotidectomy, a neck dissection, and a free skin transplantation on June 8, 2015.

In August 2015, he started with adjuvant treatment in a double-blind study CA209-238 (Efficacy Study of Nivolumab Compared to Ipilimumab in Prevention of Recurrence of Melanoma after Complete Resection of Stage IIIb/c or Stage IV Melanoma (CheckMate 238); ClinicalTrials.gov number, NCT02388906) until April 2016. In April 2016, he was referred to the rheumatology and clinical immunology department with the following complaints: fatigue, low-grade fever with a temperature reaching 38.5 C, night sweats, and weight loss of 4 kg in 2 weeks. He had also experienced continuous pain for 4 weeks in his jaws and mastoid muscles. The right temple and masseter muscle were painful upon palpation, and his pain increased upon chewing. He had no hair pain or visual problems. He developed also new-onset pain and stiffness in his upper legs, neck, and shoulders. He had no pain or stiffness in his smaller joints, excluding a diagnosis fitting with arthritis.

On physical examination, he was fatigued, his blood pressure was 140/70 (upon measurement in both arms), his height was 1.78 m, and his weight 62 kg. His right temporal artery was painful, and his left temporal artery was not palpable (status after radical surgery). His shoulders and upper legs were painful upon movement. He had no infectious, gastrointestinal, or skin symptoms. His blood tests showed an elevated ESR of 93 mm/h, CRP of 52 mg/L, a hemoglobin level of 7.8 mmol/L (in October 2015, before immune checkpoint treatment, these values were ESR of 37 mm/h, CRP of 1.6 mg/L, and a hemoglobin level of 8.1 mmol/L).

An ultrasound of the temporal and axillary arteries, a PET/CT scan, and a temporal artery biopsy were performed. No halo fitting with GCA was observed upon US of his temporal and axillary arteries and muscles. The PET/CT did not show signs of large vessel vasculitis (LVV), myositis, infections, or metastasis, but did show some uptake surrounding both hips that would fit with a diagnosis of PMR. An additional MRI was performed, which did not show cerebral or leptomeningeal metastasis, and the masseter and temporal muscle and temporal and facial artery on the right side appeared to be normal. The ophthalmologist and the neurologist found no signs and symptoms that would fit the diagnosis of GCA and also ruled out trigeminal neuralgia.

The complaints of the patient were progressive, and his ESR and CRP remained high, while his right temporal artery increased in size and remained painful upon palpation. On May 23, 2016, the patient underwent a temporal artery biopsy from his right temporal artery, which revealed a transmural inflammation of the adventitial, medial, and intimal layers of the temporal artery with a fragmented internal and external lamina elastic, diagnostic for GCA (**Figure 1**). On May 24, the patient started with high-dose prednisolone (60 mg/day), which was tapered to 30 mg/day on May 25 (due to severe side effects) and gradually tapered to 2.5 mg/day on November 3, 2016. Disease activity of GCA was monitored according to the BSR definition that a disease relapse should be suspected in patients with a return of symptoms of GCA, ischemic complications, or unexplained constitutional or

TABLE 1 | Reported cases of vasculitis developed after immune checkpoint inhibitor treatment.

Reference	Reported case
van den Brom et al. (7)	Granulomatosis with polyangiitis induced by immune checkpoint inhibition (α-CTLA-4 and α-PD-1)
Minor et al. (8)	Lymphocytic vasculitis of the uterus in a patient with melanoma receiving ipilimumab
Goldstein et al. (9)	Polymyalgia rheumatica/giant cell arteritis occurring in two patients after treatment with ipilimumab
Hodi et al. (10)	Giant cell arteritis in a patient with metastatic melanoma receiving bevacizumab plus ipilimumab
Calabrese et al. (11)	Rheumatic immune-related adverse events of checkpoint therapy for cancer (PMR-like syndrome in three patients: two receiving α-PD-1 and one receiving α-CTLA-4 and α-PD-1)

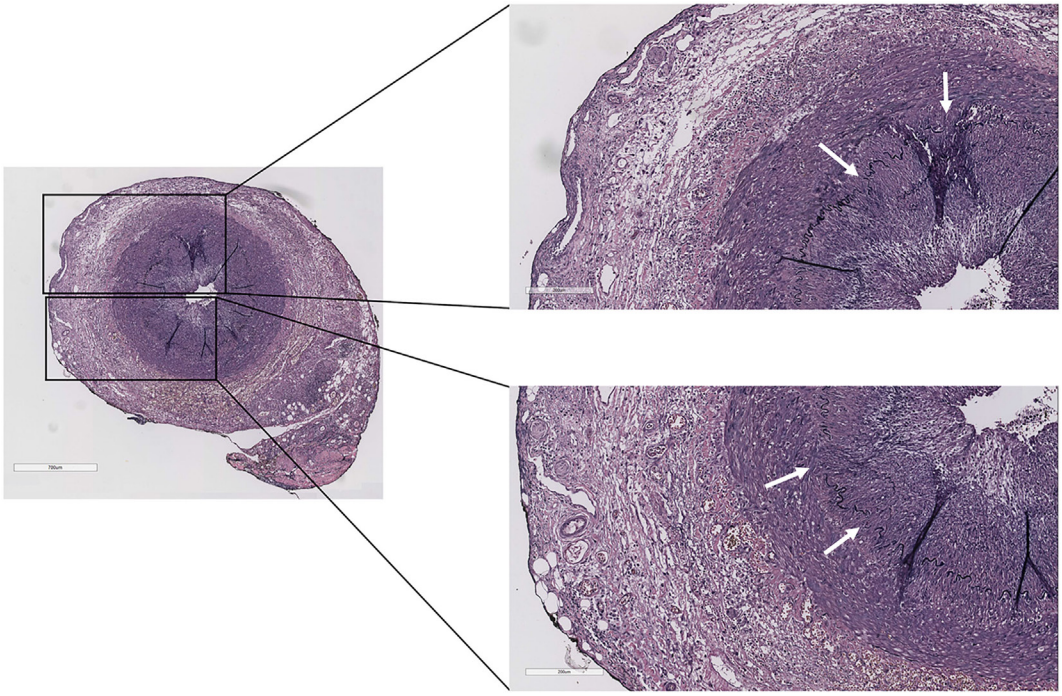

FIGURE 1 | Temporal artery biopsy of the case report patient showing transmural inflammation of the adventitial, medial, and intimal layers with a fragmented internal and external lamina elastic (white arrows) (Verhoeff–Van Gieson staining).

polymyalgic symptoms. (Relapse is usually associated with an increase in erythrocyte sedimentation rate/C-reactive protein, but may occur with normal inflammatory markers.)

Unfortunately, in October 2016, he had developed metastasized melanoma (lymph nodes and lung), and his previous adjuvant treatment was deblinded (not allowed to mention in this article nivolumab or ipilimumab as the study is not yet deblinded). On November 3, he started with a different checkpoint inhibitor. He had some persistent smoldering low-grade GCA complaints, which increased on this treatment. The complaints consisted of a headache on his left side and pain and stiffness in his neck and upper legs, and he had a painful temporal artery on his left side. The ESR of 37 mm/h and CRP of 7 mg/dL were slightly increased, suggesting a GCA relapse. The prednisolone dose was increased to 10 mg/day. Infusions with checkpoint inhibition were continued, and he was advised to take an increased prednisolone dose of 20 mg at day 2 and 3 after these infusions.

In May 2017, he still had signs and symptoms that fit with active GCA, especially jaw complaints upon chewing but no headache. The ESR was 4 mm/h and CRP was <0.3 mg/dL. He was advised to taper the prednisone to 7.5 mg/day to control the GCA without giving too much immunosuppression. A schematic representation of GCA development induced by immune checkpoint blockade is given in **Figure 2**.

The case described above is a prime example of an adverse consequence upon immune checkpoint therapy, illustrating that removing the natural brakes of the immune system may lead to a breach of tolerance and development of autoimmunity, such as LVV in this example (**Figure 3**). In this case, the patient was treated with in total two ICIs. ICIs are FDA-approved drugs in the treatment of advanced melanoma. Ipilimumab was the first checkpoint inhibitor approved by the FDA in 2011 for the treatment of advanced melanoma (12), and it showed improved efficacy and survival benefits compared to other chemotherapeutic agents (13). PD-1 inhibition with pembrolizumab and nivolumab also has proven to be effective in advanced melanoma (14–17) and was approved by the FDA in 2014.

Besides anti-PD-1 agents, the FDA has also recently approved antiprogrammed death-ligand 1 (PD-L1) agents for the treatment of patients with several types of cancer (18, 19). In the coming years, the approval of new ICIs or a combination of checkpoint-targeting agents that are currently under investigation in oncology clinical trials is expected. Approval of these drugs will translate into an increased use of immunotherapies, prompting the investigation of the underlying mechanisms of immune checkpoint regulation to avoid unwanted adverse events such as the one presented in the case above.

Although there is an increased awareness of the more common irAEs upon immune checkpoint therapies, rare but severe and potentially life-threatening autoimmune manifestations, such as vasculitis, should be taken into account when evaluating the benefit of tumor destruction and the associated risks of immunotoxicity. Some of the toxicities related to immune checkpoint therapy reported in multiple studies are summarized in **Table 2** (16, 20, 21). The reported rate for the more common irAEs, which involve the skin, gastrointestinal system, and endocrine system

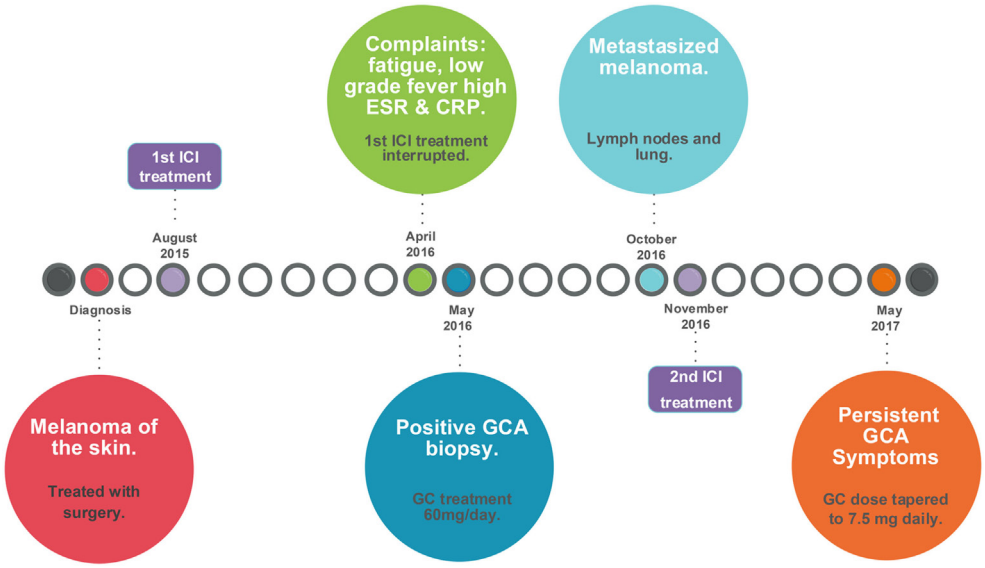

FIGURE 2 | Timeline of events leading to the development of GCA induced by checkpoint immunotherapy. CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; GC, glucocorticoids; GCA, giant cell arteritis; ICI, immune checkpoint inhibitor. Surgery included a modified radical dissections including a parotidectomy, a neck dissection, and a free skin transplantation on June 8, 2015, for stage IIIb melanoma, which was followed by inclusion in the CA209-238 study [Efficacy Study of Nivolumab Compared to Ipilimumab in Prevention of Recurrence of Melanoma After Complete Resection of Stage IIIb/c or Stage IV Melanoma (CheckMate 238); ClinicalTrials.gov number, NCT02388906].

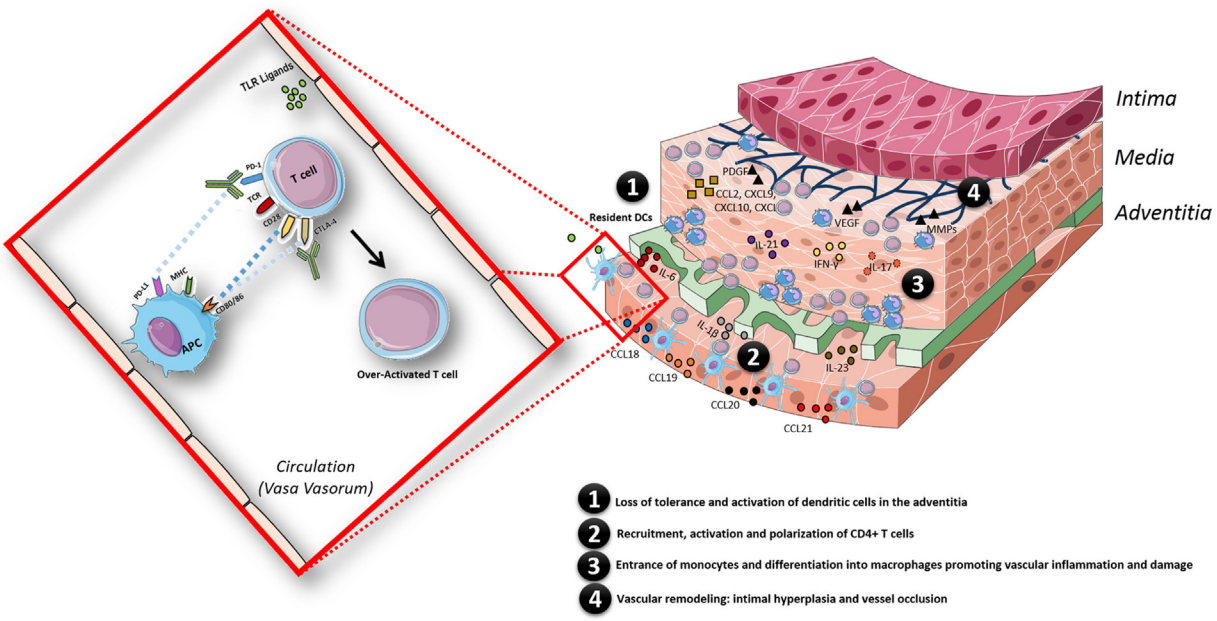

FIGURE 3 | Schematic model of the pathogenesis of giant cell arteritis, facilitated by the state of chronic inflammation in aged individuals and in addition by an overactivated immune system triggered by immune checkpoint inhibitor treatment. The inflammatory response in the arterial wall is initiated when resident dendritic cells (DCs) sense danger signals *via* pattern recognition receptors such as toll-like receptors. Activated DCs produce chemokines (CCL18, CCL19, CCL20, and CCL21), which recruit CD4+ T cells; once recruited in the arterial wall, CD4+ T cells are activated by DCs presenting still undefined antigen(s). The presence of pro-inflammatory cytokines (IL-6, IL-1β, IL-23, IL-18, and IL-12) in the microenvironment polarizes CD4+ T cells toward Th1 and Th17 cells, which produce large amounts of IFN-γ and IL-17. Eventually, monocytes enter the vascular wall and differentiate into macrophages promoting vascular inflammation by secreting cytokines and vascular damage *via* secretion of matrix metalloproteinases (MMPs). Macrophages, giant cells or injured VSMC also produce growth factors such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). This results in vascular remodeling: intimal hyperplasia and vessel occlusion. The whole process is facilitated by a state of chronic inflammation as observed in aged individuals and additionally by an overactivated immune system triggered by immune checkpoint therapy treatment in this case.

TABLE 2 | Frequency of selected immune-related adverse events associated with immune checkpoint inhibitor treatment.[a]

Type of disorder	Anti-CTLA-4 (ipilimumab)	Anti-PD-1 (nivolumab or pembrolizumab)	Combination therapy
Skin (rash)			
Robert et al. (16)[b]	14.5	14.7	NR
Postow et al. (20, 37)[c]	26	NR	41
Larkin et al. (21)[d]	32.8	25.9	40.3
Range	15–33%	15–26%	40–41%
Gastrointestinal (diarrhea)			
Robert et al. (16)[b]	22.7	16.9	NR
Postow et al. (20, 37)[c]	37	NR	45
Larkin et al. (21)[d]	33.1	19.2	44.1
Range	23–37%	17–19%	44–45%
Endocrine (hypothyroidism)			
Robert et al. (16)[b]	2	10.1	NR
Postow et al. (20, 37)[c]	15	NR	16
Larkin et al. (21)[d]	4.2	8.6	15
Range	2–15%	9–10%	15–16%
Others (arthralgia)			
Robert et al. (16)[b]	5.1	9.4	NR
Postow et al. (20, 37)[c]	9	NR	11
Larkin et al. (21)[d]	6.1	7.7	10.5
Range	5–9%	8–9%	10–11%

[a]Values are the percentage of treated patients who experienced adverse events of any grade (based on the common terminology criteria for adverse events grading system).
[b]Ipilimumab (N = 256), anti-PD-1 agent used: pembrolizumab (N = 278).
[c]Ipilimumab (N = 46); combination therapy used: nivolumab plus ipilimumab (N = 94).
[d]Ipilimumab (N = 311); anti-PD-1 agent used: nivolumab (N = 313); combination therapy used: nivolumab plus ipilimumab (N = 313).
NR, not reported.

are comparable when using only one ICI, but the reported rate for these irAEs significantly increases when a combination of therapies is used. For those types of disorders that are not as common, the reporting rate is very low, even when combination therapy is used. The frequency of autoimmune complications may be underestimated due to the fact that follow-up in clinical trials is usually short, and the development of autoimmune toxicities can have a delayed onset (22).

To better understand the mechanisms of action of ICIs and the adverse consequences derived from their use, it is essential to consider the various immune functions that these checkpoints control; this issue is addressed in the following sections.

COINHIBITORY CHECKPOINT PATHWAYS

The two inhibitory checkpoint pathways that have been most widely studied in oncology are the CTLA-4 and PD-1 pathways. Immune responses are negatively regulated by these pathways at different levels and by different mechanisms.

CTLA-4 Pathway

The ability of the immune system to protect from harm and prevent unnecessary tissue injury is maintained by a delicate balance between costimulatory and coinhibitory molecules. One example of this delicate balance is the interaction between the coinhibitory molecule CTLA-4 and its counterpart, the costimulatory molecule CD28. Both CD28 and CTLA-4 are expressed on T cells and control the early stages of T cell activation (23–25). Once antigen recognition occurs through engagement of the T cell receptor (TCR) with the cognate antigen–MHC complex, presented by antigen-presenting cells (APCs), CD28 binds to CD80 and CD86; this binding strongly amplifies TCR signaling to activate T cells (25–28). Within 48 h of activation, expression of CTLA-4 is upregulated on activated T cells (3). As CD28 and CTLA-4 share identical ligands, the latter dampens T cell activation by outcompeting the former in binding to CD80 and CD86 (24, 29–31). CTLA-4 can further decrease activation by sending a signal to APCs to reduce CD80/86 expression (32) and secrete indoleamine 2,3-dioxygenase (IDO), an enzyme that catalyzes tryptophan degradation (33), disabling T lymphocytes to proliferate due to tryptophan shortage (34). Activated CD8+ T cells also express CTLA-4, which suppresses helper T cell activity and enhances the immunosuppressive activity of regulatory T (Treg) cells (35). Treg cells constitutively express CTLA-4, which on the one hand leads to Treg cell proliferation and enhanced production of IL-35, IL-10, TGF-β, and IDO. On the other hand, on effector T (Teff) cells, CTLA-4 engagement causes a decreased activation and proliferation (6, 36).

Collectively, as CTLA-4 regulation takes place early in the process of T cell activation and augments Treg function, it is likely that its blockade leads to an unrestrained non-specific activation of the immune response. This broad activation may explain the wide variety of adverse events seen when this pathway is blocked (25, 37).

PD-1 Pathway

Although both CTLA-4 and PD-1 are negative checkpoints, PD-1 exerts its function at different levels and *via* different mechanisms. Upon engagement to either PD-L1 (also known as CD274 and B7-H1) or programmed death-ligand 2 (PD-L2; also known as CD273 and B7-DC), tyrosine phosphorylation of the PD-1 cytoplasmic domain occurs and tyrosine phosphatase SHP-2 is recruited, resulting in disruption of the TCR signaling cascade (38–41). These effects ultimately block T cell proliferation, diminish cytokine production and cytolytic function, and impair T cell survival (3, 42, 43). The cellular expression of PD-1 is broader than that of CTLA-4; for example, B cells and natural killer cells also express and upregulate PD-1 upon activation (25, 44), thereby temporarily dampening their effector functions (39). Another important subset of T cells that highly expresses PD-1 is Treg cells, and it has been demonstrated that PD-1 ligation on these cells enhances their immunosuppressive activity (43, 45). Both the PD-L1 and PD-L2 ligands are expressed on APCs and other hematopoietic and non-hematopoietic cell types (46).

In preclinical models, PD-1/PD-L1 pathway inhibition also generates antitumor activity and enhances autoimmunity (47). However, the autoimmune phenotypes of mice with PD-1 or CTLA-4 deficiencies are different. CTLA-4 deficiency results in a more severe, non-specific autoimmune phenotype as it affects both cell-intrinsic activities (on Teff cells) and cell-extrinsic activities (on Treg cells) (48). By contrast, PD-1 deficiency results in a mild and chronic autoimmune phenotype since it is mainly manifested as cell-intrinsic alterations of Teff cells (3, 48). Since

PD-1 activation suppresses the immune response during the effector phase of T cell activation and upon repeated antigen exposure, PD-1 blockade probably targets a more restricted assortment of T cells than CTLA-4 blockade (3).

LESSONS LEARNED FROM ONCOLOGY

The cancer immunity cycle described by Chen and Mellman in 2013 has become a useful framework for immunotherapy research. Briefly, the authors refer to seven steps, which need to be initiated and allowed to proceed and expand iteratively for an anticancer immune response to effectively kill cancer cells. These steps involve: step 1: the release of cancer antigens, step 2: presentation of those antigens through APCs and dendritic cells (DCs), step 3: T cell priming and activation within the lymph node, step 4: T cell trafficking to tumors, step 5: T cell infiltration into the tumor, step 6: recognition of cancer cells by T cells, and finally, step 7: cancer cell killing, which restarts the cycle (49). In each step described above, as in all of the immune system processes, checks and balances are required to perform optimally, which in cancer patients are ablated due to cancer's many strategies to evade recognition by the host immune system. Obstacles encountered in one or several steps of the cancer-immunity cycle are the target of immunotherapy; therefore, combination of approaches with therapies stimulating various and different steps of the cycle may result in higher response rates (50) and consequently more irAEs.

Effect of Immunotherapies on Checkpoint Molecule Expression and Function

In cancer patients, anti-CTLA-4 treatment lowers the threshold required for T cell activation, which leads to an expansion of circulating low-avidity T cells (51), resulting in a sustained immune response. In addition, it has been shown that anti-CTLA-4 therapy promotes antitumor activity by a selective reduction of intratumoral Treg *via* Fc-γR-mediated depletion (52), impairing Treg cell survival and function along with concomitant activation of Teff cells (35, 53). In addition, Th17 cells, which are implicated in many autoimmune and chronic inflammatory disorders (54) and in tumor eradication (55) processes, are also affected by CTLA-4 blocking. In cancer patients, it has been demonstrated that upon anti-CTLA-4 treatment, the number of circulating Th17 cells in patients increases, especially in those patients who developed clinically relevant inflammatory and autoimmune toxicities (56).

Recently, Wei et al. confirmed that distinct cellular mechanisms underlie anti-CTLA-4 and anti-PD-1 checkpoint blockade. The authors concluded that both checkpoint blockade therapies targeted only specific tumor-infiltrating exhausted-like CD8 T cells and that the effect of these agents primarily differed in the expansion of inducible costimulator (ICOS) + Th1-like CD4 effector cells induced by the anti-CTLA-4 agent (57). Furthermore, additional studies in cancer patients show that after targeting CTLA-4 with ipilimumab, responding patients have increased ICOS + T cells (58, 59). Several research groups have reported that there appears to be a compensatory upregulation of alternative checkpoints following immune checkpoint blockade (60–62). Very recently, a study by Gao et al. demonstrated that the inhibitory immune checkpoint molecules PD-L1 and V-domain Ig suppressor of T cell activation are both upregulated in CD4+ and CD8+ T cells and CD68+ macrophages of prostate cancer patients in response to ipilimumab therapy (62). The upregulation of alternative checkpoints as a compensatory mechanism might explain the lack of response or partial tumor regression observed in preclinical models (60, 61) and in cancer patients when treated with anti-CTLA-4 or anti-PD-1 monotherapy (16, 62, 63).

Such compensatory mechanism by which the immune system strives toward balance is supported by increasing evidence, indicating that basic signaling mechanisms of several immune checkpoint pathways are intertwined with each other forming a complex network that regulates the immune response. Kamphorst et al. found that CD28 signaling is essential for T cells to effectively respond to PD-1 blockade during chronic viral infection (64). Through conditional gene deletion, they showed a cell-intrinsic requirement of CD28 for CD8 T cell proliferation after PD-1 therapy (64). Moreover, Hui et al. reported that CD28 is strongly preferred over the TCR as a target for dephosphorylation by PD-1-recruited SHP-2 phosphatase, revealing that signaling through PD-1 occurs mainly by inactivating CD28 signaling (65). These data suggest that there is a broader interaction between PD-1 and CD28 than previously assumed, and such interaction might serve as a general mechanism for enhancing normal T cell responses and revitalizing exhausted T cells (66).

The unprecedented clinical success of cancer immunotherapy and the subsequent development of irAEs seen with these therapies have enabled researchers to study the underlying mechanisms of the early stages of autoimmunity. The expression of inhibitory receptors has been reported to be altered in many autoimmune diseases (67, 68), which suggests that signaling by inhibitory receptors is involved in the etiology of autoimmune diseases (67, 69). However, whether defective expression and/or function of immune checkpoints is a cause or consequence of autoimmunity and the ensuing autoimmune diseases is largely unknown. One factor that may be involved is age since aging is known to alter many aspects of the immune system and increases the susceptibility for the development of autoimmune diseases.

IMPACT OF AGING AND IMMUNOSENESCENCE ON CHECKPOINT MOLECULE EXPRESSION

As a result of aging-related changes in the immune system, the human body becomes more susceptible for developing cancer, autoimmune diseases, infections, and cardiovascular diseases (70–73). Aging impacts both the innate and adaptive constituents of the immune system, which lead to a dysregulated immune and inflammatory response contributing to the increased incidence of chronic immune-mediated diseases in elderly individuals (74).

The immune system of aged people shows an accumulation in the frequency of highly differentiated T cells of which, due to a greater homeostatic stability, CD4+ T cells are being less affected by the age-associated phenotypic and functional changes than CD8+ T cells (75, 76). These changes include loss of the cell surface costimulatory molecules CD27 and CD28, CD8+

T cells losing CD28 first followed by CD27 and vice versa for CD4+ T cells (77). Loss of the costimulatory molecule CD28 is a hallmark of the age-related decline of T cell function, which has been associated with a less-efficient capability to mediate immune responses in old individuals (78).

In addition to the loss of costimulatory molecules, there is an increase in the expression of inhibitory receptors, which adds to T cell dysfunction during aging (79). The expression of the inhibitory checkpoint molecule, CTLA-4 increases with age (80), whereas the expression of PD-1 is considered to be dependent on viral status rather than age and may also serve as a useful marker on viral-specific CD8+ T cells to indicate the degree of T cell exhaustion (41). In chronic viral infections and tumor microenvironments, PD-1-expressing exhausted cells lose their ability to produce IFN-γ and TNF-α and therefore become dysfunctional (81–83).

The age-related changes and deterioration of the immune system have been linked to immunosenescence (84), a term referring to the continuous remodeling of lymphoid organs, which leads to reduced immune function in elderly people (85). One of the major factors that fuels immunosenescence appears to be the lifelong chronic antigen load (86, 87) including leakage of microbial products from the gut to the circulation, resulting in continuous stimulation of both innate and adaptive immunity. Altogether, these changes lead to a chronic pro-inflammatory state favoring the development of age-associated (auto) inflammatory diseases (88).

ROLE OF IMMUNE CHECKPOINTS IN THE DEVELOPMENT OF IMMUNE-MEDIATED VASCULITIS

Vasculitides are a heterogeneous group of inflammatory disorders characterized by inflammation of the blood vessel wall. The clinical manifestations are determined by the localization, the type of vessel involved, and the nature of the inflammatory process (89). The Chapel Hill nomenclature classifies non-infectious vasculitides mainly according to the type of vessel affected: LVV, medium vessel vasculitis (MVV), and small vessel vasculitis (SVV). LVV affects the aorta and its main branches, and the primary vasculitides in this group are GCA and Takayasu arteritis. MVV affects the main visceral arteries and its branches; examples of diseases in this group are polyarteritis nodosa and Kawasaki disease. Finally, SVV is further subdivided into antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) and immune complex SVV. The major clinicopathologic variants of AAV are GPA, microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA) (90).

Antineutrophil cytoplasmic antibody-associated vasculitis is predominantly disease of the elderly. The incidence of AAV increases with age, peaking in those aged 65–74 years (91–93). A hallmark of the AAV is the presence of autoantibodies directed at neutrophil cytoplasmic constituents (ANCA) (94, 95). The target antigens of ANCA in the AAV are proteinase 3 (PR3) and myeloperoxidase (MPO) where GPA is primarily associated with PR3-ANCA and MPA and EGPA with MPO-ANCA. The immunopathological model of AAV in the acute effector phase is centered around ANCA and pro-inflammatory stimuli, most likely of infectious origin, which synergize in initiating a destructive inflammatory process (94, 95). A central event in this process is ANCA-mediated neutrophil activation resulting in the generation of reactive oxygen species (ROS), degranulation and cytokine production, a process that is greatly facilitated by minor (pro-)inflammatory stimuli that prime the neutrophil to interact with ANCA. Upon disease progression, acute vasculitis lesion transform into lesions that predominantly contain macrophages and T cells.

Although data on checkpoint expression in AAV patients are scarce, Wilde et al. reported increased expression of PD-1 on circulating T helper cells of GPA patients, whereas T cells in renal lesions mostly lacked PD-1 (96). The authors found that PD-1 expression was positively correlated with expansion of memory T cells, $CD28^{null}$ T cells, as well as with T cell activation. In addition, PD-1 expression was found to be enhanced on pro-inflammatory IFN-γ T cells in GPA patients. These observations suggested that increased PD-1 expression on T cells might counterbalance persistent T cell activation (96).

Furthermore, Slot et al. analyzed single-nucleotide polymorphisms in the genes encoding PD-1 and CTLA-4 describing SNP frequencies in GPA patients that could explain hyperreactivity of T cells in these patients (97). Interestingly, in 2016, our group reported for the first time the development of GPA after sequential immune checkpoint inhibition with anti-CTLA-4 and anti-PD-1 treatment, as well as the first report of vasculitis observed after anti-PD-1 treatment (7). In that case report, we hypothesized that anti-CTLA-4 treatment induced PR3-ANCA production, which created the conditions necessary for the development of GPA, a process that was rapidly amplified by anti-PD-1 treatment (7).

GCA, the most common vasculitis after 50 years of age (98, 99), is thought to be caused by both changes in the aging vessel wall and in the immune system. The immunopathological model of GCA can be divided into four phases: in phase 1, there is a loss of tolerance (cause unknown) and activation of resident DCs of the adventitia, which results in the recruitment, activation, and polarization of CD4+ T cells (phase 2). Once recruited and activated in the arterial wall, the presence of pro-inflammatory cytokines (e.g., IL-12, IL-18, IL-23, IL-6, and IL-1β) in the microenvironment polarizes CD4+ T cells toward Th1 and Th17 cells. Th1 and Th17 are responsible for the production of large amounts of IFN-γ and IL-17, respectively, which ultimately leads to the recruitment of CD8+ T cells and monocytes (phase 3). Vascular remodeling (phase 4) starts when the IFN-γ-stimulated monocytes differentiate into macrophages and vascular smooth muscle cells differentiate into myofibroblasts producing IL-6, IL-1β, TNF-α, and vascular endothelial growth factor (99). This amplifies the local inflammatory response causing the release of toxic mediators for the arterial tissue such as ROS and matrix metalloproteinase, which eventually results in remodeling processes leading to intima proliferation and vascular occlusion (99, 100).

Accumulating evidence, including the case herein reported, points to an important role of immune checkpoints in the development of GCA. This is also emphasized by the demonstrated efficacy of abatacept; a new treatment for GCA (99, 101). This

agent is a soluble fusion protein consisting of the ligand-binding domain of CTLA-4 and the Fc region derived from IgG1. CTLA-4-Ig binds to the APC B7 (CD80/86) molecule, thereby blocking B7 interaction with the CD28/CTLA-4 receptor on the T cell (102). By contrast, ipilimumab antagonizes the action of CTLA-4, thus enhancing immune reactivity by releasing this immunosuppressive checkpoint.

The involvement of immune checkpoints in the development of autoimmune side events is further supported by evidence from oncology, which shows that both CTLA-4 and PD-1 blockade result in enhanced Th17 cell responses and impaired Treg survival and function (52, 53, 56, 103). In addition, PD-1 blockade results in enhanced Th1 cell responses and increased production of cytokines such as IL-6 and IL-17 (103). This T cell functional flexibility and plasticity might be one of the mechanisms involved in the induction of autoimmune side effects (6).

In addition to CTLA-4 involvement in GCA, a recent study indicates that the immunoprotective PD-1/PD-L1 signaling pathway is affected as well. The study showed that tissue-residing DCs of GCA patients were low in PD-L1, whereas the majority of vasculitic T cells at the site of inflammation expressed PD-1 (104). Moreover, the *in vivo* vasculitogenic potential of PD-1 blockade was demonstrated using a humanized mouse model system of vasculitis, the Human Artery-Severe Combined Immunodeficiency Mouse Chimera model. Briefly, human axillary arteries were engrafted into NSG mice, and PBMCs from GCA patients or healthy individuals were adoptively transferred into the chimeras; chimeras were randomly assigned to treatment with PD-1 antibody or isotype control antibody. In this model, the authors confirmed that inhibiting PD-1/PD-L1 interaction enhanced tissue inflammation as GCA PBMCs but not healthy PBMCs were able to induce vasculitis. More specifically, PD-1 blockade enabled very few healthy T cells to enter the vascular wall, while PBMCs from GCA patients induced vessel wall inflammation. These observations suggested that T cells from GCA patients are especially vulnerable to PD-1 blockade (104, 105).

Zhang et al. demonstrated that in GCA a breakdown in PD-1/PD-L1 checkpoint resulted in unleashed vasculitic immunity and that such breakdown was responsible for the pathogenic remodeling of the inflamed arterial wall (104). The authors reported that PD-1 blockade gave rise to T cells producing IFN-γ, IL-17, and IL-21, which sustained multifunctional effector functions associated with the rapid outgrowth of hyperplastic intima and the induction of microvascular neoangiogenesis (104). Worthy of note, T cells producing IFN-γ, IL-17, and IL-21 play an important role in GCA and contribute to the pathogenesis of the disease (106, 107). Furthermore, PD-1 blockade biased T cells toward increased T-bet and RORC expression and diminished FoxP3 expression (104).

CONCLUDING REMARKS

During the past decade, the introduction of ICIs has revolutionized cancer therapy and has proven to be a very effective strategy in inducing durable antitumor responses in multiple cancer types. Increasing evidence supports the idea that immune checkpoints cannot be regarded as separate pathways but as a complex network functioning in concert to maintain the delicate balance in the immune system. However, despite the clear therapeutic benefit, it is undeniable that the induction of irAEs is a serious disadvantage. It has become clear that data on safety of immune checkpoint therapies need further study in elderly individuals (85). It might be that the patient's age is a relevant risk factor for irAEs (108) as the immune system of an elderly person is likely to demonstrate age-associated changes in checkpoint expression and function, which may be altered due to the chronic, low-grade inflammation. These changes imply that elderly patients will respond differently to ICI therapy than do younger patients evaluated in clinical trials.

Collectively, age-related changes and alterations in signaling pathways are complex and interconnected. These changes are likely to influence DC, Teff, and Treg pathways, increasing the likelihood of T cell suppression in the elderly (79). Indeed more research is needed to understand the link between age-related cellular and molecular changes and their potential influence on DC and T cell pathways leading to the development of autoimmunity. Nonetheless, lessons learned from the oncology field are valuable, enabling researchers to realize that the immune system is capable of reconfiguring the immune checkpoint complex network after modulation using ICIs. The altered expression of inhibitory receptors as seen in vasculitis patients, such as the abnormalities in the PD-1/PD-L1 pathway (105), hints at the involvement of immune checkpoints in disease development. Perhaps the use of agonistic inhibitory checkpoint molecules to halt self-damaging responses could restore the checks and balances, which are reported to be deficient in vasculitis.

ETHICS STATEMENT

Written informed consent was obtained from the patient prior to presenting the case.

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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REVIEW
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Influence of Inflammation in the Process of T Lymphocyte Differentiation: Proliferative, Metabolic, and Oxidative Changes

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T lymphocytes, from their first encounter with their specific antigen as naïve cell until the last stages of their differentiation, in a replicative state of senescence, go through a series of phases. In several of these stages, T lymphocytes are subjected to exponential growth in successive encounters with the same antigen. This entire process occurs throughout the life of a human individual and, earlier, in patients with chronic infections/pathologies through inflammatory mediators, first acutely and later in a chronic form. This process plays a fundamental role in amplifying the activating signals on T lymphocytes and directing their clonal proliferation. The mechanisms that control cell growth are high levels of telomerase activity and maintenance of telomeric length that are far superior to other cell types, as well as metabolic adaptation and redox control. Large numbers of highly differentiated memory cells are accumulated in the immunological niches where they will contribute in a significant way to increase the levels of inflammatory mediators that will perpetuate the new state at the systemic level. These levels of inflammation greatly influence the process of T lymphocyte differentiation from naïve T lymphocyte, even before, until the arrival of exhaustion or cell death. The changes observed during lymphocyte differentiation are correlated with changes in cellular metabolism and these in turn are influenced by the inflammatory state of the environment where the cell is located. Reactive oxygen species (ROS) exert a dual action in the population of T lymphocytes. Exposure to high levels of ROS decreases the capacity of activation and T lymphocyte proliferation; however, intermediate levels of oxidation are necessary for the lymphocyte activation, differentiation, and effector functions. In conclusion, we can affirm that the inflammatory levels in the environment greatly influence the differentiation and activity of T lymphocyte populations. However, little is known about the mechanisms involved in these processes. The elucidation of these mechanisms would be of great help in the advance of improvements in pathologies with a large inflammatory base such as rheumatoid arthritis, intestinal inflammatory diseases, several infectious diseases and even, cancerous processes.

Keywords: inflammation, T lymphocytes, differentiation, metabolic reprogramming, exhaustion, redox balance

INTRODUCTION

Inflammation is the process in which leukocytes and plasma proteins are recruited from blood into tissues, accumulated and then activated to elicit an adequate immune response. Inflammation is triggered by recognition of pathogen-associated molecular patterns and damage-associated molecular patterns from injured tissues during innate immune responses and it is refined and prolonged during adaptive immune responses. Many of these reactions involve cytokines which are produced by dendritic cells, macrophages, and other types of cells during innate immune reactions. The leukocytes that are mainly recruited in inflammation are neutrophils, and monocytes (**Figure 1A**).

Inflammation can be sensed in the nearby lymph nodes and thus influence recruitment and activation of lymphocytes in the nodes. Peripheral tissue inflammation, which usually accompanies infections, causes a significant increase of blood flow into lymph nodes and consequently an increase in T lymphocyte influx into lymph nodes draining at the site of inflammation. T lymphocytes are fully activated only when a foreign peptide is recognized in the context of the innate immune system activation by a pathogen or by some other causes of inflammation. In this pro-inflammatory environment, co-stimulatory ligands and increase in the expression MHC class I and II molecules are induced in antigen-presenting cells (APCs), which are necessary for an optimal T lymphocyte activation to occur. There are also many inflammatory mediators and cytokines that attract T lymphocytes, activating them through their antigenic receptors (1).

Although innate immune stimuli may contribute to chronic inflammation, the adaptive immune system may also be involved because T lymphocyte-producing cytokines are powerful inducers of inflammation. In this scenario, macrophages are activated by type 1 helper T lymphocytes (Th1 cells), both through cell contact and through IFN-γ secretion (2).

When cells that responded to the inflammatory environment cannot eliminate pathogens, the acute inflammatory condition can become a chronic condition (**Figure 1B**). In addition to a local or systemic inflammatory status, this chronic phase is

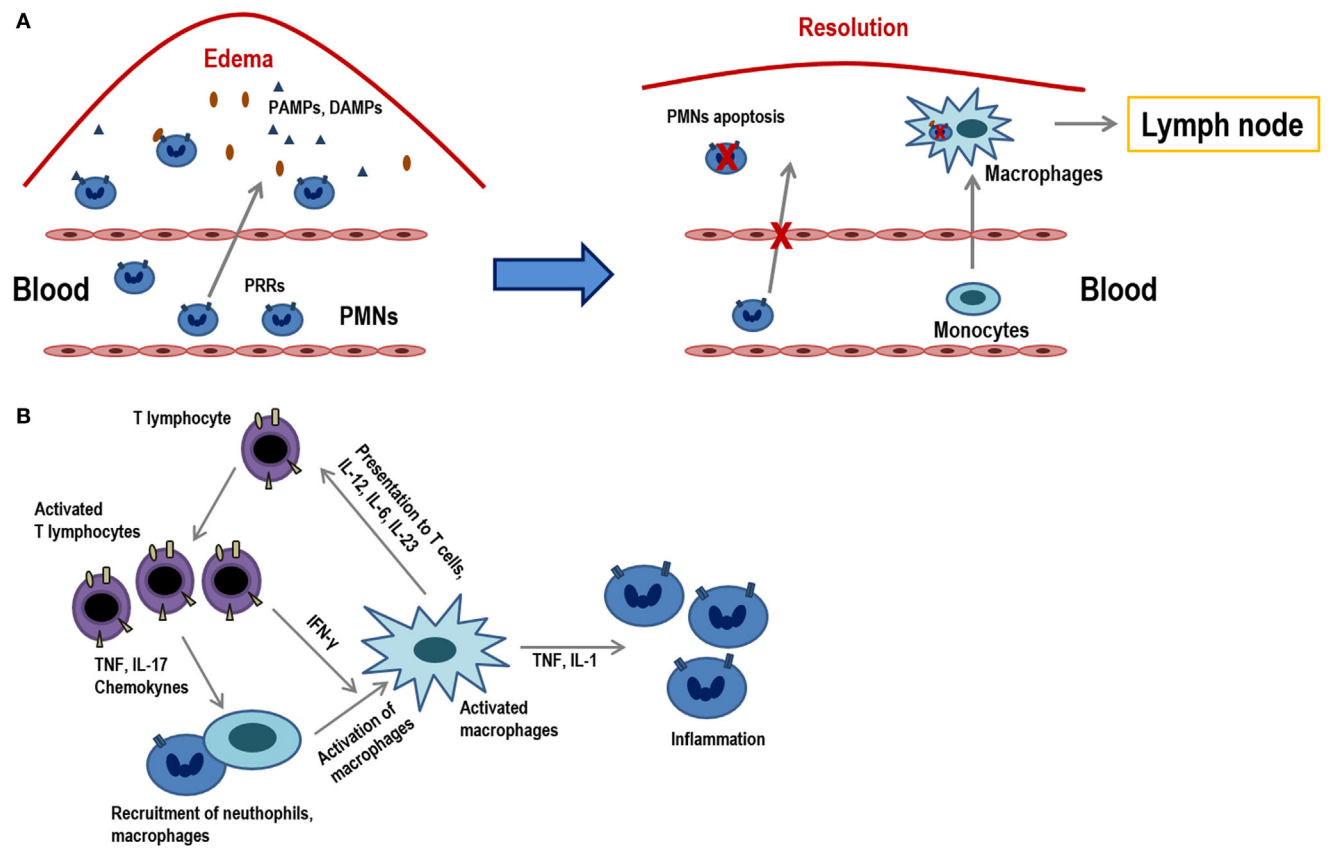

FIGURE 1 | Acute inflammation vs. chronic inflammation. **(A)** The onset of acute inflammation is characterized by the accumulation of polymorphonuclear neutrophils (PMNs) and monocytes that rapidly convert into tissue macrophages, as well as the appearance of edema due to damage in the inflamed tissues. Damage-associated molecular patterns and pathogen-associated molecular patterns are recognized by pattern recognition receptors (PRRs) and attract PMNs to the place of infection. PMNs represent the first line of defense in infected tissues as they eliminate much of the pathogens or harmful materials by phagocytosis. When inflammation occurs, neutrophils suffer apoptosis and they are ingested by macrophages that migrate to the lymph nodes where they will present the antigens. **(B)** Activated T lymphocytes produce cytokines (TNF, IL-17, chemokines) that recruit macrophages and others (IFN-γ) which activate them. T lymphocyte subpopulations (Th1, Th2, Th17, etc.) produce diverse types of cytokines and, in turn, activated macrophages and stimulate T lymphocytes *via* the presentation of antigens and through different cytokines (IL-12, IL-6, IL-23). These macrophages also act on neutrophils by releasing molecules, such as TNF and IL-1.

characterized by a maintained leukocyte infiltrate within the injured tissues. This low-grade inflammation is prevalent during aging and it is, therefore, denominated as "inflammaging." Furthermore, this phenomenon can also be observed in chronic infections, autoimmunity diseases, other chronic inflammatory pathologies or cancer. Consequently, all of them are characterized by persistent antigens that induce a sustained inflammation concomitant with a marked differentiation of the adaptive immunity, mainly in T lymphocytes. These highly differentiated cells in turn contribute to perpetuate the process by producing increased levels of proinflamatory cytokines. During the last stages of differentiation, the pro-inflammatory environment may be responsible for an inefficient response of T lymphocytes, as it is shown in older individuals (3). Indeed, it has been demonstrated that a reduced cytokine-related JAK–STAT signaling is correlated with chronic inflammation and age-associated morbidities (4).

T lymphocytes are produced in the bone marrow from where they migrate to the thymus for completing the maturation process. Then, naïve T lymphocytes recirculate between blood and secondary lymphoid organs until they contact their specific antigen adequately and properly. Upon contact, they proliferate and acquire properties to assemble an appropriate immune response. After antigen elimination, part of these cells remains as memory cells, with its own homeostasis and proliferation, though most of the effective cells die. Memory cells display a series of migratory and functional features that allow them to mount a quick response after the reencounter with the antigen. Therefore, the adaptive immune response presents two main advantages for the individual. On the one hand, it allows to create a specific immune response against the invading pathogen, with which it will finish it in a very effective way. On the other hand, a set of memory cells is formed to endure for many years thus affording protection from new reinfection by the same pathogen.

T lymphocytes can be categorized using a combination of different surface markers (CD45RA, CCR7) in distinct groups depending on their functionality. These categories are the naïve (CD45RA+CCR7+), effector memory (EM, CD45RA−CCR7−), central memory (CM, CD45RA−CCR7+), and effector memory RA (EMRA, CD45RA+CCR7−) populations (5). The effector T lymphocytes are a quite heterogeneous population and the use of two markers (CD27 and CD28) allows categorizing this population into other subpopulations; (CD28−CD27−) is the population more differentiated of all (6).

The step from naïve T lymphocytes to effector and memory T lymphocytes is one of the most fundamental processes in the T lymphocyte-mediated immunity and requires proliferative, metabolic, and oxidative adaptations.

T LYMPHOCYTE PROLIFERATION

Naïve T Lymphocyte Homeostasis

The number of naïve T lymphocytes remains stable in number and diversity along the time, when there are no involved powerful immune responses. However, T lymphocytes are neither a lethargic nor an immovable cellular population and indeed this naïve T lymphocyte pool is continuously interacting with other cells through homeostatic signs. The number of T lymphocytes in the periphery is almost constant although many new naïve cells appear every day from the thymus, especially during the early ages of the individual. Therefore, this homeostasis requires a strict control as it is very important to maintain this constant number along the time. Furthermore, the half-life of naïve T lymphocytes, roughly over 50 days, is quite longer than that of other cellular populations (7–9). Survival of naïve T lymphocytes requires signals mediated by the interaction of T lymphocyte receptor (TCR)–peptide–MHC and some cytokines, principally IL-7. This cytokine is particularly important for the correct T lymphocyte homeostasis but its concentration is very low. Thus, all the naïve T lymphocytes including the recent thymic emigrants (RTEs) compete for IL-7. If these cells do not receive enough IL-7 signal, they would die (**Figure 2A**). The necessity of soluble mediators to intervene in the homeostasis of naïve T lymphocytes has been evidenced by the capacity of various cytokines to avoid apoptosis of naïve T lymphocytes. Among these cytokines are IL-4, IL-6, lymphopoietin, and IL-7 (10–12), the latter being the one which plays a main role (13–15). Supporting this, when an IL-7 blocking antibody is injected or naïve cells are transfer into IL-7-deficient mice, survival of this subset is greatly diminished (13, 16–18). It is also believed that IL-7 is a limiting factor for determining the final size of the total lymphocyte pool, as it has been verified in several experiments in which the number of lymphocytes significantly increases in IL-7-overexpressing mice (19, 20). RTEs express low levels of IL-7 receptors, but they are more responsive to the cytokine than mature naïve T lymphocytes. Mechanistically, it has been demonstrated that IL-7 signaling in RTEs preferentially upregulates the antiapoptotic protein Bcl-2 expression, resulting in a decrease in cell apoptosis, but without an increase in proliferative effects. In contrast, mature naïve cells show a decrease in Bcl-2 expression. However, mature naïve cells have a greater proliferative response in the presence of IL-7 (21).

During lymphocyte development in the thymus, thymocytes reacting to self-molecules are eliminated or believed to be induced to produce regulatory T lymphocytes. But, on the other hand, in order to overcome positive selection, thymocytes must be able to recognize low affinity self-peptide–MHC so they can in turn leave the thymus as naïve mature cells (22). This reactivity to self-peptide–MHC is decreased after positive selection (23, 24), but does not disappear in naïve T lymphocytes, since the TCR ζ-chain continues to be phosphorylated and this can only occur if the TCR meets self-peptide–MHC complexes (25, 26). Therefore, consistent data from multiple studies confirm that self-peptide–MHC complex interactions are important for long-term naive T lymphocyte survival; however, these interactions raise much discussion and controversy. Even though there is a wide knowledge about the interaction between IL-7R and TCR in the homeostasis of naïve T lymphocytes, interestingly many of the routes that define the interactions between them are not well defined, but it is clear that it must include a mutual regulation between these two routes. This is a particularly interesting topic and should receive attention in further studies.

There are several works focused on the effect of acute inflammation, for example after bacterial sepsis, in the population of naive T lymphocytes. Most of these studies state that the function

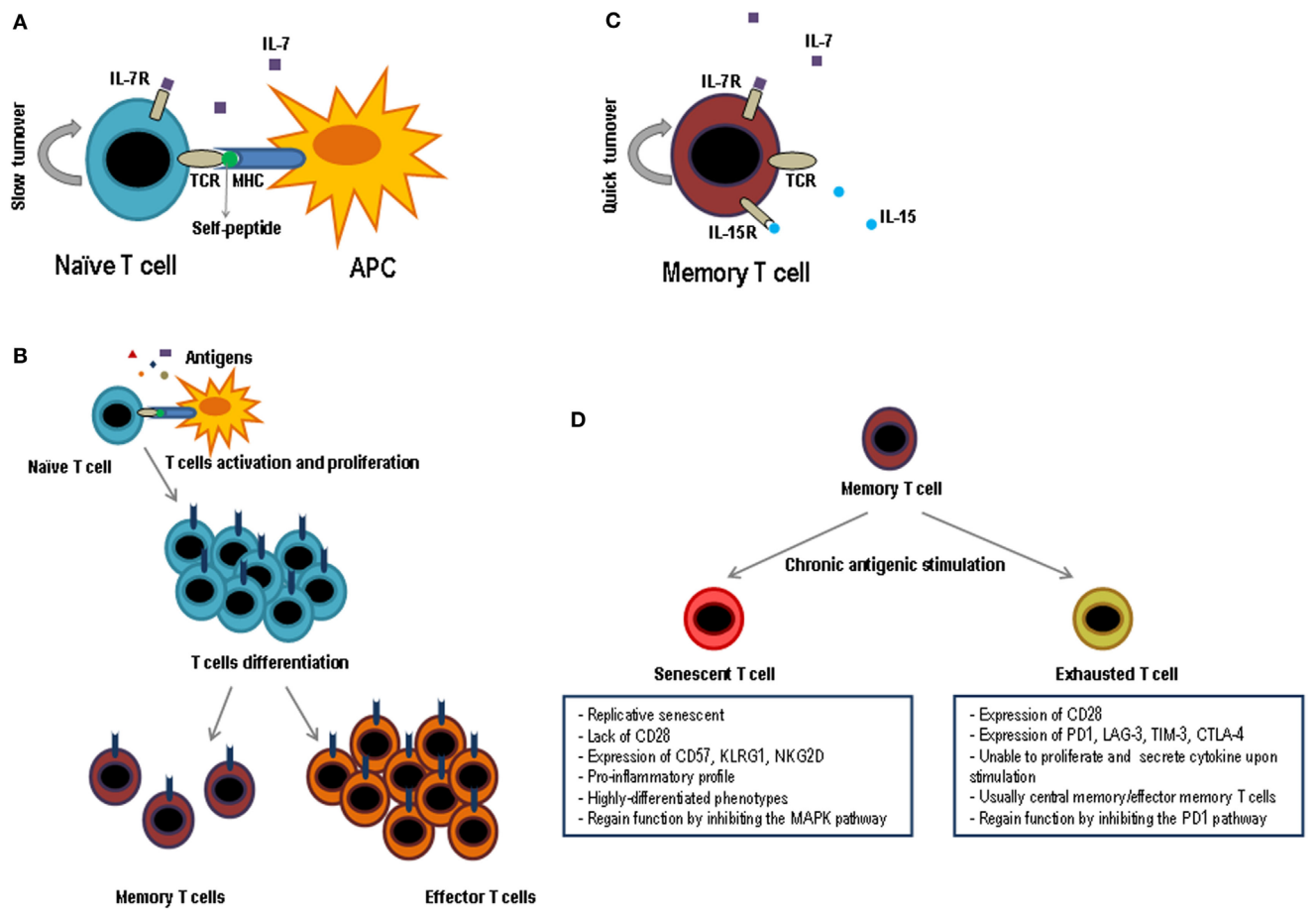

FIGURE 2 | Functional phases of T lymphocytes. Schematic representation of the key factors for survival and maintenance of naïve cells **(A)** and memory **(C)**; while naïve cells are dependent on IL-7 and the contact with self-peptides by MHC, memory cells are dependent on IL-7 and IL-15 and do not need the contact with autoantigens. **(B)** After contact with its specific antigen, naïve T lymphocytes proliferate exponentially and give rise to two differentiated populations, effector T lymphocytes and memory T lymphocytes. Effector cells will be eliminated as soon as the pathogen is destroyed, while memory cells will last a long time and they will be responsible for protecting the individual against new infections caused by the same pathogen. **(D)** When the memory cells are subjected to multiple cycles of activation by means of chronic antigens they can generate two types of T lymphocytes, exhausted T lymphocytes and senescent T lymphocytes. The principal characteristics of both cell types are shown in the figure.

of these cells remains unchanged, but concomitant with a decrease in their count after being exposed to high concentrations of pro-inflammatory cytokines (27–29). A few recent studies have shown that the sensitivity of CD8+ T lymphocytes to antigens is greatly increased if cytokines, such as IL-12, IL-18, and IFN-γ, are present prior to antigenic recognition (30, 31). However, only a very few studies have approached the effect of persistent inflammation on naive T lymphocytes, as it occurs in the context of aging or in the pro-inflammatory tumor microenvironment. Most of them suggest that there is a loss in the number of naïve T lymphocytes and a decrease in their functionality, although it is not clear the molecular mechanisms involved and more studies must be carried out (3, 32).

Clonal Expansion in Response to Specific Antigens

Naïve T lymphocytes present an amazing capacity to react against specific antigens through massive proliferation and differentiation to effector T lymphocytes. They are able to migrate to the infection sites and eliminate the triggering pathogen. Encounter with the antigen takes place in the secondary lymphoid organs, where APCs show them to the T lymphocytes. Then, a differentiation process begins; it is destined to produce large amounts of effective cells to fight against the pathogen with a clonal proliferative process but without coming to exhaustion. Interaction between T lymphocytes and APCs continues in tissues and so does the expansion and cellular differentiation, in an effort to contain the infection without damaging the tissues of the affected individual. Therefore, antigenic recognition by T lymphocytes provides a series of changes that leads to a clonal expansion of differentiated and effective T lymphocytes. The activation of T lymphocytes through its specific TCR can be detected seconds after antigenic contact, and it continues during hours and days in the context of pathology (33, 34) (**Figure 2B**).

The activation of lymphocytes after recognizing a peptide in the context of a MHC molecule (priming of naïve T lymphocytes)

carries a series of processes of various types (genetics, proliferative, differentiation, biochemical) that lead to the formation of specific clones of effective lymphocytes, some of which will remain for long time in the form of memory cells. Before antigen exposure, the frequency of naive T lymphocytes specific for any antigen is 1 in 10^5 to 10^6 lymphocytes. After antigen exposure, the frequency of CD8+ T lymphocytes specific may increase to as many as 1 in 3 CD8+ T lymphocytes, representing a >50,000-fold expansion of antigen-specific CD8+ T lymphocytes, and the number of specific CD4+ cells increases up to 1 in 100 CD4+ lymphocytes may increase up to 5,000-fold.

It is well known that the single contact of T lymphocyte with its antigen is not enough to generate a cellular response but it rather causes the cell to enter into a refractory state and does not respond to any stimulus (35). This discovery led to the hypothesis that there should be other additional stimulatory signals that would enable T lymphocytes to become activated and exert their functions. Thus, when the CD28 molecule was identified as a co-stimulator of T lymphocyte function, the theory of the "two signals" was then reported, these two signals being necessary for the T lymphocytes activation. However, numerous evidences have suggested that other membrane-bound and soluble inflammatory signals are necessary to complete the activation of T lymphocytes, thus enforcing the "three signals" and "four signals" alternative theories. All experimental data seem to highlight the significant role of inflammatory mediators in the process of T lymphocytes differentiation into the effector population, resulting in a more adequate tool to respond to the aggression suffered (36, 37). On the other hand, there is also a great deal of data suggesting that TNF superfamily receptors (CD30, 41BB, OX-40, CD27) interact with their ligands in APCs (CD30L, 41BBL, OX-40L, CD70), which favors the survival of the activated cells and their passage into memory cells (38, 39).

T lymphocytes also have a large number of inhibitory molecules that help to regulate the cellular response, in a way that keeps this response not to be exaggerated and that would end up being harmful to the body. These inhibitory molecules act both, by limiting the co-stimulatory signals and by binding to the appropriate co-stimulatory receptors. The main inhibitory receptors belong to the CD28 family, including cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death 1 (PD-1), both being involved in the phenomenon of tolerance. CTLA-4 is an inhibitory molecule expressed in activated T lymphocytes which causes an increase in the intracellular phosphatase activity, thus producing a decrease in the signals generated by the TCR and CD28 molecule. On the other hand, CTLA-4 acts also as a competitive receptor for the CD80/CD86 receptor, but with a higher affinity for these receptors than CD28 itself (40, 41). As a result, depending on their level of expression on the cell surface, CTLA-4 may interfere with CD80/CD86 binding. Several more inhibitors, such as lymphocyte activation gene 3, and V-domain Ig suppressor of T lymphocyte activation have been described, blocking these inhibitors by specific antibodies are being studied to increase the immune response in numerous cancers (42, 43).

The scenario in which the T lymphocyte response occurs may change in situations such as aging or in tumor microenvironment, where systemic or local inflammation is present (**Figure 3**).

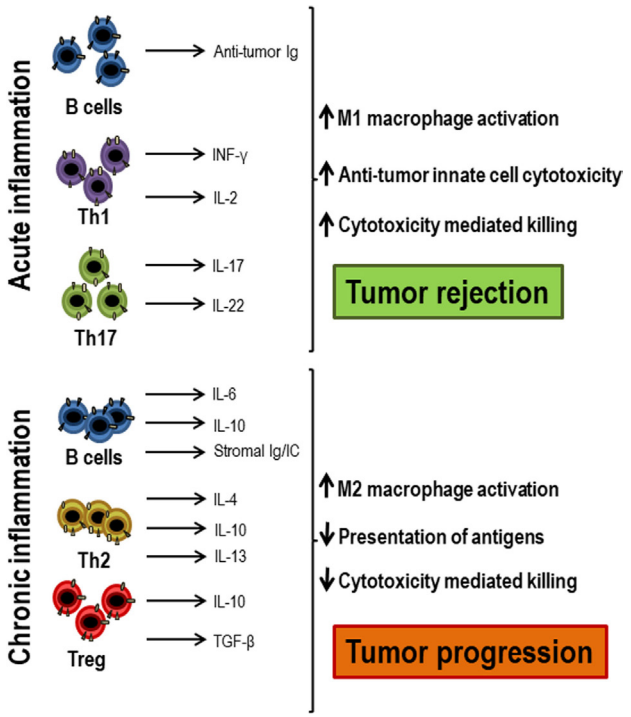

FIGURE 3 | Acute and chronic inflammation participation in antitumor responses. When acute inflammation is activated by tumor cells, Th1 cells secrete antitumor cytokines, such as IFN-γ and IL-2, which, together with antitumor antibodies produced by B lymphocytes, exert an antitumor response and lead to tumor rejection, attracting innate immune cells and cytotoxic T lymphocytes. However, when chronic inflammation occurs in response to tumors, there is often an increase in regulatory T lymphocytes, Th2 cells, and activated B lymphocytes that secrete growth factors, such as IL-4, IL-6, IL-10, IL-13, TGF-β, and immunoglobulins that decrease both antigenic presentation and the activation of cytotoxic cells, favoring tumor progression.

The levels of pro-inflammatory cytokines, such as IL-6, IL-1β, TNF-α, or GM-CSF, increase in these cases and this influences the lymphocyte response (3, 44). Dendritic cells are essential for T lymphocytes to be activated and differentiated as they present the antigen, producing co-stimulatory signals and essential cytokines. All these processes are highly dependent on the inflammatory environment mainly in chronic situation (45, 46). In the immune response during aging, myeloid dendritic cells in inflammatory environments have a decreased ability to present the antigens to CD4+ and CD8+ T lymphocytes (47). Moreover, most studies describe a reduced ability to produce cytokines by dendritic cells stimulated through toll-like receptors *in vitro*, a defect related to low response to vaccination in elderly (48, 49). TNF-α directly affects the immune response, in part by reducing the expression of the co-stimulatory molecule CD28 (50, 51). The pro-inflammatory environment may also be responsible for the attenuated response of T lymphocytes to cytokines, possibly due to the activation of negative regulatory pathways (4). According to the inflammatory environment in which lymphocytes are, the polarization of T lymphocytes will be different. In inflammatory environments, there is no evidence that Th1, Th2, or Th17 cells

are diminished, but follicular T lymphocytes have been found to be less common in the elderly with systemic inflammation in response to vaccination (52, 53). It has been shown that CD4+ T lymphocytes activated in inflammatory environments are less responsive to type I interferons due to recruitment by the IFNR signaling complex of SHP1 (54). In conclusion, the inflammatory environment must be taken into account when evaluating immune responses. Individuals with high systemic and/or local inflammatory levels, as it occurs in the elderly and in cancer patients, may have an impaired response to cytokines and a poor response to antigens. In these cases, an anti-inflammatory treatment could be beneficial in trying to restore a correct immune response.

Maintenance of Memory T Lymphocytes

The T lymphocyte-specific antigen response is characterized by clonal expansion, followed by a contraction in the number of specific cells and the formation of memory T lymphocytes. In this process, T lymphocytes acquire many key features to afford protection against re-infections, which are potentially deadly. Memory T lymphocytes are found in greater numbers than naïve T lymphocytes and in the presence of IL-7 and IL-15 these cells can be maintained for long periods of time without antigenic stimulation (55, 56) (**Figure 2C**). Central and effector memory T lymphocytes are less dependent on the contact with the MHC–peptide complex for survival than naïve T lymphocytes do (57). In addition, their self-renewal is three to four times faster than in naïve T lymphocytes and has a high proliferation rate in lymphopenia (58). The large number of memory cells, their high ability to reactivate, produce cytokines, and kill after antigenic stimulation and their distribution by almost all tissues makes memory T lymphocytes capable of protecting the individual much better than naïve T lymphocytes.

As previously mentioned, memory T lymphocytes can be divided into two populations, effector memory cells (EM, CD45RA−, CCR7−) and central memory cells (CM, CD45RA+, CCR7+), but other subpopulations can be established from the expression of CD27 and CD28 (5). EM are preferentially located in non-lymphoid and mucosal tissues and have a lower response threshold than CM, which are found mainly in secondary lymphoid organs and have a greater expansion capacity than EM.

There is no unanimity to describe the way by which T lymphocytes become effector and central memory cells (59). The most convincing hypothesis suggest a developmental trend from naïve T lymphocytes to memory cells, in which most memory cells have gone through a phase of effector cells. Some effector cells might then evolve into memory cells since some microarray studies have shown that passage of naïve cells to effectors and memory is a gradual step (59–61). Besides, several laboratories have used murine models of labeled effector T lymphocytes to track them, showing that most memory cells have indeed derived from these effector cells (62, 63). Thus, at least a set of memory cells that appear after an infection are generated from the effector cells.

Numerous studies have shown that exposure to a restricted inflammation increases the appearance of memory-like T lymphocytes when the intensity of inflammatory signals is controlled (59, 64). Several inflammatory molecules, such as interferon type I and IL-12 can be considered to act as the third signal (in addition to TCR and co-stimulation) and thus, to promote differentiation of T lymphocytes to effector cells (65).

According to several studies, prolonged and/or intense exposure to the pro-inflammatory cytokine IL-12 promotes a preferential differentiation toward effector cells, rather than memory cells (66, 67). The effect of other inflammatory molecules on T lymphocytes may be indirect. For instance, an interesting study found a blockage in the contraction of T lymphocytes when there was a deficiency of IFN-γ (68). In this scenario, we believe that IFN-γ offers a competitive advantage for the appearance of memory T lymphocytes, in some types of immune responses (69). Some studies suggest that at least some level of inflammatory signal is necessary for the differentiation to memory T lymphocytes. T lymphocytes deprived of this inflammatory signal (third signal) or deficient in T-bet transcription factor showed a decreased ability to produce long-lived memory cells. T-bet is needed for the expression of the CD122 molecule, that is the beta chain of the cytokine receptors IL-15 and IL-2, and to be able to react to homeostasis mediated by IL-15 (66, 70, 71). The cytokine IL-15 not only can promote the division and proliferation of differentiated and memory T lymphocytes but it is also capable of increasing its functional capacities (72, 73).

The differentiation of memory T lymphocytes in the course of chronic infections or in the presence of persistent antigens (autoimmune diseases, tumors, or atherosclerotic diseases) is different from differentiation in acute infections and leads to a defect in the functionality of T lymphocytes. These cells phenotypically acquire expression of several typical cytotoxic cell markers, such as NKG2D, PD-1, CD56, CD16, KLRG1, etc. (74, 75). Unlike memory cells generated after the elimination of an acute infection, memory cells in situations where the antigen is present in a chronic form, and in an inflammatory environment, proliferate and increase in number because the continuous stimulus (76, 77). These subpopulations, in turn, have propensity to secrete pro-inflammatory cytokines, such as IFN-γ, IL-1, TNF-α, and IL-6, contributing to increase the systemic or local inflammation (78).

New Encounters with Specific Antigens and Telomere Maintenance

One of the most remarkable characteristics of the immunological memory system is the capacity of the antigen-primed T lymphocytes to carry out a rapid response after they encounter the same antigen again (79). Co-stimulation signals have been recognized as critical for optimal T lymphocyte responses and result from important interaction between receptors on the surface of T lymphocytes and their ligands on APCs. However, memory T lymphocytes exhibit more reduced dependency to co-stimulation, and a significant lower threshold to respond.

T lymphocytes must be able to grow exponentially upon the first, and especially the second and the following, encounter with an antigen, and when they are no longer needed some of them enter apoptosis and disappear. Like most normal cells, lymphocytes are able to pass through a limited number of division cycles. Limited number of cell cycle divisions is associated to cell senescence or cell stress, which ends up in cell death triggering. Similar to what

occurs to normal cells that evolve toward a neoplastic state acquiring a high rate of proliferation, lymphoid cells are also able to constitutively activate telomerase (80). Upregulation of telomerase and consequently elongation of telomeres allows extending the cells lifespan (81, 82). When the mechanisms that compensate telomeric shortening disappear and when shortening reaches a critical point known, cells enter a state of growth arrest termed senescence (83, 84). Telomere size can be determined by analyzing the terminal restriction fragments (TRF) that contains the TTAGGG region. The length of the TRF varies depending on cell population and each chromosome within the same cell. The critical point for cells to enter senescence appears when the size of TRFs reach less than 6 kb (85). The overall finding from different studies is that T lymphocytes in humans can carry out a certain number of divisions, after which they can no longer be divided (86, 87). Importantly, the reach of the replicative senescence stage by T lymphocytes does not imply a loss of cell viability. Moreover, senescent T lymphocytes under suitable conditions can remain alive and metabolically active for a prolonged period of time (88, 89). This transitional state of lymphocytes is not, however, shared with stem cells or malignant tumor cells, which do not reach replicative senescence and have stable chromosomes despite intense division. Stem and malignant T lymphocytes maintain telomerase activity and, therefore, replicative capacity throughout their lives (90). Nevertheless, by using mice lacking telomerase, it has been demonstrated that telomere shortening shunts premalignant T lymphocytes into the senescent state, therefore, reducing tumorigenesis (91).

It is very important to distinguish undifferentiated and highly differentiated T lymphocytes in order to study telomerase activity in these two populations. The undifferentiated T lymphocytes (CD27 +CD28+) display longer telomeres than highly differentiated T lymphocytes (CD27−CD28−) while intermediate populations (CD27−CD28+ or CD27+CD28−) have telomere lengths between both, undifferentiated and highly differentiated cells (92, 93). In addition, the proliferation ratio in T lymphocytes is higher in highly differentiated CD45RA−CCR7− cells that have a lower telomeric length (92, 94). Telomerase activity correlates with the length of telomeres, thus this activity is greater in the more undifferentiated cells and much lower in the cells with high differentiation. In addition, as the cell ages, the ability to induce telomerase expression and activation is lost (92, 93, 95). Moreover, differences in the behavior of telomerase have been found between CD4+ and CD8+ T lymphocytes. Cultures of CD4+ and CD8+ T lymphocytes from the same subject that have encountered an antigen for the fourth time were unable to increase telomerase production. However, the CD4+ T lymphocytes had much higher telomerase activity than that of the CD8+ T lymphocytes from the same donor (96). Several studies have shown that homeostasis of CD4+ T lymphocytes is much more rigorous than that presented by CD8+ T lymphocytes. In addition, aging in lymphocytes was previously described in CD8+ T lymphocytes, since the changes observed in immunosenescence occur much earlier in these CD8+ T lymphocytes (97–99).

The signaling *via* the TCR and the co-stimulation with other molecules such as CD28 is essential for the induction of telomerase activity. This activity peaks 4–5 days after the TCR has been stimulated, but presents a decrease in its activity after 10 days (92, 100, 101). T lymphocytes can proliferate under stimulation of various cytokines without the mediation of TCR. This is homeostatic proliferation, the mechanism by which naïve and memory T lymphocytes are maintained in the periphery (8). The cytokines IL-7 and IL-15 have been related to telomerase activity in the CD4+ and CD8+ T lymphocytes, respectively (102, 103). Our laboratory has demonstrated that IL-15 has a preferential effect on the CD4+CD28− T lymphocyte population, which causes an increase in proliferation and specific responses of these cells (73). It has also been found inhibitory effects on telomerase activity by some cytokines such as IFN-α and TGF-β (104, 105).

Factors that promote repeated T lymphocyte stimulation, such as persistent antigen and chronic inflammation, appear to drive telomere loss and replicative senescence. Elderly individuals and cancer patients, often exhibit chronic inflammation characterized by immune system dysregulation with increased inflammatory cytokine production (73, 74, 106, 107) and redox imbalance due to reduced antioxidant defenses and overproduction of reactive oxygen species (ROS) (108). It is now recognized that chronic inflammation is a major risk factor for several age-associated diseases, including chronic obstructive pulmonary disease, neurodegeneration, obesity, and vascular disease (109, 110). Premature telomere erosion in peripheral blood lymphocytes is a common characteristic of these diseases as well as autoimmune syndromes (103). These findings suggest that telomere loss increases susceptibility to autoimmune disease and may be a predisposing factor for age-related inflammatory disease.

Restoring telomerase activity in T lymphocytes would have a great impact on human lives by restoring telomere shortening and, in turn, avoiding the deleterious effects of aged T lymphocytes. Several studies have shown that if the telomerase activity is preserved, the telomeric length is stabilized and replicative senescence can be delayed (111, 112). One possible solution to stimulate telomerase activity would be to eliminate senescent T lymphocytes from the bloodstream or bring them into apoptosis. Telomere loss could be stopped by inhibiting cytokines, such as TNF-α, mediating telomere shortening.

T Lymphocyte Exhaustion

In a viral infection or cancerous environment where there is a permanent exposure to certain antigens and high inflammation, memory cells are highly affected (113). This alteration, known as T cell exhaustion, presents a series of characteristics: progressive loss of effector functions, upregulation and co-expression of many inhibitory receptors, alteration of transcription factors, dysfunctional metabolism, failure to enter a quiescent state, and response to normal homeostasis (114) (**Figure 2D**). Although the appearance of exhausted T lymphocytes was first described in an environment of viral infection, it has also been observed in the presence of cancer and in various inflammatory diseases. Initially, exhausted T lymphocytes have a defect in proliferative progression and a defect in the expression of telomerase, but the rest of their functions are completely conserved (115). Next, exhausted T lymphocytes, mainly due to continuous antigenic stimulation and a pro-inflammatory environment, enter into a state of

differentiation where they gradually lose their effector functions, such as cytokine production and cytotoxic capacity, which prevents these cells from being effective against cancer or microbial infections (113). Exhausted T lymphocytes normally arise during high-grade chronic infections in highly pro-inflammatory environments, where the level and duration of antigenic stimulation are critical for this process to occur (116, 117).

Viral infections and cancerous tumors can cause the appearance of exhausted T lymphocytes, but not all cases lead to an exhausted T lymphocyte (113, 118, 119). The difference in the ability to produce exhausted T lymphocytes may be due to different T lymphocyte specificities. The most effective specificities in stopping pathogens inactivate them faster and destroy them quickly. Exhausted cells regain functionality and become typical memory T lymphocytes when viral infection is under control and in turn, antigen concentration decreases (120).

Exhaustion of T lymphocytes is accompanied by an increase in expression of inhibitory molecules, including PD-1, CTLA-4, LAG3, 2B4, CD160, and T lymphocyte immunoreceptor with immunoglobulin and ITIM domains (TIGIT). PD-1 is an inhibitory protein that intervenes in self-tolerance by inhibiting the activation of T lymphocytes using a similar mechanism as describe for CTLA-4 (121). After PD-1 interaction with its ligands, namely PD-L1 or PD-L2, TCR signaling is blocked by recruiting SHP-2 phosphatase and subsequent dephosphorylating of the antigen receptor (122). Interestingly, both ligands are often overexpressed in many tumor cells, but PD-1 is highly expressed in T lymphocytes from patients with different types of cancer. PD-1 ligand levels from tumor cells and PD-1 levels from T lymphocytes are usually correlated with the tumor aggressiveness and poor prognosis (121).

The proliferation of exhausted cells is partially compensated with PD-1 suppression, but telomerase activity is not restored (123). Many causes leading to T lymphocyte exhaustion are shared with those leading to T lymphocyte aging. Several epigenetic studies in naïve and central memory T lymphocytes reveal an age-associated loss of access of T lymphocytes promoters to their chromatin site of action, particularly to the NRF1-binding sites (124). This loss seems to intervene in the reduced expression of the genes of the mitochondrial respiratory chain and, therefore, in the deficient oxidative phosphorylation that can lead to death of the T lymphocytes (125). Repression of the molecule PGC1α, a cofactor of NRF1 activity, is an event that appears early in exhausted T lymphocytes, indicating a mechanistic overlap between T lymphocyte aging and exhaustion (126). Effector memory T lymphocytes of elderly individuals compared to effector memory cells in young people show few differences with respect to the accessibility of chromatin promoters. Thus, the memory T lymphocytes in elderly individuals do not exhibit the epigenetic marks of the exhausted T lymphocytes (124, 127). This observation may indicate that T lymphocyte aging affects mainly to naïve cells and central memory, possibly through pathways similar to those involved in T lymphocyte exhaustion. On the other hand, effector T lymphocytes in the elderly do not show any signs of exhaustion.

The epigenetic phenotype of the exhausted T lymphocytes remains stable when PD-1 is blocked and the restoration of function is, therefore, only transient in most T lymphocytes (128). This evidence leads us to consider whether treatment with PD-1 blockers decreases along with age due to aging of the exhausted T lymphocytes or rather because the responsive fraction of exhausted T lymphocytes to treatment decrease with age.

The appearance of exhausted T lymphocytes prevents the possibility of an adequate control of infections and malignant tumors. Therefore, if overexpressing pathways in the exhausted T lymphocytes could be modulated, for example, by inhibiting PD-1 and CTLA-4, their dysfunctional state could be reversed and immune responses invigorated.

METABOLIC REPROGRAMMING

T lymphocytes must carry out metabolic strategies throughout their differentiation process and because of the changing microenvironment in which they are located. The purpose of these adaptations is to meet energy and structural needs in the different stages of proliferation and to achieve functional responses according to the availability of nutrients.

Naïve T lymphocytes, since they exit the thymus as mature cells and during their travel throughout the secondary lymphoid tissues until encountering their specific antigen, show a reduced rate of cell division. Even more, it is considered that they are in a functional quiescence, which does not require a high-energy consumption. They use the available nutrients trying to obtain the highest energy yield. Glucose, fatty acids, and amino acids are metabolized until they enter into the tricarboxylic acid (TCA) cycle where ATP and reducing equivalents are generated, which subsequently increase the production of ATP upon entering the pathway of oxidative phosphorylation (129, 130).

When naïve T lymphocytes encounter APCs carrying their specific antigenic peptide, activation takes place in a lymph node. Clonal expansion of the antigen-specific T lymphocytes is mainly mediated by IL-2 and the subsequent functional differentiation adapts the response to the specific triggering pathogen. Proliferation leads to generate sufficient amount of specific T lymphocytes capable of eradicating the pathogen. Thus, T lymphocyte activation not only requires energy but also the production of precursors that support the explosive proliferation through the biosynthesis of the required cellular components, proteins, lipids, nucleic acids, etc. These processes imply a high increase in energy demands, resulting in a switch in the metabolic pathways of nutrient utilization. Initially, there is an increase in glucose uptake, by increasing the expression of glucose transporter (GLUT) such as GLUT-1 (131). Glucose is, therefore, essentially metabolized to lactate by aerobic glycolysis, a phenomenon known as "Wargurg Effect," which was initially described in tumor cells and, more recently, also in T lymphocytes (132–134). It is currently considered that "T lymphocyte activation-induced metabolic reprogramming is reminiscent of the metabolic changes associated with oncogenic transformation" (135, 136). More than 90 years ago, Otto Warburg described that tumor cells, even in the presence of oxygen, showed a high rate of glycolysis. This so-called aerobic glycolysis is now recognized as one of the new hallmark of cancer capabilities (5). In differentiated cells, glucose renders through the TCA cycle an energetic

yield of 36 molecules of ATP. And yet, cancer cells rather prefer to compromise a high ATP production in order to obtain other benefits, in terms of building blocks for their new daughter cells (137–139). Recently, a similar program has also been observed in all proliferative cells including T lymphocytes upon antigen activation. This metabolic adaptation ensures a high glycolytic rate, which is not inhibited by the production of mitochondrial ATP. Moreover, glycolysis inhibits apoptosis and contributes to the maintaining of mitochondrial membrane potential (137, 140). Regarding the immune system, metabolic reprogramming has also been linked to the acquisition of certain functional properties by T lymphocytes such as the secretion of IFN-γ (134, 141).

Other functional parallelism found is glutaminolysis, which also increases in response to T lymphocyte activation as well as after transformation of cancer cells. Glutamine is rapidly consumed by tumor cells, playing a key structural role as a nutrient in the biosynthesis of nucleotides. The concentration of glutamine has been shown to be limiting in the progression of the cell cycle. Glutamine deprivation leads to cell cycle arrest in some cell types. Glutamine is not only an important source of carbon and nitrogen for the synthesis of other amino acids but the α-ketoglutarate generated is an anaplerotic substrate of the TCA cycle. Thus, it may contribute to the generation of ATP when glucose is primarily derived toward this so-called aerobic glycolysis (142).

On the other hand, pentose phosphate pathway (PPP) is the main catabolic route that generates ribose, necessary for the synthesis of nucleotides, and NADPH, essential for proliferation as it provides the reduction equivalents for fatty acid and cholesterol biosynthesis. Furthermore, PPP plays an essential role in balancing the redox status by regulating the production of glutathione (143). The utilization of PPP is usually elevated in cancer cells and in proliferative T lymphocytes. The key enzymes in the pathway are overexpressed in cancer and oncogenes and tumor suppressors have been shown to regulate PPP activity.

Metabolic decisions are dependent on the co-stimulatory signals received by T lymphocytes, in particular on CD28, which mediates many of these processes *via* the PI3K–Akt–mTOR pathway. However, in cancer cells intracellular programs combine with extracellular signals, to achieve a significant independence from external requirements. Genetic alterations as common as PI3K and its negative regulator PTEN as well as gen amplifications of upstream receptor tyrosine kinases result in an increase in glucose uptake and in the metabolic reprogramming in several cancer cells (144). It is noteworthy to mention that the metabolic state of cancer cells has a potential influence on the surrounding cells. Thus, within the tumor microenvironment, cancer cells alter the metabolic composition of the extracellular milieu, affecting the signaling pathways that influence the infiltration of immune cells, including T infiltrating lymphocytes. mTOR is a central part of various signals that come from the immune microenvironment because mTOR works as a sensor of extracellular medium conditions. mTOR is an evolutionarily conserved serine/threonine kinase, which forms two complexes, mTORC1 and mTORC2, determined by the association with different adapters and scaffolding proteins. mTOR is responsible for integrating different responses upon receiving environmental signals and for the control of various cellular functions, such as growth, apoptosis, actin reorganization, metabolism, and ribosome genesis (145, 146). mTOR activation targets T lymphocyte metabolism, switching it toward glycolytic metabolism by induction of two major transcription factors, namely HIF1α and c-MYC (147, 148). HIF1α is stabilized under hypoxia but it can also be activated by mTOR even under aerobic conditions. It increases the expression of GLUTs and glycolytic enzymes, such as pyruvate dehydrogenase kinase 1, limiting the entry of pyruvate into TCA cycle and favoring its reduction to lactate. The deregulation of multiple elements of the mTOR pathway has been reported in many types of cancers, implying significant effects on tumor progression. Likewise, mTORC1 signaling controls transcription of many genes, some of which are involved in metabolic and biosynthetic pathways (149).

Signals derived from anaerobic conditions and nutrients availability may modulate the cytokine profile of T lymphocytes (150). CD4+ T lymphocyte differentiation into a specific effector phenotype (mainly Th1, Th2, Th17, Treg) will depend fundamentally on the cytokines present in the immunological microenvironment at the time of antigenic presentation. mTOR plays a key role in the differentiation into the effector phenotypes, but not into Treg, so mTOR1 is needed for differentiation toward Th1 and Th17, whereas mTOR2 regulates Th2 differentiation (151). Thus, mTOR coordinates the metabolic pathways and the differentiation of each subpopulation of CD4+ T lymphocytes (146). High levels of HIF1α direct the CD4+ T lymphocyte metabolism to the glycolytic pathway, favoring the activation of RORγt transcription factor and its differentiation toward a Th17 inflammatory phenotype. Inhibition of this pathway, even under conditions that promote Th17 differentiation, results in Treg lymphocytes (152, 153). Th1 also possess a high glycolytic rate, concomitant with a higher surface location of GLUT1 (154). In fact, there is a coordinated regulation between T lymphocyte metabolism and the IFN-γ production by Th1. GAPDH is attached to UA-rich regions located at the 3′ in the untranslated region of the IFN-γ mRNA in non-activated cells. When glycolytic metabolism is activated, this enzyme is involved and does not bind to the mRNA, allowing its translation and IFN-γ production (134).

It has also been shown that the limited availability of glutamine in the extracellular medium favors Treg differentiation, even in the presence of cytokines involved in Th1 differentiation (155). The reason seems to be the decrease in intracellular levels of α-ketoglutarate, a mTORC1 activator, the expression of T-bet transcription factor and, as a result, differentiation into Th1. Similarly, the production of GLUT1 receptor, essential for the development of Th1 responses, is increased, whereas Tregs are unaffected by deficiency in this receptor and maintain their inhibitory capacity on T lymphocytes independently of GLUT1 (156).

In CD8+ T lymphocytes, mTORC1–HIF1α is activated by a PI3K–Akt-independent pathway, through phosphoinositide-dependent kinase 1. Metabolically, it promotes the activity of glycolytic enzymes and at functional level, it activates cytolytic capacity, controls the migration, and inhibits the generation of memory cells. Meanwhile, mTORC2 activates oxidative metabolism (157, 158).

In other way, long-lived specific memory cells that circulate between the secondary lymphoid organs, blood, and tissues

do not proliferate significantly and have a quiescent functional state with scarce or no cytokine production. The main difference with naïve cells is that memory cells need to be prepared to respond quickly and efficiently to a new contact with the antigen. Their metabolism is based on the oxidation of glucose and fatty acids and they are characterized by a high content of large mitochondria, which are generated by fusion of the individual organelle that support the energetic requirements in reactivations (159, 160). In addition, ATP obtained from glucose oxidation is used to synthesize fatty acids, which in turn will be oxidized. The motive of this futile cycle could be the maintenance of the mitochondrial activity in order to be ready to respond quickly to specific-antigen re-stimulation (161–163).

Following multiple antigenic challenge, mainly in the case of chronic viral and tumor antigens and in situations of chronic inflammation, the specific T lymphocytes go through successive phases of clonal division, which as mentioned above, changes its degree of differentiation and, in parallel, its phenotype and functional capacity. In this stage, metabolic switching occurs in favor of an oxidative phenotype and is unambiguously associated with increased mitochondrial ROS production. On the other hand, inhibition of fatty acid oxidation decreases NADPH and glutation (GSH) levels. However, ROS levels increase, suggesting that control of fatty acid oxidation, in addition to PPP, regulates NADPH levels, which is essential to regenerate GSH pools from the glutathione disulfide (164, 165).

Finally, T lymphocytes reach replicative senescence, characterized by constitutive p38 mitogen-activated protein kinase activation, telomeric shortening, the loss of telomerase activity, and reduced proliferative capacity in response to stimulation (123). This new situation is also associated with metabolic adaptations. Again, a link between T lymphocytes aging and bioenergetic status has been proposed, since glucose deprivation in non-senescent T lymphocytes induces the activation of p38, and its constitutive activation inducer, the metabolic sensor of intracellular levels of ATP AMPK (5′-monophosphate activated protein kinase). These processes lead to a reduction in telomerase activity and proliferation similar to those observed in senescent T lymphocytes (166, 167). The main metabolic pathway used by these cells at this stage is glycolysis. Senescent T lymphocytes show mitochondrial dysfunction and consequently produce higher levels of ROS and defective mitochondrial biogenesis, which may justify their metabolic switch. P38 inhibition leads to mitophagy and thereby nonfunctional mitochondria are eliminated and ROS production is reduced. However, the increase in energy needed to sustain proliferation continues to be obtained from glycolysis and not from oxidative phosphorylation (168). As mentioned previously, high levels of inflammation in certain chronic viral infections and in cancer, induces an exhausted stage, similar to happen in senescence (113, 169, 170). There are numerous links between the inhibition of T lymphocytes by CTLA-4 and PD-1 and metabolic signaling pathways. CTLA-4 interacts with PP2A (protein phosphatase 2), a negative regulator of AKT, mTOR, and MAPK signaling, whereas PD-1 inhibits AKT phosphorylation by preventing CD28-mediated activation of PI3K (171). It has been shown that PD-1 inhibits glycolysis and amino acid metabolism and promotes lipid metabolism, whereas CTLA-4 inhibits both processes and mitochondrial biogenesis in memory cells (172, 173). Since the main metabolic pathway of T lymphocytes during activation is the aerobic glycolysis, these molecules could be blocking differentiation into effector T lymphocytes, at least partially, by metabolic regulation. The increase in β-oxidation of fatty acids could be an explanation for the maintenance of these cells, despite being exhausted, and in addition to its ability to recover functionality when interaction between PD-1 and its ligands hangs (173). **Figure 4** shows a diagram of the main lymphocyte metabolic pathways in their different differentiation stages.

REDOX CONTROL OF CELLULAR FATE

Relationship between oxidative stress and inflammation has been widely documented (174). Oxidative stress plays a pathogenic role in many chronic inflammatory diseases. Lower levels of GSH, an intracellular thiol antioxidant, causes ROS production, which results in imbalanced immune response and inflammation. Moreover, protein oxidations turn into release of inflammatory signal molecules and inflammatory stimuli induce the release of peroxiredoxin 2, a redox-active intracellular enzyme (175).

Basal levels of ROS generated in response to endogenous and exogenous stimuli are crucial mediators of multiple cell processes such as growth, differentiation, or migration, but excessive production might induce cell death, apoptosis, and/or senescence. Oxidative stress triggered by the excessive ROS production, cause oxidative damage to cellular components such as DNA, proteins, or lipids, which is closely related to the pathogenesis of various diseases including cancer. In addition to exogenous ROS, major intracellular sources of ROS are NADPH oxidases and particularly mitochondria. Usually, physiologically generated ROS are balanced by non-enzymatic and enzymatic systems, such as, GSH, superoxide dismutases (SOD1/2), thioredoxins (Trx1/2), catalase, or peroxidases. In addition, NADPH, is one of the main thiol-dependent electron donors system in the cell and plays a critical role in the regulation of cellular redox environment and in a wide range of cellular pathways, including activation of transcription factors such as NF-κB, activator protein-1, p53, HIF-1, or the redox factor 1 (176). Elevated levels of ROS production have been considered an adverse event, playing an important role in tumor initiation and progression, but also in promoting inflammatory environments. However, ROS are now more widely recognized as important signaling molecules (177, 178). Redox signaling in cells by ROS such as hydrogen peroxide (H_2O_2) occurs through the reversible oxidation of cysteine thiol groups. A major cellular target of ROS is the thiol side chain (RSH) of cysteine, Cys sulfenic (Cys-SOH) and sulfinic (Cys-SO2H) acids have emerged as important mechanisms for regulation of protein function. These residues modifications result in reversible structural alterations that can modify protein function, which may imply either inactivation or gain of function (179).

The importance of ROS in immunity is exemplified by their generation and release in the form of an "oxidative burst" by phagocytic cells as part of the innate immune cell network to effectively destroy pathogens and clear debris. However, ROS exert, as it does in other cells, a dual role on T lymphocyte biology. Mild

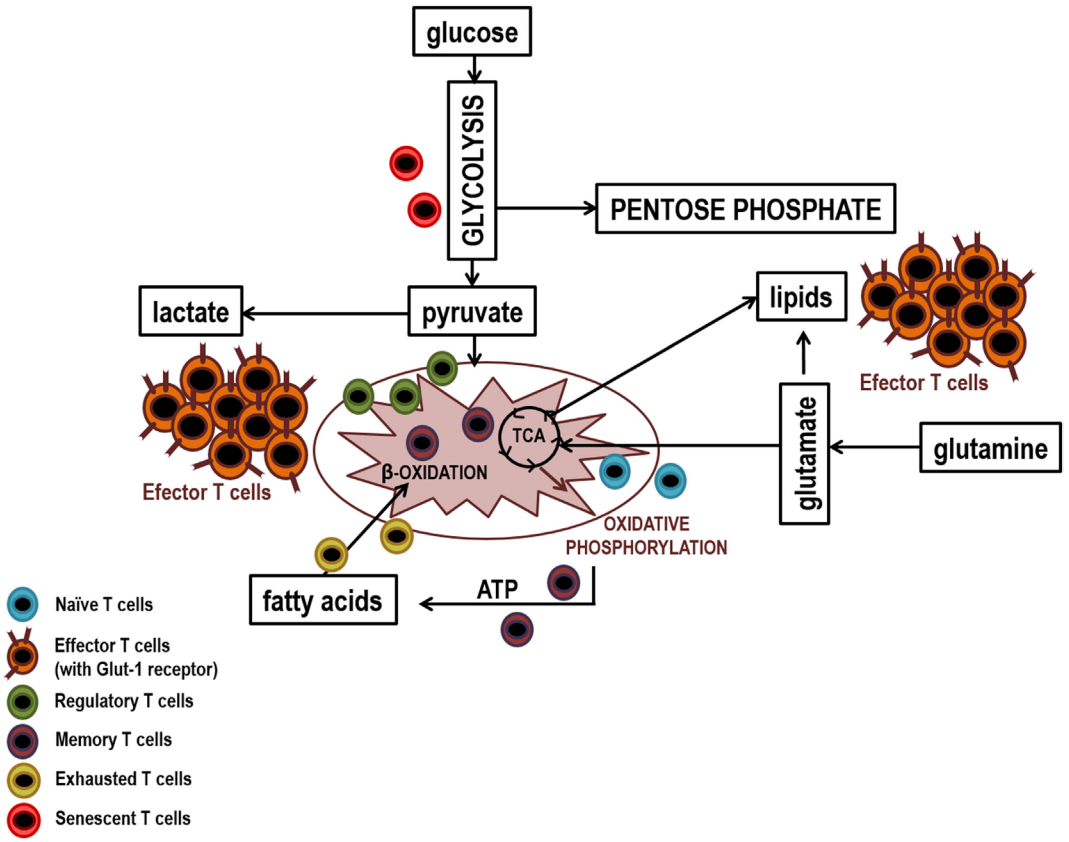

FIGURE 4 | Main metabolic pathways of lymphocyte subpopulations. Naïve, memory, and regulatory T lymphocytes uptake low levels of glucose which it is metabolized by glycolysis and then the pyruvate generated is fully oxidized to CO_2 in the mitochondria by the Krebs cycle. In contrast, effector lymphocytes have a large demand for glucose to maintain the biosynthetic processes that facilitate cellular growth, proliferation, and the synthesis of substantial quantities of effector molecules. Thus, effector lymphocytes show higher production of glucose transporters (GLUTs) and higher activity of glycolytic enzymes that facilitates elevated rates of glucose uptake and glycolytic flux. To sustain high rates of proliferation, pyruvate is converted to lactate and released out of cells. This prevents the accumulation of pyruvate which could result in the inhibition of the glycolysis pthway. Due to the high levels of glycolytic flux, glycolytic intermediates can be diverted into biosynthetic pathways to generate amino acids, lipids, and nucleotides in order to generate biomass. Senescent T lymphocytes use glycolysis extensively, partly because they have dysfunctional mitochondria and exhausted T lymphocytes mainly use lipid metabolism to carry out their poor cellular functions.

ROS are essential for T lymphocyte activation, expansion, and effector function (180–183). Still, elevated rate of ROS production or exposure and defective neutralization by antioxidant cellular systems, causes oxidative stress that compromises T lymphocyte proliferation and activity (182, 184). Balance between both situations may be fragile and the studies yield results that seem to be contradictory, probably due to different experimental conditions.

Regulatory Effect of Oxidation on T Lymphocytes

Generation of ROS and Ca^{2+} release from intracellular stores are direct consequences of TCR/CD28 stimulation. Both are essential for TCR signaling, particularly in activation-induced CD95L expression (185). 5-lipoxygenase, NOX-2 and mitochondrial complexes are the most important sources of ROS in T lymphocytes (186–188). Oxidative signals originated from Complex I of the ETC regulate T lymphocyte activation-induced expression of IL-2 and IL-4, whereas Complex III is required for CD4+ activation and antigen-specific T lymphocyte expansion (189, 190). It is well known that ROS can activate the transcription factor NF-κB, whereas chronic exposure to oxidative stress inhibits its phosphorylation and the activation of T lymphocytes (191, 192). Related to this, translocation of NF-κB to the nucleus occurs in a cytoplasmic oxidative environment; however, binding to DNA requires reducing environment. Very high levels of ROS might affect both compartments and in such circumstances NF-κB pathway will be inhibited (193, 194). On the contrary, reduced ROS production is associated with decreased phosphorylation of JNK and NF-κB and, therefore, low IFN-γ and CD39 expression in CD8+ T lymphocytes (195). Equivalent results have been found in other regulatory pathways, since the exposure to low levels of ROS stimulates mTORC1 while high concentrations or long-term ROS treatment decrease mTORC1 activity (196).

Reactive oxygen species are also implicated in T lymphocyte differentiation, and murine models with specific knockouts of NOX-2, such as $gp91^{phox}$ and $p47^{phox}$, have been employed to test this association. $p47^{phox}$ deficiency leads to Th17 differentiation, because mice $p47^{phox-/-}$ have diminished expression of T-bet, STAT-1, and STAT-4 transcription factors, but increased phosphorylation of STAT-3. Additionally, it has been

shown a reduced production of IL-2, IL-4, IFN-γ, TNF-α, and GM-CSF, but increased IL-10, IL-17, and TGF-β (188). On the contrary, lack of gp91^{phox} leads to a Th1 phenotype with reduced GATA-3 expression and STAT-5 and STAT-6 phosphorylation but increased T-bet expression. These T lymphocytes produce less IL-4 and IL-5 but more IL-17 and IFN-γ (189, 197). Then, NOX-2-deficienT lymphocytes showed a decreased in IL-4 but an increased IL-17 production. Interestingly, NOX-2 is not required for the proper activation of primary murine T lymphocytes, as gp91$^{phox-/-}$ T lymphocytes have no defect in CD25 and CD69 expression, IL-2 production, or proliferation (187, 197).

CD4 T lymphocyte plasticity, switching from one lineage to another, may be affected by the oxidative microenvironment (37). As mentioned before, an oxidative microenvironment exerts opposite effects on cytokine secretion by Th1 compared to Th2 cells. When *in vitro* derived Th1 and Th2 clones or employed T lymphocytes derived from autoimmune thyroiditis to examine their ability to expand and produce cytokines in response to oxidative stress, low levels of H_2O_2 are able to reduce IFN-γ production by activated Th1 clones but to increase IL-4 secretion by activated Th2 clones (198). Besides, mitochondrial ROS can control T lymphocyte activation by upregulating IL-2 and IL-4 expression, and using T lymphocytes isolated from patients with atopic dermatitis, the inhibition of Complex I-mediated ROS blocks disease-associated spontaneous hyperexpression and TCR-induced expression of IL-4 (189).

Oxidative Stress on T Lymphocytes

In opposite to regulatory role of mild oxidation, oxidative stress shows important effects during T lymphocyte development and differentiation. Thymus-specific elevation of mitochondrial superoxide $\left(O_2^{\bullet -}\right)$ disrupts normal T lymphocyte development and impairs the function of the mammalian adaptive immune system (199).

The stage of differentiation largely determines sensitivity of individual T lymphocyte subsets to oxidative stress. The susceptibility of T lymphocytes to oxidative stress varies greatly depending on which stage of differentiation they are in (**Figure 5**). Effector cells are exposed to low oxidative environment, while memory cells are T lymphocytes found in the most oxidative environments. Some secreted cytokines can cause oxidative stress in T lymphocytes and in cancer cells. For example, tumors associated macrophages have been shown to induce sub-lethal oxidative stress in murine mammary cancer cells, maybe through the secretion of TNF-α. On the other hand, extracellular superoxide dismutases might finely tuning the levels of H_2O_2 in the extracellular milieu altering the proliferation and differentiation of immune cells (200). In fact, ROS may induce decreased viability in CD4+ T lymphocytes and the inhibition of DNA synthesis (181, 201). The latter is associated with alterations in the TCR signaling, including conformational changes of TCRζ and LCK, reduction of PLCγ-1 phosphorylation and calcium flux, and increased ERK phosphorylation. Moreover, it has been known that prolonged exposure to H_2O_2 suppress tyrosine phosphorylation, calcium flux, NFAT, and NF-κB activation, and IL-2 production (191).

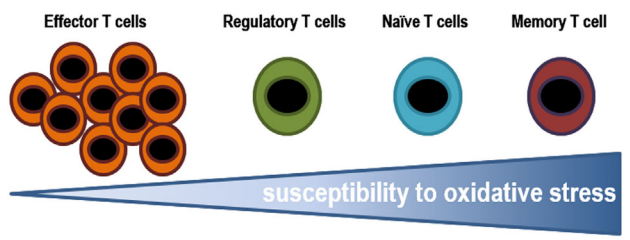

FIGURE 5 | Susceptibility of T lymphocytes to oxidative stress. Effector cells are exposed to low oxidative environment, while memory cells are T lymphocytes found in the most oxidative environments. The ability of T lymphocytes to stand such oxidative conditions, in either an infectious disease state or a tumor microenvironment, might determine the pool of T lymphocytes.

In vitro assays testing the resistance of different subsets T lymphocytes to H_2O_2, it has been shown that it decreases from effector, to regulatory, naïve, and finally memory T lymphocytes (201). Effector T lymphocytes are able to withstand higher concentrations of ROS, which is probably essential to play their role in helping to phagocytes to eliminate pathogens (184). Whereas, human Tregs have higher thiol content and as a result, they are more resistant to cell death induced by H_2O_2 secreted by granulocytes than conventional T lymphocytes (201). Tregs suppress GSH synthesis and cysteine release by DCs in a CTLA-4-dependent manner. The resulting decrease in intracellular GSH leads to reduction in DNA synthesis in conventional T lymphocytes (191, 202), reduced levels leads to membrane displacement of LAT (central adapter protein in the TCR), and responsiveness T lymphocytes. A recent study showed that Tregs modulate GSH metabolism in T lymphocytes *via* cell contact and antigen-dependent, but not by an antigen-specific mechanism during suppression (202). The mechanism has been not identified yet but it could involve NADPH oxidase. Macrophages have also been shown to suppress T lymphocyte activation *in vitro* and *in vivo* through ROS (203) and, recent data demonstrates that macrophages induce Tregs *via* a ROS-dependent pathway that can be blocked by the NADPH oxidase inhibitor apocynin (204). In addition, scavenging enzymes that reduced oxidative intracellular milieu influence the regulation of T lymphocyte activity. Mitochondrial superoxide dismutase (MnSOD/SOD2) reduces T lymphocyte differentiation and functional ability by decreasing ROS levels (199, 205). Glutathione peroxidase-4 inhibits lipid peroxidation and plays a central role in the survival and the expansion of T lymphocytes.

In aging, the increase in oxidative stress and accumulated damage in the leukocytes appears to be related to the age-related deterioration of immune functions. A study by De la Fuente et al. reveals that the greater the cellular oxidative state and oxidative damage observed in immune cells of aged mice as well as in peripheral blood of elderly humans, were related with impaired immune responses (phagocytosis, chemotaxis, lymphoproliferation, etc.). However, in healthy centenarian individuals and in very long-lived mice, with preserved immune functions, they all presented a decreased expression of different inflammatory genes and highly controlled oxidative stress in their immune cells, which can partly explain their longevity (206–209). It is

very important to emphasize in this context, that phagocytes, are postulated as the main responsible for oxidation-chronic inflammation stress that is associated with age and with immunosenescence (210). In the end, as a result of the oxidative damage that is related to aging, these cells could lose the ability to regulate their redox and inflammatory state, with the result of producing more and more oxidizing and inflammatory compounds, and thus contribute to the increase of oxidative and inflammatory stress. On the other hand, there are several studies performed on macrophages and peripheral blood neutrophils, in mice and humans, which have shown that these cells produce higher levels of oxidized compounds than those produced by lymphocytes and these levels increase with age. Furthermore, all these oxidative and inflammatory disbalances have been related to functional dysfunction of T lymphocytes.

On the other hand, different sources of ROS are involved in the activation-induced cell death (AICD) expression of Fas ligand (FasL) of T lymphocytes, and re-exposure to the specific antigen increases T lymphocyte sensitivity. First, H_2O_2 produced by DUOX-1 upon TCR serves to amplify proximal signaling events downstream of the TCR. Second, $O_2^{\bullet-}$ released from mitochondrial Complex I, potentially in response to ERK signaling, triggers the expression of FasL. Finally, Fas ligation activates NOX-2, which probably contributes to the execution of the apoptotic program *via* H_2O_2-mediated activation of AKT and the inhibition of MEK. Moreover, cell-intrinsic antioxidants, such as glutathione, vitamin E, MnSOD, and CuZnSOD, interfere with FasL expression, thus counteracting AICD (184).

The selective cell death of effector cells with the memory phenotype may influence the size of the eventual memory T lymphocyte pool and consequently the functional ability of the responding cells. More specifically, CTLs exhibiting an EM phenotype were preferentially sensitive to AICD, compared CTLs with a CM. This increased sensitivity of EM T lymphocytes to TCR-induced AICD might correlate to the reduced levels of thiols in CD45RO+ T lymphocytes as compared to CD45RA+ memory T lymphocytes (211, 212). The loss of thiols after proliferation on repeated TCR stimulation may relate to apoptosis susceptibility. In this way, naïve T lymphocytes have higher levels of surface thiols and higher production of intracellular GSH compared to the antigen-experienced T lymphocytes (213–215). In addition, scavengers could reduce ROS-induced apoptosis of naïve and memory T lymphocytes. In fact, the increase levels of reduced thiol groups and intracellular GSH in a T lymphocyte subset could be responsible for its increased ability to persist in an oxidative stress microenvironment.

CONCLUSION

Inflammation, both acute and chronic, both localized and systemic, plays a key role in the differentiation and ontogeny of T lymphocytes. From the moment of formation in the bone marrow until arrival to exhausted or senescent status, inflammation influences the development of T lymphocytes, and in turn the adaptive immune responses in the human body. The homeostasis and the way in which T lymphocytes respond to a specific antigen are influenced by the level of inflammation of the environment where the T lymphocytes are located. All the changes that occur along the ontogeny and differentiation of T lymphocytes require metabolic and oxidative adaptations. Despite the profound influence of inflammation in all these processes, little is known about the mechanisms through which it influences T lymphocytes; therefore, this is a research field with great practical applications to explore.

AUTHOR CONTRIBUTIONS

All authors have contributed equally to the elaboration of the manuscript.

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Next-Generation Sequencing Analysis of the Human TCRγδ+ T-Cell Repertoire Reveals Shifts in Vγ- and Vδ-Usage in Memory Populations upon Aging

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Immunological aging remodels the immune system at several levels. This has been documented in particular for the T-cell receptor (TCR)αβ+ T-cell compartment, showing reduced naive T-cell outputs and an accumulation of terminally differentiated clonally expanding effector T-cells, leading to increased proneness to autoimmunity and cancer development at older age. Even though TCRαβ+ and TCRγδ+ T-cells follow similar paths of development involving V(D)J-recombination of TCR genes in the thymus, TCRγδ+ T-cells tend to be more subjected to peripheral rather than central selection. However, the impact of aging in shaping of the peripheral TRG/TRD repertoire remains largely elusive. Next-generation sequencing analysis methods were optimized based on a spike-in method using plasmid vector DNA-samples for accurate TRG/TRD receptor diversity quantification, resulting in optimally defined primer concentrations, annealing temperatures and cycle numbers. Next, TRG/TRD repertoire diversity was evaluated during TCRγδ+ T-cell ontogeny, showing a broad, diverse repertoire in thymic and cord blood samples with Gaussian CDR3-length distributions, in contrast to the more skewed repertoire in mature circulating TCRγδ+ T-cells in adult peripheral blood. During aging the naive repertoire maintained its diversity with Gaussian CDR3-length distributions, while in the central and effector memory populations a clear shift from young (Vγ9/Vδ2 dominance) to elderly (Vγ2/Vδ1 dominance) was observed. Together with less clear Gaussian CDR3-length distributions, this would be highly suggestive of differentially heavily selected repertoires. Despite the apparent age-related shift from Vγ9/Vδ2 to Vγ2/Vδ1, no clear aging effect was observed on the Vδ2 invariant T nucleotide and canonical Vγ9–Jγ1.2 selection determinants. A more detailed look into the healthy TRG/TRD repertoire revealed known cytomegalovirus-specific TRG/TRD clonotypes in a few donors, albeit without a significant aging-effect, while *Mycobacterium tuberculosis*-specific clonotypes were absent. Notably, in effector subsets of elderly individuals, we could identify reported TRG and TRD receptor chains from TCRγδ+ T-cell large granular lymphocyte leukemia proliferations, which typically present in the elderly population. Collectively, our results point to relatively subtle age-related changes in the human TRG/TRD repertoire, with a clear shift in Vγ/Vδ usage in memory cells upon aging.

Keywords: TCRγδ+, development, aging, repertoire, next-generation sequencing

INTRODUCTION

Immunological aging, also referred to as immunosenescence, is a complex phenomenon consisting of senescence and exhaustion processes, which are characterized by different functional and marker expression profiles (1, 2). Immunosenescence acts on different levels in the immune system, e.g., reduced antigen-specific responses (3), thymic shrinkage, and a significantly reduced naive T-cell output (3–5), convergence of the innate and adaptive immunity (6), and ultimately T-cell exhaustion (1). Immunosenescence is believed to play a major role in shaping of the antigen receptor repertoire of T-cells.

T-cells develop in the thymus, where they undergo commitment, rearrangement, selection and maturation processes. The main event during T-cell development is the rearrangements of the variable (V), diversity (D), and joining (J) genes of the T-cell receptor (TR) loci, in order to establish a large diversity of antigen receptors (7, 8). Two main types of T-cells are generated; first, TCRγδ+ thymocytes, through early TR delta and gamma (TRD, TRG) rearrangements, then followed by TCRαβ+ thymocytes upon TR beta and alpha (TRB, TRA) rearrangements (8). TCRαβ+ thymocytes undergo positive selection through TCR signaling to subsequently mature into functional T-cells, followed by negative selection in order to eliminate self-reactive T-cell precursors (9). In contrast, TCRγδ+ thymocytes do not undergo positive and/or negative selection in the thymus (10), but extrathymic development and peripheral (antigenic) selection of TCRγδ+ T-cells have been described (11).

TCRγδ+ T-cells appear to be the first functional population of circulating T lymphocytes in both murine and human peripheral blood (PB) [reviewed in Ref. (12)]. In the human fetal and neonatal situation these functional circulating TCRγδ+ T-cells mainly concern Vδ1+ cells. Readily after birth and during further development to adulthood a switch occurs in the circulating TCRγδ+ T-cell population with the number of Vδ1+ cells decreasing and Vγ9/Vδ2 cells becoming the predominant TCRγδ+ T-cell types (13). This process is believed to be the result of peripheral antigenic selection, exemplified by the presence of an invariant T nucleotide in the majority of the selected Vδ2–Jδ1 rearrangements (13–15). Furthermore, epitopes from pathogens or other antigens that could stimulate and select TCRγδ+ T-cell types have been described: *Mycobacterium tuberculosis* has been found to be a major stimulator of Vγ9/Vδ2 cells in both infected lungs and PB (16), whereas non-Vγ9/Vδ1 cells are known to be stimulated by viruses, such as cytomegalovirus (CMV) (17, 18) and Epstein-Bar virus (EBV) (19). TCRγδ+ T-cells do not only recognize antigens *via* their receptor, but they also respond to lipid antigens presented on CD1d-molecules, and that are associated with stress, inflammation and cancer [reviewed by Ref. (20)]. Most TCRγδ+ T-cells recognizing these CD1d-lipid antigen complexes are Vδ1 or Vδ3 cells, commonly located in the gut (21). TCRγδ+ T-cells can also recognize butyrophilins, tumor-antigens, endothelial antigens, antigen-presenting cells, and Toll-like receptors [reviewed in Ref. (22)], all of which are postulated to contribute to shaping of the TCRγδ+ T-cell repertoire.

TCRγδ+ T-cell recognition and selection has been mostly described in the context of the developing immune system from fetus to neonate and adulthood, but—contrary to the TCRαβ+ T-cell repertoire—effects of aging on the TCRγδ+ T-cell repertoire have not been extensively addressed. Since it has been found that TCRγδ+ T-cells follow the classical aging model as found in mainly CD8+ TCRαβ+ T-cells (23), we hypothesized that the naive mature TCRγδ+ T-cell repertoire would depict a broad spectrum of rearrangements and that it would show a more skewed pattern during further development from neonates to young adults and eventually elderly individuals. Furthermore, in view of the fact that T-cell large granular lymphocyte (LGL) leukemia typically presents as a proliferation of effector cells in elderly, we were interested to compare our TRG/TRD repertoire findings to the LGL clonal repertoire. To this end, we investigated the developing and aging TRG/TRD repertoire in TCRγδ+ T-cell subsets, using an optimized experimental next-generation sequencing (NGS) procedure to minimize technical biases of PCR-based methods. Our data show subset- and donor-specific TRG/TRD repertoires, suggestive of selection, with significant differences in the combinatorial repertoire in especially memory populations between young and elderly individuals. When looking closer into TRG/TRD clonotypes, TCRγδ+ T-LGL leukemia receptor chains could be traced in especially the effector subsets of elderly individuals, which would fit the current idea that TCRγδ+ T-LGL leukemia cells originate from the normal healthy antigen-experienced TCRγδ+ T-cells.

MATERIALS AND METHODS

Subjects and Materials

Blood from healthy blood donors from Sanquin Blood Bank (Amsterdam, The Netherlands) in the age range of 20–35 years (young adults, $N = 11$) and 56–70 years (elderly, $N = 12$) was used upon written informed consent at the blood bank (project number NVT0012.01) and anonymized for further use. The maximum age to donate blood is 70 years. Healthy neonatal cord blood (CB) was obtained postpartum or after Caesarian section through collaboration and upon written informed consent at the Departments of Obstetrics and Hematology. CB was drawn using CB Collect bags containing citrate phosphate dextrose solution as anticoagulant. Thymic lobes were removed upon heart surgery in individuals under the age of two years upon written informed consent from parents. Both CB and thymus material was obtained under Medical Ethics Committee approval (project number hmPOO2004-003). Whole thymic material was sliced and prepared prior to cryopreservation. Peripheral blood mononuclear cells (PBMCs) and cord blood mononuclear cells (CBMCs) were obtained through Ficoll density gradient separation. Isolated PBMCs, CBMCs, and thymocytes were cryopreserved in Iscove's Modified Dulbecco's Medium (Lonza, Basel, Switzerland) with dimethyl sulfoxide and stored in vials at −180°C until further use. All studies were conducted in accordance with the principles of the Declaration of Helsinki.

Cell Sorting

Cryopreserved material was thawed and sorted using CD3, CD45, TCRαβ, TCRγδ, CD45RA, CD45RO, CD27, and CD197 antibodies (Table S1 in Supplementary Material) to obtain TCRγδ+ naive (CD45RA+ CD27+ CD197 +), central memory (CD45RA-CD45RO+ CD27+ CD197+), effector memory (Temro population defined as CD45RA-CD45RO+ CD27− CD197−), and effector

(Temra population, CD45RA+ CD27− CD197−) T-cells (Figure S1 in Supplementary Material). Cell sorting was performed with FACS Aria I and III instruments (BD Biosciences, San Jose, CA, USA).

DNA Isolation

Following isolation, cells were lysed and subjected to DNA isolation using the DNA/RNA/miRNA AllPrepKit according to the manufacturer's protocol (Qiagen, Hilden, Germany). DNA concentration and quality (A260/A280 absorption ratio) were determined by Nanodrop measurements (Thermo Fischer Scientific, Waltham, MA, USA).

Primer Design

Primers for cloning and Illumina-based sequencing were largely based on those reported in BIOMED-2 assays (24). The Vδ3 primer was redesigned to better fit amplicon length of PCR products generated with the existing Vδ1 and Vδ2 primers. The Jγ1.2 primer was newly designed, as this primer was not included in the BIOMED-2 TRG assay. Vγ1F and Jγ1.3/2.3 primers were adjusted compared with the BIOMED-2 protocol (Table S2 in Supplementary Material). Primers were adapted for Illumina-based sequencing by adding Illumina forward (5′-ACACTCTTTCC CTACACGACGCTCTTCCGATCT-3′) and reverse (5′-TCGCGA GTTAATGCAACGATCGTCGAAATTCGC-3′) overhang adaptor sequences to the respective primers. The second PCR, by means of these overhang adaptor sequences, attaches sample-specific dual indices for sample identification and Illumina sequencing adaptors using primers from the Illumina TruSeq Custom Amplicon Index Kit (Illumina, San Diego, CA, USA).

Plasmid Pool Preparation

Primer validation and titration was done using plasmid vectors with cloned TRD and TRG gene rearrangements. All possible V–J gene combinations were PCR amplified and cloned from immature T-cell lines (25) and thymus DNA into the pGEM T-Easy vector in a 3:1 insert:vector ratio according to the manufacturer's protocol (Promega, Madison, WI, USA). Composition of the plasmid pools is summarized in Table S3 in Supplementary Material.

Assay Optimization Experiments

PCRs were first tested in singleplex and multiplex settings with varying primer concentrations, annealing temperatures and PCR cycle numbers. Each initial PCR mix contained GeneAmp PCR Buffer II (1×), magnesium chloride (2.5 mM), dNTPs (2.0 mM), and AmpliTaqGold (1 U) (Thermo Fischer Scientific). Total forward and reverse primer(s) amounts were generally 10 pmol. The PCR protocol was largely based on the BIOMED-2 publication (23), with varying annealing temperatures (Tm = 58/59/60/62) and different numbers of cycles (20 and 25 cycles). Primer concentration adjustment, and optimization of annealing temperatures and number of PCR cycles were based on the results of iterative optimization experiments as summarized in Table S4 and Figures S2 and S3 in Supplementary Material.

Amplicon Preparation

Amplicons from the first step PCR were purified using the Agencourt AMPure XP bead purification kit (Beckman Coulter, Fullerton, CA, USA), whereafter concentrations were measured with the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fischer Scientific), after which the amplicons were adjusted to similar concentrations. The second step PCR was performed with primers from the Illumina TruSeq Custom Amplicon Index Kit (Illumina) using the KAPA HiFi HotStart PCR Kit (Kapa Biosystems, Wilmington, MA, USA). Second PCR amplicons were evaluated *via* agarose gel electrophoresis or PicoGreen concentration measurement. Library pool preparation was subsequently performed based on the gel image or PicoGreen measurement results. The library pool was further purified with Agencourt AMPure XP beads and normalized for Illumina-based sequencing, according to the manufacturer's protocol (Illumina).

Next-Generation Sequencing

Paired-end NGS (2 × 221 bp) was performed on the MiSeq platform (Illumina, San Diego, CA, USA) with the use of an Illumina MiSeq Reagent Kit V3, according to the manufacturer's protocol (Illumina).

Bioinformatic Data Analysis

Illumina NGS data were obtained in FASTQ format. Paired-end reads were combined using the FASTQ-join tool in the Erasmus MC Galaxy Server (26), with the use of usegalaxy.org (27–29) converted from FASTQ to FASTA with the converter tool (30). Sequencing annotations were made *via* the IMGT High V-quest database (31–34). Calculation of the clonality score for multiple replicates was based on the algorithm described by Boyd et al. (35). Clonal type definition was based on V and J gene usage and CDR3-region at the nucleotide level. Rearrangements were visualized using Circoletto plots [www.circos.ca (36)]. CDR3 amino acid compositions were visualized using WebLogo online tool [www.weblogo.berkeley.edu (37, 38)].

The NGS TRG-TRD data set has been submitted to the BioProject repository (BioProjectID: PRJNA434217, submissionID SUB3660187; http://www.ncbi.nlm.nih.gov/bioproject/434217). Sequencing details can be accessed through SRA database accession SRP133150 (https://www.ncbi.nlm.nih.gov/sra/SRP133150).

Statistical Analysis

Data were checked for normal distributions using the Hartigan's Dip Test Statistic for Unimodality package (39–41) in R version 3.4.1 (42). All statistical analyses were performed with Prism 5 (GraphPad, La Jolla, CA, USA).

RESULTS

Multiplex PCR Assay Fine-Tuning Leads to an Optimized, Bias-Free NGS Assay for Reliable Quantification of the TRG/TRD Repertoire

The multiplex PCR assay to be analyzed by NGS was optimized and fine-tuned for more accurate quantification and receptor diversity analysis of the TRG and TRD loci using a diverse set of artificial DNA spike-in samples and primer concentration titration experiments (Tables S4 and S5 and Figures S2 and S3 in Supplementary Material). Each artificial DNA sample, represented by plasmid

vector DNA, contained a mixture of known V(D)J rearrangements, cloned from either immature T-cell lines or thymus DNA in equimolar proportions (Table S4 in Supplementary Material). After several rounds of fine-tuning (Figures S2 and S3 in Supplementary Material), and repeated technical validation with plasmid spike-in pools we established the most optimal PCR conditions for both TRG (**Figure 1A**) and TRD (**Figure 1B**) multiplex assays in view of unbiased NGS data. Remaining small differences between observed and expected read frequencies are introduced by chance in the PCR reaction and/or due to inevitable interassay variation. These were reduced to a minimum by using four replicates for each sample, which included four differently pipetted mixes to reduce pipetting bias and the use of four different PCR machines to reduce machine-dependent bias. These optimization experiments resulted in variable primer concentrations and defined annealing temperatures and cycle numbers for the TRG and TRD multiplex PCR reactions (Table S5 in Supplementary Material).

The TRG/TRD Repertoire Is Diverse in Immature Thymus and CB, and More Skewed in Mature Circulating TCRγδ+ T-Cells

In order to determine changes in TCRγδ+ T-cell repertoire in healthy individuals, we first investigated TRG/TRD repertoire diversity during ontogeny using purified TCRγδ+ T-cells from different compartments, i.e., thymus (Thy) and neonatal CB. In addition, we sequenced the total mature TCRγδ+ T-cell population of healthy adult PB samples. TRG rearrangements in Thy and CB samples were highly diverse (**Figure 2A**, upper two rows), and the intersample variation of especially Thy samples was low, in keeping with the non-selected character of the TCRγδ+ T-cells in these compartments. These findings were in strong contrast to PB samples, which showed a high level of skewing and predominance of certain receptors (including Vγ9–Jγ1.2 sequences) were observed (**Figure 2A**, bottom row), albeit with high inter-individual differences, illustrating the dominant role of (antigenic) selection. TRD diversity was less apparent, although in Thy and CB samples (**Figure 2B**, upper two rows) all three predominant Vδ-genes were identified. Again, intersample variation was low, illustrating the non-selected character of Thy and CB cells. Intersample variation was more evident for the PB samples (**Figure 2B**, bottom row), with predominance of Vδ2 usage, but also Vδ3 usage in some cases, reflecting different types of (antigenic) selection between individuals.

Collectively, these data confirmed our hypothesis of a broad and diverse TRG/TRD repertoire in the immature Thy and CB samples and a more skewed TRG/TRD repertoire in mature circulating TCRγδ+ T-cells in adults, thereby validating our optimized multiplex PCR-based TRG/TRD NGS assays.

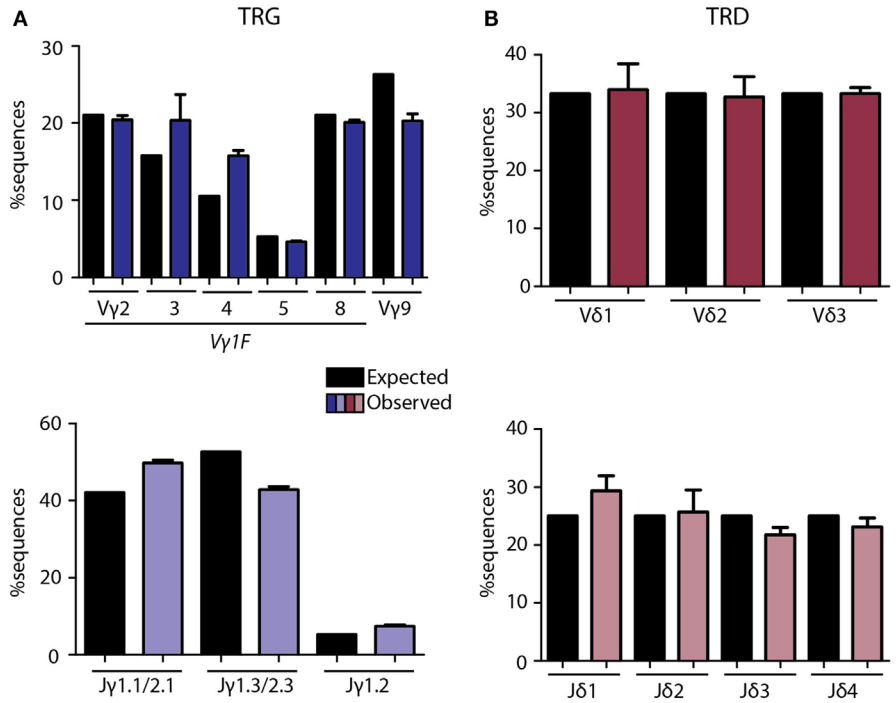

FIGURE 1 | Technical optimization of next-generation sequencing assays for TRG/TRD loci. Multiplex PCR assays were optimized with balanced primer concentrations, annealing temperatures, and cycle numbers as summarized in Table S3 in Supplementary Material. Plasmid pools were used as spike-in samples to determine the percentage expected sequences per V and J gene vs. the observed percentage after sequencing (Table S2 in Supplementary Material). Expected percentages are indicated in black bars, observed percentages are indicated in colored bars. TRG assays showed high overlap between frequencies of expected and observed sequences for Vγ and Jγ **(A)** genes, with some variation due to single primers covering multiple genes (Vγ1F covering Vγ2-8, Jγ1.1/2.1 covering Jγ1.1 and Jγ2.1, and Jγ1.3/2.3 covering Jγ1.3 and Jγ2.3). TRD assays showed nearly similar percentages of expected and observed sequences for Vδ and Jδ **(B)** genes. Error bars represent SD of PCR replicates ($N = 4$).

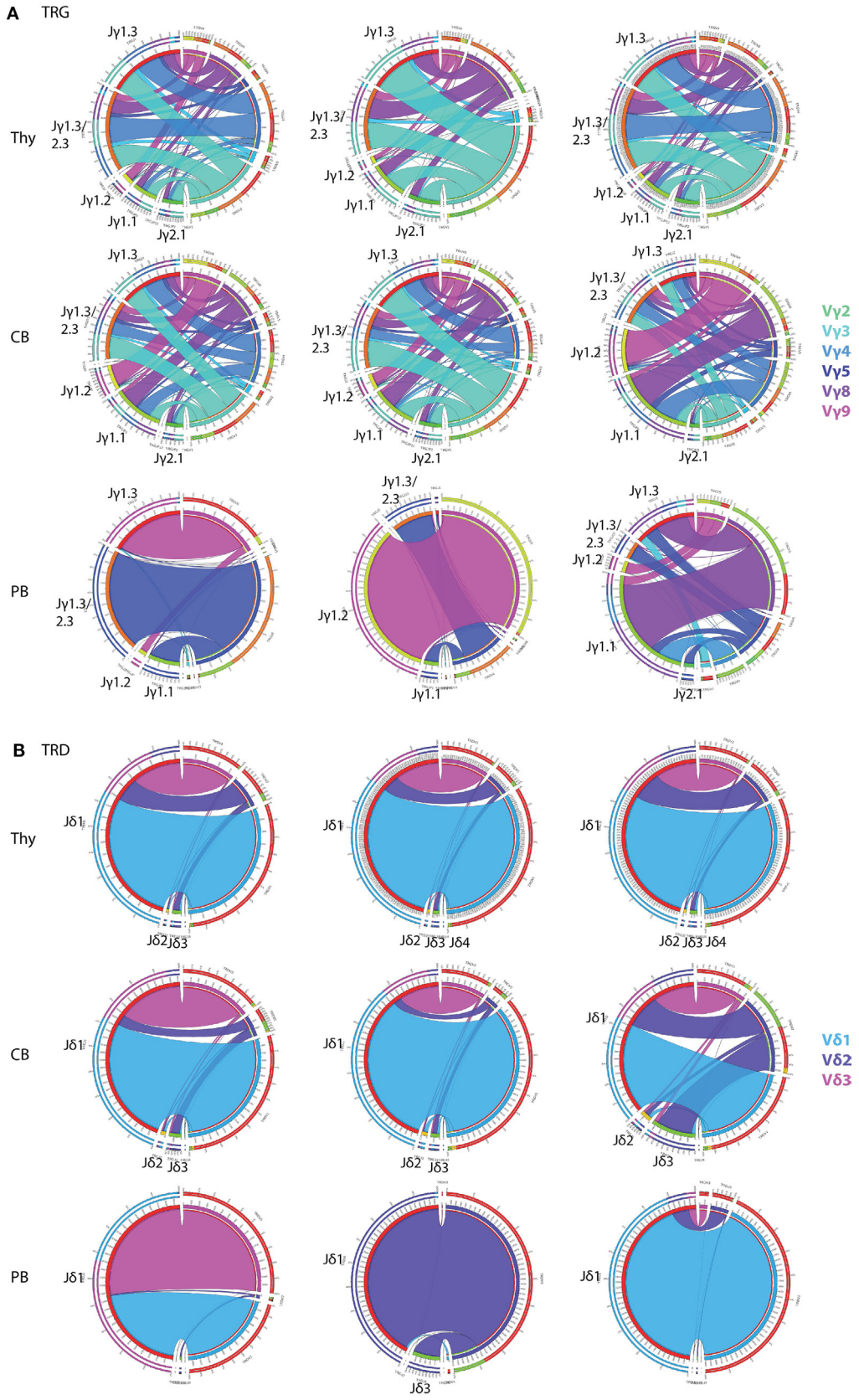

FIGURE 2 | Continued

FIGURE 2 | Circoletto visualization of the TRG/TRD repertoire during ontogeny. Optimized multiplex PCR next-generation sequencing assays were applied on total TCRγδ+ T-cells sorted from thymus (Thy), neonatal cord blood (CB), and adult peripheral blood (PB). TRG assays showed high repertoire diversity in both Thy and CB samples, with low interindividual variation, while adult PB samples showed individual-specific repertoire patterns with less receptor diversity **(A)**. TRD assays showed high dominance of Vδ1 (light blue bars), which was also observed in CB samples, both with low intersample variation. Adult PB samples showed donor-specific patterns with sometimes skewing toward Vδ2 and even Vδ3 **(B)**. Three representative samples of each samples type are visualized: Thy04-10, Thy05-13, Thy10-03, CB2, CB3, CB4, PB30, PB31, and PB50. Plots were made using the Circoletto online software tool [http://www.circos.ca (35)]. Each band represents a V–J rearrangement, with colors based on V-gene usage.

Upon Aging Memory TCRγδ+ T-Cells Show Shifts in V-Gene Usage, Whereas Naive and Effector Populations Do Not

As it has become evident that aging plays a major role in shaping the elderly immune system (43), we next evaluated the role of aging on the combinatorial TRG/TRD repertoire. To this end, we sorted TCRγδ+ T-cells from healthy young ($N = 11$; age range 20–35) and elderly ($N = 12$; age range 56–70) individuals into four subsets: naive (CD45RA+ CD45RO− CD27+ CD197+), central memory (CD45RA− CD45RO+ CD27+ CD197+), effector memory (Temro; CD45RA− CD45RO+ CD27− CD197−), and effector (Temra; CD45RA+ CD45RO− CD27− CD197−) TCRγδ+ T-cells. Subset distributions of young and elderly individuals (Figure S4 in Supplementary Material) correlated with those from our previous aging study, from which it is known that TCRγδ+ T cells show little CCR7 expression fitting with low absolute and relative numbers of naive and central memory cells (44) (Table S6 in Supplementary Material). Even though the spectrum of V–J combinations for both TRG and TRD varied in a donor-specific way between individuals (Figure S5 in Supplementary Material), the overall TRG/TRD combinatorial diversity appeared to be mostly determined by differences in Vγ/Vδ usage rather than Jγ/Jδ gene usage.

Naive TCRγδ+ T-cells of both young and elderly individuals showed a relatively diverse TRG repertoire, which was in strong contrast to (central and effector) memory TCRγδ+ T-cells that showed dominant Vγ9 gene usage. Effector TCRγδ+ T-cells of both age groups were more diverse again. Of note, significant differences between young and elderly could be observed in mainly the memory populations, as reflected by a significantly higher Vγ2-usage in central memory TCRγδ+ T-cells in elderly, as well as significantly lower Vγ2-8-usage and significantly higher Vγ9 gene usage in effector memory cells of elderly (**Figure 3A**).

When comparing TRD combinatorial profiles in the different subsets between young and elderly individuals, significantly higher Vδ1 and significantly lower Vδ2 gene usage was observed in memory populations of elderly individuals. This effect was also observed in the effector population. In the effector memory cells of elderly Vδ3 gene usage was also significantly higher (**Figure 3B**).

Overall, these data show clear differences in the TRG/TRD combinatorial repertoire between naive TCRγδ+ T-cells on the one hand and especially memory TCRγδ+ T-cells on the other hand. Notably, the clear dominance of Vγ9 and Vδ2 usage in memory and effector TCRγδ+ T-cells in young individuals was less prominent in elderly individuals, who on average showed significant shifts toward more Vγ2 and Vδ1 gene usage in addition to Vγ9 and Vδ2. Most significant differences between young and elderly were identified in central and effector memory populations.

The TRG/TRD Junctional Region Repertoire Shows Signs of Selection in Memory and Effector Cell Populations of both Young and Old Individuals

For a more detailed view of the TRG/TRD repertoire, we then studied CDR3-regions, which reflect the most relevant antigen-binding part of the antigen receptors. These CDR3-length distributions are indicative of the junctional repertoire. TRG/TRD CDR3-length distributions of Thy TCRγδ+ T-cells showed Gaussian profiles, just like the TRD CDR3-length distributions of CB TCRγδ+ T-cells; TRG CDR3-lengths of CB TCRγδ+ T-cells showed less clear Gaussian distributions and more prominent peaks, probably reflecting low-level selection (Figure S6 in Supplementary Material). The effect of selection became even more evident in adult individuals; naive TCRγδ+ T-cells showed mostly Gaussian CDR3 profiles, in contrast to memory and effector TCRγδ+ T-cells of young individuals, which showed dominant peaks for both the TRG and TRD CDR3-regions (**Figures 4A,B**). Elderly individuals did not show clear Gaussian profiles, and even prominent peaks in all subsets, thus reflecting a more heavily selected repertoire (**Figures 4A,B**). The average TRG and TRD CDR3-lengths were not markedly different between young and elderly individuals.

TRG Canonical and TRD Invariant T Selection Determinants are Detectable in Normal TCRγδ+ T-Cells but Do Not Increase upon Aging

During development selection of TCRγδ+ T-cells is known to be associated with so-called selection determinants, which represent molecular fingerprints in the CDR3-regions of TRG and TRD chains. In circulating TCRγδ+ T-cells a high frequency of Vγ9–Jγ1.2 recombinations with preferential joining at the GCA sequence has been noted (**Figure 5A**). We therefore studied this so-called canonical Vγ9–Jγ1.2 rearrangement, characterized by a defined CDR3-length and amino acid composition (**Figure 5A**), in different subsets of young and elderly healthy controls. Approximately 10–20% of all productive Vγ9–Jγ1.2 rearrangements contained the canonical sequence (**Figure 5B**). The frequencies of canonical Vγ9–Jγ1.2 sequences did not clearly differ between different subsets in young and elderly (**Figure 5B**). In TCRγδ-receptors the canonical Vγ9–Jγ1.2 chain is frequently combined with a Vδ2-derived chain, especially resulting from Vδ2–Jδ1 recombination. These Vδ2–Jδ1 rearrangements often contain a so-called invariant T nucleotide, a selection determinant at the relative second position of the first codon of the junctional region (**Figure 5C**), translating into leucine (L), valine (V),

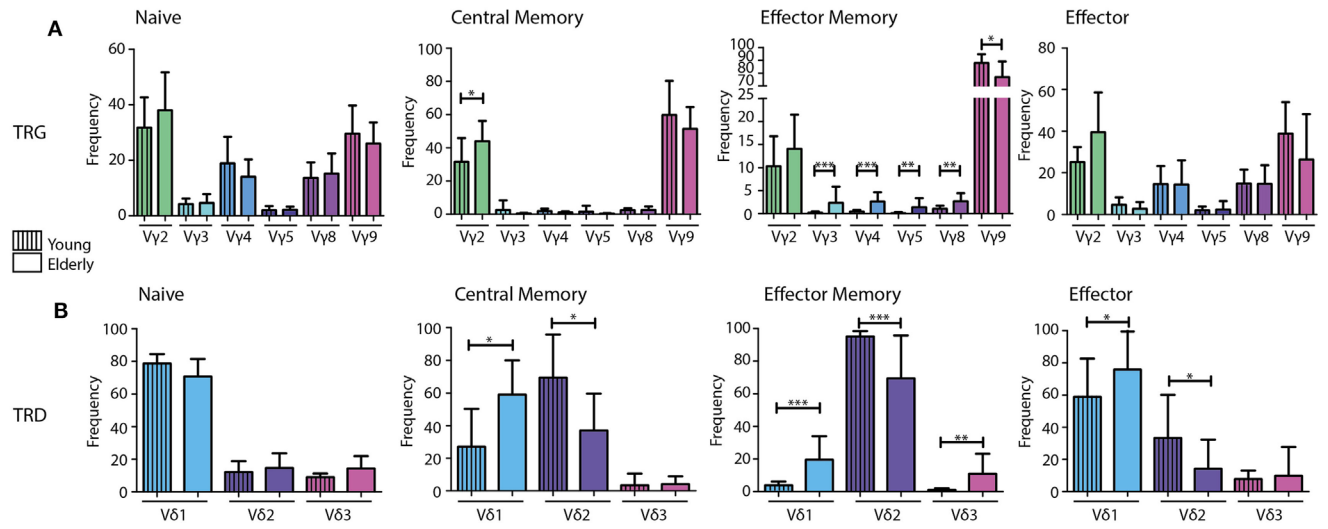

FIGURE 3 | V gene diversity in the combinatorial TRG/TRD repertoire of different TCRγδ+ T-cell subsets from young and elderly individuals. V-gene usage per subset visualized in bar graphs indicating a diverse repertoire in naive and effector subsets of both young and elderly, with skewing toward Vγ2 and Vγ9 in memory populations **(A)**. Naive populations showed diversity in Vδ usage with Vδ1 dominance in young and elderly. Young individuals showed relatively higher Vδ2 usage in especially the memory and effector subsets, whereas elderly individuals showed clear Vδ1 dominance in all subsets, except for effector memory cells **(B)**. Median V-gene frequencies of productive sequences with SD bars were indicated in bar graphs for young ($N = 11$) and elderly ($N = 12$) individuals. Color legends are indicated in the figure. Statistical significance was tested using the Mann–Whitney *U*-test. Level of significance is indicated in the plots: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.0001$.

or isoleucine (I) amino acids at that first codon in the junction. The invariant T was observed in all individuals (**Figure 5D**, outer gray circles), and resulted in L, V or I amino acids at this position (**Figure 5D**, inner blue pie charts). The invariant T was present at higher frequency in memory and effector subsets compared to naive TCRγδ+ T-cell subsets. On average, invariant T frequencies per subset did not differ much between young and elderly individuals, although the percentage of invariant T-containing sequences at the nucleotide level of naive TCRγδ+ T-cells of young individuals was clearly lower than that of elderly naive TCRγδ+ T-cells (**Figure 5D**).

Taken together, the most common selection determinants described in TCRγδ+ T-cells (i.e., the Vγ9–Jγ1.2 canonical sequence and the Vδ2–Jδ1 invariant T nucleotide) were readily identified in different TCRγδ+ T-cell subsets in our healthy control cohort, albeit that frequencies did not clearly differ between young and elderly.

Analysis of TRG/TRD Clonotypes Shows the Presence of TCRγδ+ T-LGL Leukemia-Related Clonotypes in Especially Effector Cells of Elderly

In view of TCRγδ+ T-cell selection processes, we then studied the possible recurrence of specific TRG/TRD clonotypes in the repertoire of young and elderly individuals, as a sign of activated TCRγδ+ T-cell clones. To this end multiple replicates ($N = 3$) of each TCRγδ+ T-cell subset were studied in independent PCR reactions and the number of so-called coincident sequences was determined (35, 46). In all subsets, both young and elderly, the frequency of clonotype sequences found in only one of the replicates was the highest, while the frequencies of coincidences found in two or three replicates were relatively low for both TRG and TRD (Figure S7 in Supplementary Material). When comparing young and elderly, small shifts leading to higher numbers of coincidences in two or three replicates were seen in the latter (Figure S7 in Supplementary Material). We then only focused on the coincidences present in all three replicates, since these sequences best reflect the individuals' repertoire selection. Especially in the effector memory population absolute numbers of sequences found in all three replicates were higher, while in naive subsets from both young and elderly these numbers were lower, except for a few cases (Table S7 in Supplementary Material).

To understand whether the recurrence of clonotypes would be associated with particular infections, we next evaluated receptor clonotypes linked to pathogens such as *M. tuberculosis* (16, 47) and herpes viruses such as CMV (18). Whereas in our healthy controls no *M. tuberculosis*-specific clonotypes could be identified, CMV-specific TRG or TRD clonotypes were found in most controls, and in one case even a complete CMV-specific TCRγδ receptor could be identified (data not shown). However, there were no evident differences between young and elderly individuals.

Finally, as leukemic TCRγδ+ T-cells typically arise in the elderly population and are associated with specific clonotypes, we retrospectively reviewed our TCRγδ+ T-cell LGL leukemia database of clonal TRG/TRD sequences (13, 47) and searched for these LGL clonotypes in the normal TCRγδ+ T-cell repertoire of young and elderly healthy individuals. Interestingly, two TCRγδ+ T-LGL leukemia-associated TRG and TRD clonotypes were found in four older individuals and in one young individual

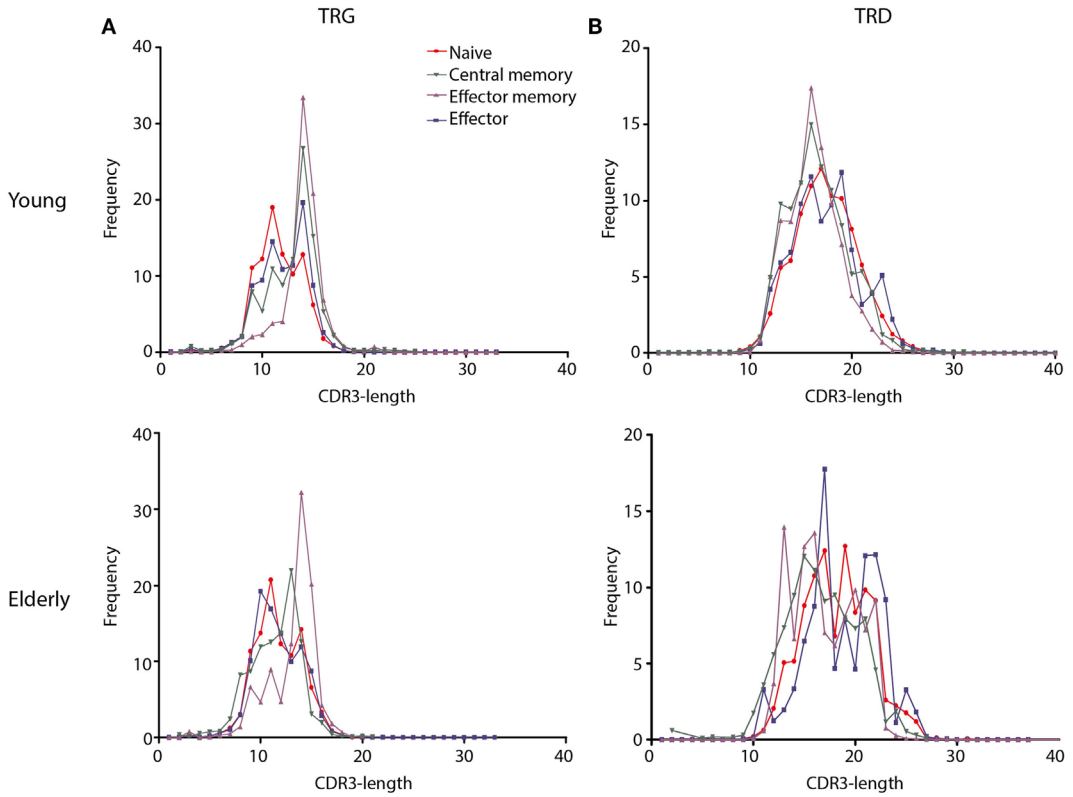

FIGURE 4 | CDR3-length distributions of TCRγδ+ T-cell subsets from young and elderly individuals. Frequencies of CDR3-lengths from different TCRγδ+ T-cell subsets are summarized per T-cell receptor chain and age group. Naive subsets (red lines) showed distributions resembling Gaussian profiles, while memory subsets showed dominant peaks suggestive of selection and receptor skewing (green and purple lines). TRG CDR3-lengths showed similar distributions between young and elderly **(A)**, TRD CDR3-lengths in young individuals showed a Gaussian profile in the naive subset and more skewing in memory and effector subsets, whereas in elderly individuals all subsets showed dominant peaks **(B)**. Mean frequencies per subset were indicated for young (N = 11) and elderly (N = 12) individuals. Data normality was tested using the diptest package in R.

(**Table 1**). The Vδ3–Jδ1 receptor as identified in TCRγδ+ T-LGL leukemia case 12-098 was identified in one young individual (naive subset, 26-year-old female), and in three older individuals (naive subset, 56-year-old male; effector subset, 69-year-old female and 68-year-old male) (**Table 1**). The TCRγδ+ T-LGL leukemia-related receptor from case 10 to 200 was found twice in older individuals (naive subset, 56-year-old male; effector subset, 70-year-old male) (**Table 1**). Although the numbers are low, the fact that two TCRγδ+ T-LGL leukemia-related receptors could specifically be identified in effector cells of elderly would support the idea that TCRγδ+ T-LGL leukemia cells originate from the normal TCRγδ repertoire, especially from antigen-experienced TCRγδ+ T-cells of individuals of older age (13, 48, 49).

DISCUSSION

Aging of the immune system has become increasingly important due to increased hygiene and higher life expectancies in the Western World (3, 5, 50). Immunosenescence plays an additional role in shaping the immune repertoire. Shaping of the immune system during ontogeny and upon aging relies on continuous antigenic exposures, varying from pathogens to cellular stress. In the current study, we showed that (antigenic) selection starts during early ontogeny in the thymus and CB samples and continues in circulating TCRγδ+ T-cells in young and elderly individuals. While maintaining diversity in the naive subsets, the effect of aging is most significant in memory subsets, characterized by strong receptor skewing, and in effector subsets.

Following technical optimization of multiplex PCR assays for NGS analysis, we demonstrated highly diverse TRG, but Vδ1-skewed TCRγδ+ T-cell repertoires in precursor TCRγδ+ T-cells from thymus and CB, with low inter-sample variation. This was in clear contrast to circulating mature TCRγδ+ T-cells that showed Vγ9/Vδ2 receptor skewing with high inter-sample variation and donor-specific patterns. As we recently showed significant effects of aging on maturation profiles of TCRγδ+ T-cells (49), we investigated the immune repertoire composition of different TCRγδ+ T-cell subsets including naive, central, and effector memory, and effector cells. Even though the naive TCRγδ+ T-cell population shrinks upon aging (3, 5, 49), its diversity—being the primary source for mounting immune responses—was maintained in elderly individuals. To date, only one study documented the maintenance of the naive CD4+ TCRαβ+ T-cell repertoire until the age of 70, after which the repertoire profoundly declined (51)

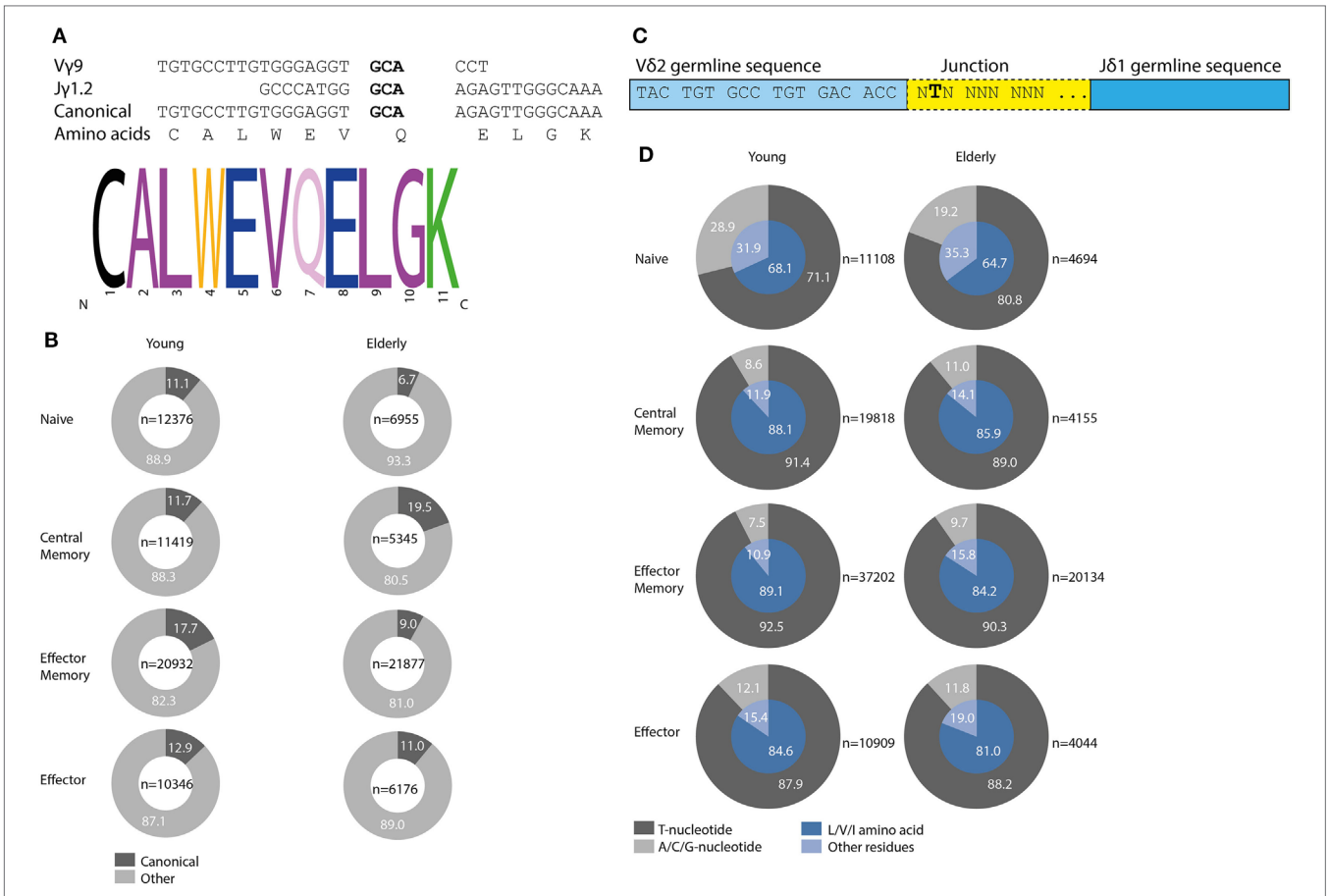

FIGURE 5 | Canonical Vγ9–Jγ1.2 sequences and Vδ2–Jδ1 invariant T selection determinant in young and elderly healthy individuals. GCA sequence in bold mediates preferential recombination of Vγ9 with Jγ1.2, resulting in specific CDR3 length and amino acid composition, adapted from Ref. (45) **(A)**. Frequencies of all productive Vγ9–Jγ1.2 sequences containing the canonical sequence per age group and subset marked in dark gray. In light gray Vγ9–Jγ1.2 rearrangements with other, non-canonical sequences are indicated. Numbers of total Vγ9–Jγ1.2 sequences are indicated in center of plots **(B)**. The invariant T selection determinant is located at the relative second position of the first codon in the junction of Vδ2–Jδ1 rearrangements, adapted from Ref. (13) **(C)**. Frequencies of invariant T selection determinant in young and elderly subsets (dark gray part outer ring), leading to L, V, or I amino acid residues (inner blue pie chart, dark blue part). Absolute number of unique productive Vδ2–Jδ1 sequences indicated next to plots **(D)**.

TABLE 1 | Complete TCRγδ+ T-LGL leukemia receptor clonotypes identified in the repertoire of healthy young and elderly individuals.

Sample information				LGL sample	TRG receptor chain		TRD receptor chain	
Donor	**Sex**	**Age**	**Subset**		**V-J rearrangement**	**CDR3 composition**	**V-J rearrangement**	**CDR3 composition**
Young individuals								
B49	F	26	Naive	LGL 12-098	Vδ3–Jδ1	CAFSSLTGGYKEYTDKLIF	Vγ9–Jγ1.3	CALWEVPNYKKLF
Elderly individuals								
B41	M	56	Naive	LGL 10-200	Vδ2–Jδ1	CACDTVGDRDTDKLIF	Vγ9–Jγ1.3	CALWEVQYYKKLF
				LGL 12-098	Vδ3–Jδ1	CAFSSLTGGYKEYTDKLIF	Vγ9–Jγ1.3	CALWEVPNYKKLF
B51	M	70	Effector	LGL 10-200	Vδ2–Jδ1	CACDTVGDRDTDKLIF	Vγ9–Jγ1.3	CALWEVQYYKKLF
B44	F	69	Effector	LGL 12-098	Vδ3–Jδ1	CAFSSLTGGYKEYTDKLIF	Vγ9–Jγ1.3	CALWEVPNYKKLF
B60	M	68	Effector	LGL 12-098	Vδ3–Jδ1	CAFSSLTGGYKEYTDKLIF	Vγ9–Jγ1.3	CALWEVPNYKKLF

[reviewed in Ref. (52)]. These results are in line with our findings, although our cohort consisted of elderly until the age of 70. This is one drawback of our study, but the maximum age to donate blood at our national blood bank is 70. Nevertheless, it would be interesting to also study healthy individuals >70 years of age, although the high volumes of blood needed to obtain sufficient numbers of naive TCRγδ+ T-cells could complicate such studies. Low cell numbers pose serious limitations to studying the repertoire due to potentially low levels of input DNA and skewed data. To overcome such limitations and to directly link overall receptor usage for both TRG

and TRD loci, single molecule-based assays could be considered. However, these assays are rather novel and require extensive optimization and validation experiments as well. Another limitation of our study is the fact that due to limited cell material, we could not go into mechanistic and functional implications of our findings.

Age-related differences were most evident in central and effector memory populations: Vγ9 usage was highly important in young individuals, while a shift toward Vγ2 and other Vγ1-family genes was observed in effector memory TCRγδ+ T-cells of elderly. The significant increase in Vγ2 usage in elderly was accompanied by a significant decrease in Vδ2 and increase in Vδ1 usage, collectively indicating a shift from Vγ9/Vδ2 specificity in young to Vγ2/Vδ1 in elderly. These findings might suggest differences in antigenic selection, or might be due to underlying clonal expansion in these populations (53). Also, CMV is known to elicit Vδ1+ TCRγδ+ T-cell-specific responses (17). Additionally, we have recently demonstrated the effect of CMV on the TCRγδ+ T-cell immune system, through increasing Vδ1+ TCRγδ+ T-cells in elderly carrying CMV (49), and it has been shown that latent CMV carriage is related to the expansion of CMV specific T-cells (54). When zooming in on the antigen-binding part, the CDR3 region, we could indeed identify CMV-specific CDR3 regions in a few donors, albeit without a significant aging effect. This could reflect high anti-CMV responses mounted by Vδ1+ TCRγδ+ T-cells, although such responses were mainly observed in renal allograft recipients and not in healthy controls (17). Given that CMV infects mainly fibroblasts and epithelial cells (55), and that the majority of Vδ1+ TCRγδ+ T-cells reside in epithelial and mucosal tissues (56–58) these findings could indicate that healthy individuals have a local, rather than circulatory, protection by Vδ1+ TCRγδ+ T-cells against CMV. Interestingly, in a recent study Davey et al., showed that the Vδ1 population in CB is unfocused, but that in adult PB clonal expansions could be found that had directly differentiated from naive into effector phenotypes with parallel CD27 downregulation (59). In contrast, Vδ2 cells maintained their TCR expression from birth to adulthood. Together with the CMV effect upon aging, these findings could explain the higher Vδ1 usage in elderly and possibly the occurrence of clonopathies.

In this study, we also examined other dominant TRG/TRD clonotypes, such as for *M. tuberculosis*, since TCRγδ+ T-cells are known to elicit strong responses (16), but these were not identified. This could be related to the recruitment of our donors (mostly of Caucasian descent) *via* the national Dutch blood bank, and the fact that donors are tested prior to blood donation. Furthermore, open tuberculosis is not endemic in the Netherlands. Curiously, some TRG/TRD clonotypes derived from complete TCRγδ+ T-LGL leukemia receptors were identified in the healthy effector subset repertoire. These findings would be in line with earlier correlations identified between TCRγδ+ T-LGL leukemia cells and healthy effector TCRγδ+ T-cells (49) and would support the concept that TCRγδ+ T-LGL is a disease that typically arises in effector cells of elderly.

Our optimized multiplex PCR assays for NGS analysis could also be applicable to other disease states, such as TCRγδ+ T-cell lymphomas, or treatments, such as bone marrow transplantation (BMTx). TCRγδ+ T-cells have been described to reconstitute in increased numbers after BMTx in acute leukemia patients (60). The here described method would allow to investigate to what extent the TRG/TRD repertoire has changed upon BMTx, and how the TCRγδ+ T-cell compartment regenerates. Also, circulating TCRγδ+ T-cells have been described in metastatic melanomas, in which it would be interesting to distinguish pro- and anti-tumor specific TCRγδ+ T-cells (61), the latter particularly in view of tumor-eradicating effects (45, 62). Also, investigating the TRG/TRD repertoires of tissue-residing TCRγδ+ T-cells could be relevant, not only for CMV-specific responses, but also for other local antigens contributing to the TCRγδ+ T-cell repertoire. As the ability of TCRγδ+ T-cells to move in and out of tissues has not been convincingly demonstrated yet, sequencing TCRγδ+ T-cells from different tissues could provide insight in both residing, migrating and circulating properties, as well as in development of local immune repertoires and additional functions and specificities of TCRγδ+ T-cells.

In summary, using an optimized NGS assay we identified specific TRG/TRD repertoires during ontogeny and upon aging. Despite strong individual-specific repertoire compositions, significant differences in Vγ and Vδ gene usage were identified upon aging in especially the memory TCRγδ+ T-cell subsets. These age-dependent effects caused shifts from Vγ9/Vδ2 dominance in young to Vγ2/Vδ1 dominance in elderly. Additionally, some TRG/TRD clonotypes related to TCRγδ+ T-LGL leukemia were identified in normal effector TCRγδ+ T-cells of especially elderly individuals, which fits the idea that TCRγδ+ T-LGL leukemia originates from normal circulating, antigen-experienced effector TCRγδ+ T-cells.

ETHICS STATEMENT

Blood from healthy blood donors from Sanquin Blood Bank (Amsterdam, The Netherlands) in the age ranges 20–35 (young adults) and 56–70 (elderly) was used upon informed consent (project number NVT0012.01) and anonymized for further use. Healthy neonatal CB was obtained postpartum or after Caesarian section upon informed consent through collaboration with the departments of Obstetrics and Hematology. Thymic lobes were removed upon heart surgery in individuals under the age of 2 years. Both CB and thymus material was obtained under Medical Ethics Committee approval (project number hmPOO2004-003). All studies were conducted in accordance with the principles of the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

MJK, JD, and AL designed the experiments. MJK and AL wrote the manuscript. MJK, FK, MYK, and IW-T performed the experiments. MJK and AL analyzed the data and prepared the figures. PV, JD, and AL supervised the project. FK and PV revised the manuscript. All authors read the manuscript carefully.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00448/full#supplementary-material.

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Human Body Composition and Immunity: Visceral Adipose Tissue Produces IL-15 and Muscle Strength Inversely Correlates with NK Cell Function in Elderly Humans

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Natural killer (NK) lymphocyte-mediated cytotoxicity and cytokine secretion control infections and cancers, but these crucial activities decline with age. NK cell development, homeostasis, and function require IL-15 and its chaperone, IL-15 receptor alpha (IL-15Rα). Macrophages and dendritic cells (DC) are major sources of these proteins. We had previously postulated that additional IL-15 and IL-15Rα is made by skeletal muscle and adipose tissue. These sources may be important in aging, when IL-15-producing immune cells decline. NK cells circulate through adipose tissue, where they may be exposed to local IL-15. The objectives of this work were to determine (1) if human muscle, subcutaneous adipose tissue (SAT), and visceral adipose tissue (VAT) are sources of IL-15 and IL-15 Rα, and (2) whether any of these tissues correlate with NK cell activity in elderly humans. We first investigated IL-15 and IL-15Rα RNA expression in paired muscle and SAT biopsies from healthy human subjects. Both tissues expressed these transcripts, but IL-15Rα RNA levels were higher in SAT than in skeletal muscle. We also investigated tissue obtained from surgeries and found that SAT and VAT expressed equivalent amounts of IL-15 and IL-15Rα RNA, respectively. Furthermore, stromal vascular fraction cells expressed more IL-15 RNA than did adipocytes. To test if these findings related to circulating IL-15 protein and NK cell function, we tested 50 healthy adults aged > 70 years old. Plasma IL-15 levels significantly correlated with abdominal VAT mass in the entire cohort and in non-obese subjects. However, plasma IL-15 levels did not correlate with skeletal muscle cross-sectional area and correlated inversely with muscle strength. Plasma IL-15 did correlate with NK cell cytotoxic granule exocytosis and with CCL4 (MIP-1β) production in response to NKp46-crosslinking. Additionally, NK cell responses to K562 leukemia cells correlated inversely with muscle strength. With aging, immune function declines while infections, cancers, and deaths increase. We propose that VAT-derived IL-15 and IL-15Rα is a compensatory NK cell support mechanism in elderly humans.

Keywords: natural killer cell, adipose tissue, IL-15, skeletal muscle strength, aging

INTRODUCTION

Natural killer (NK) cells are classified as members of the type 1 innate lymphoid cells (1). NK cells defend against infection, both directly and by orchestrating T cell, DC, monocyte, and macrophages (Mφ) responses (2). NK cells also may eliminate cancer cells and senescent cells (3, 4). Peripheral NK cells develop in the bone marrow and secondary lymphoid organs, where they are nurtured by multiple cell types and cytokines. IL-15, which is critical for mature NK cell development, homeostasis and function (5), signals *via* a trimeric receptor comprised of IL-15Rα, CD122, and CD132. IL-15 RNA is made in the bone marrow, secondary lymphoid tissues, and many nonlymphoid tissues, including skeletal muscle and adipose tissue. Although IL-15Rα is part of the IL-15 receptor, it also is required for IL-15 secretion and appearance on cell surfaces. In Mφ, DC, and other producing cells, IL-15 and IL-15Rα bind together with very high affinity. The complex is transported to the cell surface, where it stimulates neighboring NK cells in a paracrine fashion (5, 6). IL-15/IL-15Rα complexes also circulate to act on NK cells in an endocrine fashion (7). Two observations indicate that physiological IL-15 levels are dose-limiting for NK cells homeostasis: hemizygous IL-15 mice have low NK cell number and exogenous IL-15 boosts NK cell number in both normal mice and primates (8–10).

Human NK cells are classified into two major subsets based on their CD56 surface expression. Most circulating blood NK cells are $CD56^{dim}$, while 5–15% are $CD56^{bright}$. $CD56^{bright}$ NK cells are poorly cytotoxic but secrete high levels of cytokines and chemokines in response to inflammatory cytokines. Although $CD56^{dim}$ NK cells respond weakly to inflammatory cytokines, they kill target cells (such as the erythroleukemia cell line K562) and secrete chemokines and cytokines in response to antibody-coated cells and tumor cells.

Natural killer cell numbers are maintained in healthy elderly people, but NK-mediated cytotoxicity and secretion of immunoregulatory cytokines and chemokines decline with age (11, 12). Aging-related NK defects in mice are due, at least in part, to ineffective support from stromal cells (13–15). These defects could be due to decreased Mφ and dendritic cell IL-15 production and presentation (13, 15). Decreased NK cell activity in elderly people correlates with an increased incidence and severity of viral and bacterial infections and deaths (11, 16). Moreover, low NK function was found to be associated with increased cancer rates in subsequent years (17).

The objectives of this work were to determine (1) if human muscle, subcutaneous adipose tissue (SAT), and visceral adipose tissue (VAT) are sources of IL-15 and IL-15 Rα, and (2) whether any of these tissues correlate with NK cell activity in elderly humans. We found that IL-15 and IL-15Rα RNA are expressed in muscle, SAT, and VAT, but with relatively lower IL-15Rα RNA levels in skeletal muscle. Because skeletal muscle produces high levels of IL-15 RNA, we initially hypothesized that relatively strong elderly individuals would have higher IL-15 levels and more robust NK cell response (18). Contrary to our prediction, we found that plasma IL-15 level did not associate with lean tissue mass, but rather with VAT. Additionally, NK cell response inversely correlated with muscle strength.

Abbreviations: aLM/BMI, appendicular lean mass divided by BMI; BMI, body mass index; CT, computed tomography; DC, dendritic cell; DXA, dual X-ray absorptiometry; MIP-1β, chemokine CCL4; Mφ, macrophage; SAT, subcutaneous adipose tissue; SVF, stromal vascular fraction; VAT, visceral adipose tissue.

MATERIALS AND METHODS

Subjects

In accordance with the Declaration of Helsinki (modified in 2008), all protocols were approved by the Institutional Review Board of the University of Kentucky, Lexington, KY, USA. All subjects were made aware of the design and purpose of the studies, and all subjects signed consent forms. The cohorts are summarized in Table S1 in Supplementary Material, with additional information provided in some of the figure legends. Cohort A vastus lateralis muscle and SAT biopsies from healthy research subjects were frozen in liquid nitrogen and stored at −80°C. Cohort B SAT and VAT were obtained from discarded surgery specimens, immediately put on ice for no more than 3 h, and immediately processed into stromal vascular fraction (SVF) and adipocyte fractions or stored at −80°C. Cohort C VAT, including mesenteric fat, epiploic appendages, and omentum were obtained from discarded surgery specimens, immediately snap frozen in liquid nitrogen, and stored at −80°C. Cohort D blood samples were obtained between 9:30 a.m. and 12:45 p.m. and kept at room temperature until processing within 2 h of collection.

Flow Cytometry and NK Cell Stimulation

As described in Ref. (19), whole blood was diluted with PBS and the mononuclear cells were recovered using Lymphoprep® lymphocyte separation medium (Axis-Shield, Oslo, Norway). For antibody staining, ~0.5×10^6 fresh mononuclear cells were washed and incubated with human IgG for 15 min at room temperature to block Fc-receptor binding and then stained on ice for 30 min with combinations of fluorescently labeled mAb, including those specific for CD3, CD16, and CD56 to allow for identification of $CD56^{bright}$ and $CD56^{dim}$ subsets (19). After washing, the cells were analyzed on a LSR-II flow cytometer (BD, San Jose, CA, USA), and data were processed using FlowJo software. Fresh mononuclear cells (0.5×10^6) were rested overnight and then stimulated with 1×10^6 K562 cells for 3 h at 37°C. Alternatively, mononuclear cells were cultured overnight with 0.5 μg/L IL-12 and then transferred to polystyrene plates coated with anti-NKp46 mAb for 3 h. Cells were stained with mAb to CD3, CD16, CD56, and CD107a. Cells also were fixed in 2% paraformaldehyde solution, then permeabilized (1× Permeabilization buffer, eBioscience) and stained with anti-IFN-γ and anti-MIP-1β mAb.

Body Composition and Strength Measurements

In cohort D, body composition was measured by dual X-ray absorptiometry (DXA) using a GE Lunar iDXA. Standardized methods for regional partitioning and phantom calibrations were employed to ensure data quality. Scans were analyzed using the GE Lunar software v10.0 in order to calculate fat-free mass

(kg), mineral-free lean mass (kg), fat mass (kg), and percent fat. Leanness is a significant risk factor for health outcomes in the elderly (20, 21) and was calculated as appendicular lean mass divided by body mass index (BMI). aLM was calculated as the sum of lean soft tissue in both the right and left arms and legs where limbs were isolated from the trunk by using DXA pre-defined regional lines with manual adjustment. Computed tomography (CT) is described in Supplementary Material.

Measures of strength included isometric and isokinetic knee extension testing on a Biodex System 4 dynamometer. Subjects were given a familiarization training session to acclimate to the testing protocol. During testing sessions, isometric measurements of peak torque and time to peak torque were completed with the subject seated with hip and knee angles at 85° and 90°, respectively. Peak torque was recorded as the highest torque achieved over three trials, whereas time to peak was recorded as the time in seconds to reach peak torque. Knee extensor isokinetic strength testing was completed at 90 degrees per second. We assessed peak torque, time to peak torque, total work, and average power over three trials.

IL-15 Assay

Plasma IL-15 was measured with the QuantiGlo Chemiluminescent Immunoassay kit (R&D Systems) in two independent experiments, as described in Ref. (19).

Adipose Tissue Fractionation and Muscle and Adipose Tissue RNA

Subcutaneous adipose tissue and VAT samples were either frozen at −80°C if unfractionated, or immediately processed if they were to be separated into adipocyte and SVF. Unfractionated fat (~200 mg) or muscle (100 mg) were mixed with zirconium oxide beads and 1.0 mL of TRIzol (Thermo-Fisher) in a 1.6 ml microcentrifuge tube (Thermo-Fisher) and treated with a tissue disruptor (Bullet Blender Storm 5, Next Advance) at full speed for 2 min. RNA was purified from the homogenized samples using the RNAeasy Lipid Tissue Mini kit (Qiagen). Nucleic acid (~1.0 μg) was next treated with DNase 1 (Promega). After DNase inactivation, RNA was reverse transcribed using the RNA to cDNA high capacity kit (Thermo-Fisher), following manufacturer protocol.

To prepare adipocyte and SVF fractions, 10–15 g of fresh SAT or VAT was washed thoroughly with Hanks balanced salt solution (HBSS, Sigma), minced, and after clots and connective tissue was removed, subdivided into two 50 mL polypropylene conical tubes. Each tube contained 25 mL of HBSS supplemented with 40 mM HEPES (pH 7.2), 1.0% BSA (Fraction V, Bioworld), 2 mM $CaCl_2$ and 0.1% collagenase (Type 1, Worthington, OH, USA). Tubes were gently agitated on an orbital shaker for 3 h at room temperature, centrifuged at 12 × *g* for 5 min, and the floating adipocyte layer and undigested fat was removed. This fraction was sent through a 500 μm steel mesh filter to remove undigested fat and washed once with HBSS. Approximately 0.1–0.2 mL of this fraction was treated with 0.9 mL of TRIzol. The SVF was centrifuged at 300 × *g* and then treated with 1.0 mM EDTA-HBSS in at 37 C for 15 min and centrifuged at 300 × *g*. Pelleted cells were resuspended in HBSS in 1.0 mM EDTA, sent through a 70 μm filter to remove large adipocytes, centrifuged, and solubilized with 1.0 mL of TRIzol. For both adipocytes and SVF, RNA was purified and cDNA prepared as described above.

qPCR

Primers and primer design are described in Supplementary Material. We wished to estimate the IL-15 and IL-15Rα RNA that was made by adipocytes and SVF cells. Here, we distinguish contaminating adipocytes from all other SVF cells, which likely includes pre-adipocytes. We first used differentiated adult-derived human adipose stem cells (22) to confirm that adipocytes do not contain CD45 RNA. The SVF CD45 level was only 5% of that of blood mononuclear cells. Using this very conservative 5% level, we estimated the SVF RNA contamination of the adipocyte fraction, which had a median of 2.27%. Based on this measurement, we reasoned that the adiponectin level in the adipocyte fraction would closely estimate the percentage adipocyte RNA. We then used the adiponectin RNA level in the SVF to calculate the percentage of adipocyte RNA, which was a median of 0.5% compared to adipocytes. Using the amount of adiponectin signal in each SFV, we calculated the amount of IL-15 and IL-15Rα RNA that came from SVF cells.

Statistical Analysis

All statistical tests were run using IBM SPSS Software (version 24, Armonk, NY, USA). The strength and direction of associations were evaluated using the nonparametric Spearman rank-order correlation coefficient (Spearman's correlation, for short) or the Pearson product-moment correlation coefficient (Pearson's correlation, for short). Linear regression analysis was used to quantify how well two variables relate to each other. When two or more independent variables were hypothesized to affect the outcome, multiple regression analysis was used. When sample groups were not normally distributed, differences between groups were compared by related samples Wilcoxon Singed Rank Testing. All histogram charts represent single values. Significance was set at <0.05. For box-and-whisker plots, the center lines show the medians; box limits indicate the 25th and 75th percentiles, whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. Box and whisker graphs were plotted using BoxPlotR (http://shiny.chemgrid.org/boxplotr/).

RESULTS

IL-15 and IL-15Rα RNAs Are Produced in Paired Human SAT and Muscle Samples

Natural killer cell and other innate immune functions decline with age. We hypothesized that IL-15 and its chaperone, IL-15Rα, are secreted from skeletal muscle, SAT, and VAT and influence NK number and function. Therefore, we measured IL-15 and IL-15Rα RNA levels in paired samples of vastus lateralis muscle and abdominal SAT from eight healthy cohort A adult subjects (cohorts are described in Table S1 in Supplementary Material). IL-15 RNA levels in muscle and SAT were not significantly different, but SAT expressed more of the IL-15 chaperone, IL-15Rα. These data are presented as a ratio of SAT RNA level to muscle

RNA level in the same individual (**Figure 1**). This result indicates that fat is a significant source of IL-15 and IL-15Rα RNA and that IL-15Rα RNA level is higher in SAT, compared with skeletal muscle from the same individual.

IL-15 and IL-15Rα RNAs Are Produced by both VAT and SAT

We extended these findings by measuring the IL-15 and IL-15Rα transcript levels in SAT and VAT. Non-paired samples of SAT and VAT from surgeries were processed in cohorts B and C. The levels of IL-15 and IL-15Rα transcripts are shown in **Figures 2** and **3**. As indicated in the figure legend, some VAT samples were from donors with inflammatory conditions (e.g., cancer), but transcript levels from these samples did not differ from donors without inflammatory conditions, such as hernia repair (**Figures 2** and **3**). Both SAT and VAT produced considerable IL-15 and IL-15Rα RNA (**Figure 2**). VAT from the mesentery, epiploic appendages, and omentum all produced IL-15 and IL-15Rα RNAs (**Figure 3**). IL-15 and IL-15Rα RNA levels occasionally differed between VAT depots, but the differences were not found in all subjects. For example, IL-15Rα RNA was higher in epiploic fat than in mesenteric fat in subject 21, but the opposite was true in subject 26 (**Figure 3**).

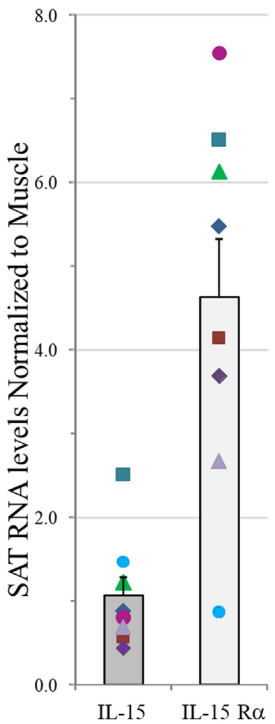

FIGURE 1 | IL-15 and IL-15Rα RNA levels in abdominal subcutaneous adipose tissue (SAT) normalized to values in paired vastus lateralis muscle samples in measured by RT-qPCR and normalized to four housekeeping genes in cohort A. Shown are means plus SEM. Each symbol for IL-15 and IL-15Rα represents an individual donor. IL-15 level did not significantly differ between tissues, but SAT expressed significantly more IL-15Rα RNA than did skeletal muscle ($p = 0.012$). Cohort A is described in Table S1 in Supplementary Material.

SVF Cells Produced More IL-15 RNA than Did Adipocytes

Adipose tissue is comprised of adipocytes, pre-adipocytes, stromal cells, and a variety of leukocytes (23), any one of which could be a source of IL-15 and IL-15Rα. To understand which cells produce IL-15 and IL-15Rα transcripts, fresh SAT and VAT received from surgery were fractionated into adipocyte fraction and SVF. **Figure 4** shows that SVF cells expressed more IL-15 RNA than did adipocytes from the same tissue sample. IL-15Rα RNA levels did not differ significantly between the paired samples. From these results, we propose that VAT IL-15 largely comes from SVF cells, which likely include Mφ.

Plasma IL-15 Level Directly Correlated with VAT

We hypothesized that non-lymphoid sources of IL-15 and IL-15Rα may influence plasma IL-15 levels and NK cell activity in the elderly. To test our hypothesis, we recruited 50 healthy adults aged >70 years old and correlated their body composition to plasma IL-15 levels and to NK cell number and function (cohort D). In these elderly subjects, IL-15 plasma levels did not differ by gender (19). IL-15 correlated strongly with CT measures of total abdominal fat and VAT (**Figure 5A**), but not with abdominal SAT (**Table 1**). The correlation between IL-15 and VAT was even stronger when analysis was limited to non-obese subjects (BMI < 30; data not shown). Because cytomegalovirus (CMV) infection is life-long and profoundly affects the human immune system (12), we tested whether the correlation between IL-15 and VAT could be explained by CMV infection status. CMV did not correlate with NK cell response to multiple different stimuli (24). Importantly, the correlation between VAT and IL-15 remained strong when CMV status was included as a factor in multifactorial analysis (**Table 1**). In multifactorial testing, the associations of IL-15 with other adipose depots were not significant when VAT was included as a factor (**Table 1**). Together, these data indicate that amount of VAT, but not other adipose depots, predicts circulating IL-15 concentration in elderly subjects.

The Elderly Subject Cohort D Showed Expected Sex Differences and Had Low C-Reactive Protein Level

As expected, men had significantly greater muscle mass, bone mineral content, fat-free mass, and android fat, whereas women had more gynoid and leg fat by DXA and more SAT by CT (data not shown). Men had more VAT than women by CT, but this difference was not significant. For all subjects, C-reactive protein levels were <10 mg/L, indicating a lack of marked inflammatory disease (data not shown). As expected, C-reactive protein level correlated with measures of adipose tissue and inversely with leanness. C-reactive protein correlated directly with IL-15 (data not shown), suggesting a link between systemic inflammation and circulating IL-15 level.

NK Cell Activity Directly Correlated with IL-15 Level

To investigate whether or not plasma IL-15 correlated with NK cell activity, we stimulated elderly cohort D peripheral blood

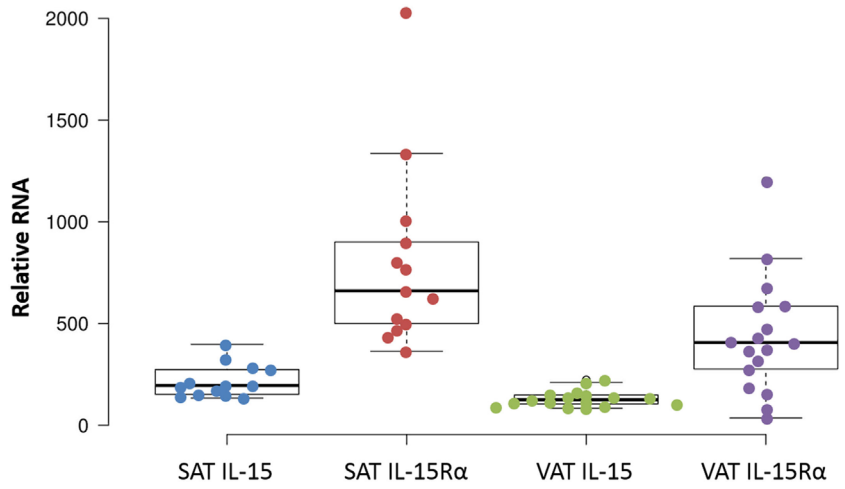

FIGURE 2 | IL-15 and IL-15Rα RNA are produced in both subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). Except for one sample, all cohort B SAT samples were from females, from surgeries for panniculectomy (7), ventral hernia (1), breast reduction (4), and mastectomy (1). VAT subjects had surgeries for hernia repair (1), nephrectomy (3), colectomy (12), and gastrectomy (1). Cohort B is described in Table S1 in Supplementary Material.

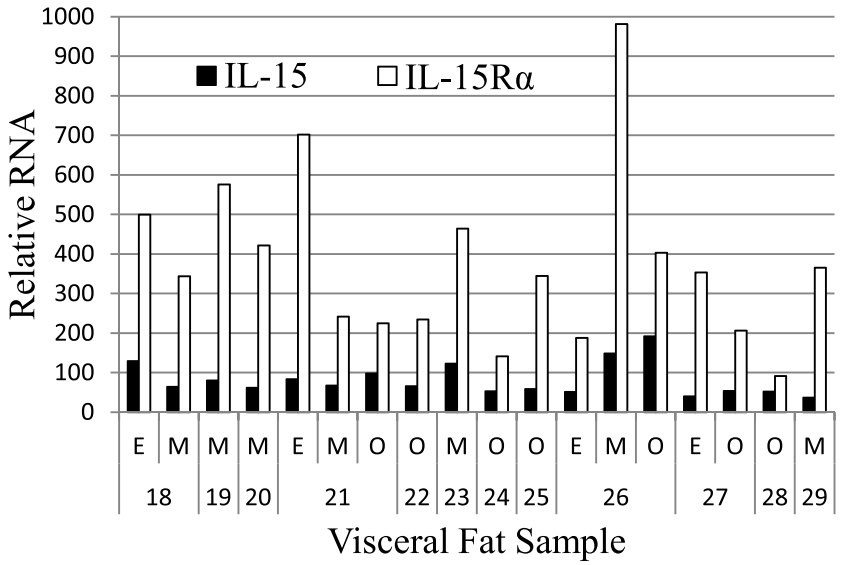

FIGURE 3 | IL-15 and IL-15Rα RNA are expressed in epiploic appendage (E), mesenteric (M), and omental (O) VAT. Cohort C subjects had surgeries for hernia repair (male #22, female #23, male #25), ostomy reversal (male #24), rectal intussusception (female #18), malfunctioning ileal conduit (male #19), large bowel obstruction (female #20), cancer (male #21, male #26, female #27, female #28), and acute appendicitis (male #29). Cohort C is described in Table S1 in Supplementary Material.

mononuclear cells *in vitro* with K562 leukemia cells or with low-level IL-12 and immobilized anti-NKp46 antibody, which are well-known NK cell stimuli. Using flow cytometry, we measured the ability of CD56^{bright} and CD56^{dim} NK cells to produce IFN-γ and MIP-1β. We also measured their cytotoxic response, as assayed by the appearance of the CD107a cytotoxic granule marker on the cell surface. Plasma IL-15 level correlated with the CD56^{dim} NK cell MIP-1β and CD107a, but not IFN-γ, responses to NKp46 crosslinking (Table S2 in Supplementary Material). The correlations between NKp46-stimulated responses and plasma IL-15 level remained significant when correcting for age and gender. The responses to NKp46 did not significantly correlate with the responses to K562 leukemia cells, indicating that these two assays measure distinct aspects of NK cell signaling (data not shown). This is not surprising because NK cell responses to K562 largely depend upon NKp30 and NKG2D (25).

NK Cell Activity Inversely Correlated with Muscle Strength

We compared NK cell responses and muscle strength. Mature CD56^{dim} NK cell responses to K562 leukemia cells showed inverse correlations with muscle strength, as measured by knee extensor

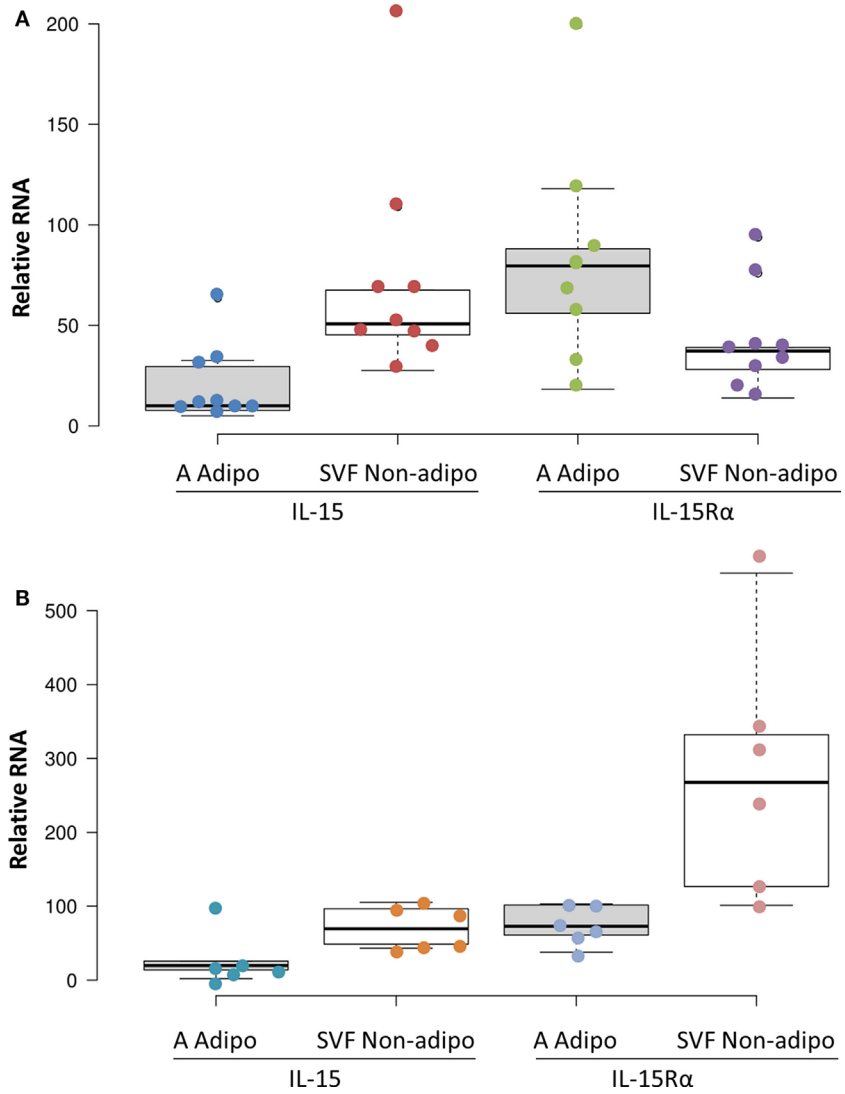

FIGURE 4 | Subcutaneous adipose tissue (SAT) **(A)** and visceral adipose tissue (VAT) **(B)** stromal vascular fraction (SVF) cells produced more IL-15 RNA than did adipocytes ($p = 0.012$ and $p = 0.043$, respectively, by Wilcoxon Signed Rank Test) in cohort B. IL-15Rα RNA levels did not significantly differ between SVF and adipocytes. Adipocyte (A Adipo) and non-adipocyte (SVF Non-adipo) were calculated to exclude contaminating cells as described in Section "Materials and Methods." All but one SAT samples were from female patients. SAT samples were from breast reductions (5), panniculectomy (4), and ventral hernia repair (1). None of the SAT cases involved cancer or other inflammatory diseases. VAT samples were from colectomies (5), exploratory laparotomy (1), and gastrectomy (1). Two VAT cases did not involve cancer. Cohort B is described in Table S1 in Supplementary Material.

peak torque (**Figure 6**; Table S2 in Supplementary Material), isometric peak torque, and average power (data not shown). Both CD56^{dim} NK cell responses to K562 target cells were significant or trended against strength after factoring in age and sex (Table S2 in Supplementary Material). As expected, skeletal muscle mass correlated with strength in this elderly cohort D (data not shown). Notably, both CD56^{dim} NK cell degranulation (as measured by CD107a) and MIP-1β responses to K562 cells robustly inversely associated with strength after factoring in either thigh muscle mass or leanness (Table S2 in Supplementary Material and data not shown). This indicates that muscle quality substantially and inversely associated with NK cell response to leukemia cells. As with other associations, CMV status did not weaken this inverse correlation (Table S2 in Supplementary Material).

Interestingly, SAT, but not VAT, correlated with the density of CD38 expression on both CD56^{bright} and CD56^{dim} NK cells (Table S3 and Figure S1 in Supplementary Material), which is described and discussed in Supplementary Material.

Strength Inversely Correlated with Plasma IL-15 in Elderly Humans

Because IL-15 is anabolic for skeletal muscle in some situations and skeletal muscle itself may be a source of IL-15, we predicted

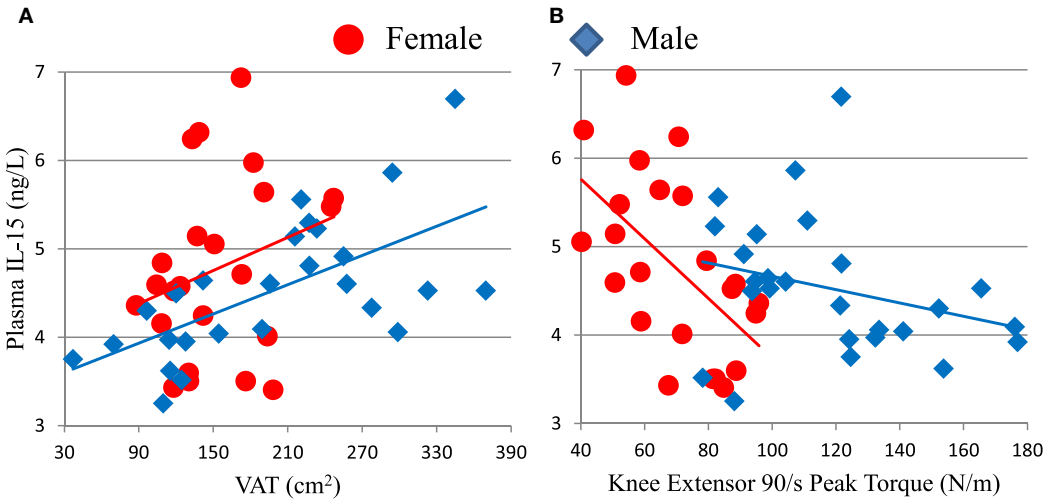

FIGURE 5 | Plasma IL-15 level associates with visceral adipose tissue (VAT) **(A)** and inversely associates with knee extensor peak torque **(B)** in cohort D. Associations of IL-15 with VAT were significant in all subjects ($\rho = 0.442$, $p = 0.001$) and in males ($\rho = 0.657$, $p < 0.001$), but not in females tested separately. IL-15 inversely associated with strength in all subjects ($\rho = -0.348$, $p = 0.014$) and in females ($\rho = -0.581$, $p = 0.004$), but not in males considered separately. Cohort D is described in Table S1 in Supplementary Material.

TABLE 1 | Correlations with plasma IL-15 level in Cohort D.[a]

	Parameter	Spearman	Reg. (age, sex)[b]	Reg. [visceral adipose tissue (VAT)][c]	Reg. (torque)[d]	Reg. [cytomegalovirus (CMV)][e]
Computed tomography (CT) fat	Abdomen	0.391, 0.005	0.325, 0.027	0.181, 0.336		
	Abdominal subcutaneous adipose tissue	0.171, 0.241	0.156, 0.355	0.135, 0.327		
	VAT	0.442, 0.001	0.450, 0.002		0.423, 0.001	0.372, 0.007
Dual X-ray absorptiometry fat	Total	0.334, 0.018	0.286, 0.053	0.160, 0.317		
	Legs	0.232, 0.105	0.125, 0.360	0.165, 0.232		
	Trunk	0.342, 0.015	0.334, 0.020	0.117, 0.507		
	Android	0.302, 0.033	0.327, 0.019	0.047, 0.805		
Other	Appendicular lean mass divided by BMI	−0.344, 0.014	−0.743, 0.001	−0.388, 0.003	−0.313, 0.100	
	Muscle area[f]	−0.244, 0.091	−0.155, 0.394	−0.417, 0.004	0.030, 0.860	
	Knee torque	−0.348, 0.014	−0.598, 0.021	−0.397, 0.002		−0.352, 0.010

[a]*The correlation coefficient and the significance are show in each cell. Significant associations are underlined.*
[b]*Linear regression corrected for influence of age and gender.*
[c]*Linear regression corrected for influence of VAT.*
[d]*Linear regression corrected for influence of knee extensor peak torque.*
[e]*Linear regression, corrected for influence of CMV infection.*
[f]*CT-measured thigh muscle cross-sectional area.*

that skeletal muscle and plasma IL-15 would directly correlate (18). However, there was no significant association between skeletal muscle mass and plasma IL-15 (**Table 1**). Because muscle mass declines less rapidly in elderly people than does strength, we searched for an association between strength and IL-15. We found a significant inverse correlation between IL-15 and knee extensor peak torque (**Table 1**; **Figure 5B**). This inverse association was confirmed when sex and age were included in multifactorial analysis; the inverse association became even more robust when VAT was included as a factor in multifactorial analysis (**Table 1**), indicating that this correlation is not confounded by VAT volume. Likewise, the correlation remained strongly negative when a role for CMV infection status was tested, suggesting that this correlation is not dependent on CMV status. When both knee extensor peak torque and thigh muscle cross-sectional area were compared, muscle strength, but not muscle cross-sectional area, was significant (**Table 1**).

DISCUSSION

The objectives of this work were to determine (1) if human muscle, SAT, and VAT are sources of IL-15 and IL-15 Rα, and (2) whether any of these tissues correlate with NK cell activity in elderly humans. We found that skeletal muscle, SAT, and VAT all

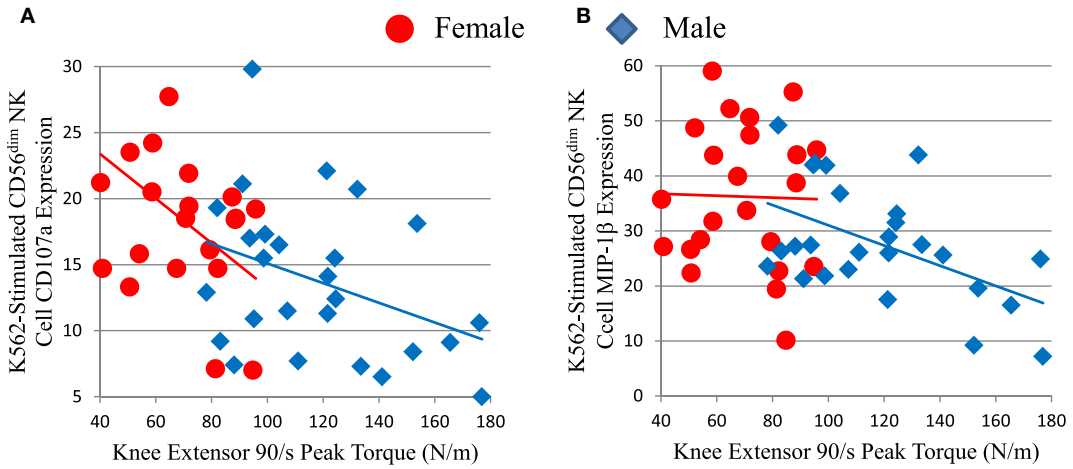

FIGURE 6 | Strength (knee extensor peak torque) correlates inversely with measures of mature CD56^{dim} natural killer (NK) cell responses to K562 leukemia cells in cohort D. CD56^{dim} NK cell CD107a **(A)** is a measure of cytotoxic granule release and correlated inversely with peak torque for all subjects ($\rho = -0.475$, $p = 0.001$), but not for each sex considered individually. CD56^{dim} NK cell MIP-1β chemokine production **(B)** correlated inversely with peak torque for all subjects ($\rho = -0.384$, $p = 0.006$), but not for each sex considered individually.

produced IL-15 and IL-15Rα RNA. Of these, only VAT correlated with IL-15 plasma levels in elderly human subjects. Our findings suggest that VAT may support NK homeostasis and activity in the elderly, when IL-15-producing immune cells have declined.

Other studies indicate that adipose tissue may be a significant source of IL-15. Dozio et al. (26) found that mouse epicardial fat, a type of VAT, expressed IL-15 and IL-15Rα RNA. Hickner and co-workers found that human abdominal SAT and skeletal muscle produce IL-15; and that SAT interstitial IL-15 level was higher in obese vs. lean young adults (27). Like us, this group found IL-15 blood levels did not correlate with total body mass (27). Another group found that IL-15 level was higher in VAT tissue homogenates from obese vs. lean middle-aged people (28). Liou et al. (29) showed that mouse adipocytes make significant amounts of IL-15 and that optimal NK cell development required adipocyte IL-15. Using parabiotic mouse pairs, O'Sullivan found that NK cells recirculate between the blood and the VAT compartments (30). This indicates that circulating NK cells are exposed to cytokines in the VAT (**Figure 7B**). Christiansen et al. found that young adult subjects placed on 12-week regimes of reduced caloric intake had significant IL-15 *declines* (31). This study suggests that negative caloric balance lowers IL-15 level and because caloric restriction usually leads to loss of fat, is consistent with our finding that increased VAT directly correlated with plasma IL-15 level (**Figure 7B**).

We attempted to determine if human skeletal muscle and fat derived IL-15 and IL-15Rα may correlate with NK activity in the elderly. Definitive experimental manipulation of human subjects is not possible, but we identified strong correlations between body composition parameters, IL-15 plasma levels, and NK cell function. We restricted our main analysis to elderly people because a prior study had suggested a correlation between BMI and NK cell number in elderly women, but not in young women (18). Additionally, because excess VAT and SAT carry different health risks, we separately analyzed correlations with IL-15 and

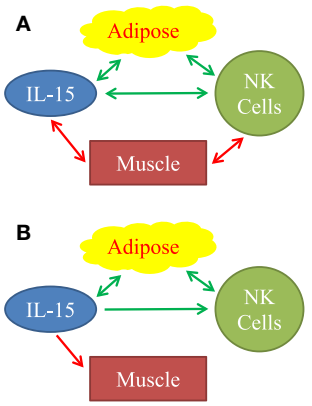

FIGURE 7 | Observed associations **(A)** and proposed mechanistic relationships **(B)** between adipose tissue, IL-15, natural killer (NK) cells, and muscle. **(A)** Positive associations are represented by green arrows. Inverse associations are represented by red arrows. All arrows are double-headed, indicating that associations do not necessarily imply mechanism. Mechanistic model **(B)** is based on our findings and on published literature. Stimulatory mechanisms are represented by green arrows and inhibitory mechanisms are represented by the red arrow. Double-headed green arrows indicate mutually positive stimulation. We propose that adipose tissue is a significant source of IL-15, especially in aging humans. IL-15 stimulates NK cells. Importantly, IL-15 forms part of a positive feedback loop between adipose tissue NK cells, other type 1 innate lymphocytes, and macrophages, as represented by the double-headed green arrows. IL-15 and other inflammatory agents weaken muscle.

NK cell activity. Our data, summarized graphically in **Figure 7A**, suggests that non-lymphoid tissues affect NK cells *via* multiple distinct interactions, but the strongest direct correlations were between IL-15 plasma levels and VAT, and an inverse correlation between NK function and muscle strength.

Our study appears to contrast with several studies of rodents and humans in which serum IL-15 level negatively associated

with VAT (32–37). As mentioned above, we found that plasma IL-15 positively associated with VAT. It is useful to consider that abdominal VAT may respond to IL-15 and that VAT itself produces IL-15 (27, 29). Most of the studies in rodents involved animals exposed to high nonphysiologic IL-15 levels *via* transgene expression or *via* injection; other studies utilized mice that were IL-15 knockouts. These extremes of IL-15 exposure might not be good models of the human condition. Prior human studies involved young adults and sometimes obese and lean groups differed significantly in age (33, 38). The contrasting outcomes in our study and past human studies may reflect age-related physiological differences. One effect of IL-15 is to increase gene expression and metabolic activity in brown adipose tissue (39). Because the amount of brown adipose tissue usually declines with age (40), many elderly individuals might not respond to IL-15 by increasing brown fat metabolic activity. Another possible explanation for this apparent contradiction is the amount of skeletal muscle mass in young and elderly adults and its correlation with adipose tissue mass. Obese young adults typically have more muscle mass than do lean young adults (41). Yet, fat mass is associated with a more rapid decline in muscle mass during aging (41). In addition, muscle strength declines rapidly in the elderly, much faster than loss of muscle mass, and may be a better measure of skeletal muscle health and function (42, 43).

Macrophages and DC functions decline with aging (13, 15, 44), making non-lymphoid sources of IL-15 relatively more important. These factors are likely to influence IL-15-depedent NK cells and memory CD8 T cells in the elderly. We propose that in elderly humans, VAT is a significant incremental source of IL-15 (**Figure 7B**) and promotes NK cell homeostasis.

Growing evidence supports the hypothesis of a positive feedback loop between type 1 innate lymphoid cells (including NK cells) and Mφ in people with a positive energy balance (30, 45–47). Adipocyte hypertrophy, fibrosis, hypoxia, and cell death cause release of inflammatory molecules, which stimulate adipose tissue Mφ (48). Stressed adipocytes express NKp46 ligands that directly stimulate NK cells and probably other type 1 innate lymphoid cells (47). In response to the inflammatory molecules, Mφ produce IL-12 and IL-15, which stimulate NK cells and other type 1innate lymphoid cells to produce IFN-γ, TNFα, and IL-6 (30, 45). These products, in turn, stimulate Mφ, setting up a positive feedback loop. Inflammatory cytokines, including IL-6 and TNFα, which affect skeletal muscle, the vasculature, and other tissues, cause pathologies associated with frailty and the metabolic syndrome (48, 49). Our data fit into this picture (**Figure 7B**). We found that IL-15 is elevated in relation to human VAT mass. Plasma C-reactive protein, a measure of inflammation, correlated strongly with plasma IL-15 (data not shown). We propose that IL-15 stimulates NK cells, both in a local paracrine fashion in VAT and in an endocrine fashion (**Figure 7B**). To explain the negative correlation between IL-15 and muscle strength, we propose that IL-15 itself and other inflammatory factors inhibit skeletal muscle (**Figure 7B**).

ETHICS STATEMENT

In accordance with the Declaration of Helsinki (modified in 2008), all protocols were approved by the Institutional Review Board of the University of Kentucky, Lexington, KY, USA. All subjects were made aware of the design and purpose of the studies, and all subjects signed consent forms. The cohorts are summarized in Table S1 in Supplementary Material, including IRB approval numbers, where applicable.

AUTHOR CONTRIBUTIONS

AA-A, SP, JC, DL, and RW performed experiments, analyzed data, and edited manuscript. MSexton and MS performed experiments and edited manuscript. PK, CP, and CL analyzed data and edited manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00440/full#supplementary-material.

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Impact of Aging, Cytomegalovirus Infection, and Long-Term Treatment for Human Immunodeficiency Virus on CD8+ T-Cell Subsets

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Both healthy aging and human immunodeficiency virus (HIV) infection lead to a progressive decline in naive CD8+ T-cell numbers and expansion of the CD8+ T-cell memory and effector compartments. HIV infection is therefore often considered a condition of premature aging. Total CD8+ T-cell numbers of HIV-infected individuals typically stay increased even after long-term (LT) combination antiretroviral treatment (cART), which is associated with an increased risk of non-AIDS morbidity and mortality. The causes of these persistent changes in the CD8+ T-cell pool remain debated. Here, we studied the impact of age, CMV infection, and LT successful cART on absolute cell numbers in different CD8+ T-cell subsets. While naïve CD8+ T-cell numbers in cART-treated individuals (N = 38) increased to healthy levels, central memory (CM), effector memory (EM), and effector CD8+ T-cell numbers remained higher than in (unselected) age-matched healthy controls (N = 107). Longitudinal analysis in a subset of patients showed that cART did result in a loss of memory CD8+ T-cells, mainly during the first year of cART, after which memory cell numbers remained relatively stable. As CMV infection is known to increase CD8+ T-cell numbers in healthy individuals, we studied whether any of the persistent changes in the CD8+ T-cell pools of cART-treated patients could be a direct reflection of the high CMV prevalence among HIV-infected individuals. We found that EM and effector CD8+ T-cell numbers in CMV+ healthy individuals (N = 87) were significantly higher than in CMV− (N = 170) healthy individuals. As a result, EM and effector CD8+ T-cell numbers in successfully cART-treated HIV-infected individuals did not deviate significantly from those of age-matched CMV+ healthy controls (N = 39). By contrast, CM T-cell numbers were quite similar in CMV+ and CMV− healthy individuals across all ages. The LT expansion of the CM CD8+ T-cell pool in cART-treated individuals could thus not be attributed directly to CMV and was also not related to residual HIV RNA or to the presence of HIV-specific CM T-cells. It remains to be investigated why the CM CD8+ T-cell subset shows seemingly irreversible changes despite years of effective treatment.

Keywords: healthy aging, cytomegalovirus, human immunodeficiency virus infection, combination antiretroviral treatment, CD8+ T-cells

INTRODUCTION

Infection with human immunodeficiency virus (HIV) leads to substantial changes in the T-cell compartment. Not only the CD4+ T-cell compartment—the decline of which forms one of the main characteristics of HIV infection—but also the CD8+ T-cell pool undergoes significant changes in HIV infection. There is a relative abundance of highly differentiated T-cells, characterized by a reduced capacity to proliferate, short telomere lengths (1) and changes in cytokine secretion capacity (2, 3). A progressive decline in naïve CD8+ T-cell numbers occurs concomitant with an increase in memory CD8+ T-cell numbers (4). Since these changes are reminiscent of the changes in the T-cell compartment observed during healthy aging (5, 6), HIV infection is often regarded a condition of premature immunological aging (7). While immune senescence during healthy aging is thought to result from the multiple rounds of activation of the immune system throughout life, the chronic immune activation induced by HIV may accelerate this aging process (8, 9).

When HIV-infected individuals are treated with combination antiretroviral therapy (cART), dramatic changes in the composition of the CD4+ and CD8+ T-cell pools are typically observed. We have previously shown that treatment with cART enables the CD4+ T-cell compartment to fully reconstitute in the majority of cases when HIV is successfully suppressed. CD4+ T-cell numbers increase gradually during the first years of cART to eventually reach age-matched control levels, and also the composition of the CD4+ T-cell pool becomes comparable to that of healthy, age-matched individuals (10). By contrast, HIV-induced changes in the CD8+ T-cell compartment seem to be more persistent. Increased CD8+ T-cell numbers have been reported even in patients on long-term (LT) cART and have been related to an increased risk of non-AIDS morbidity and mortality (11–14). The possible causes of these persistent changes remain debated and include residual viral load, residual immune activation, and CMV coinfection (12).

Interpreting alterations in the size and composition of the CD8+ T-cell pool is complicated by the influence of cytomegalovirus (CMV), a virus that is highly prevalent among healthy individuals and even more among HIV-infected individuals. The percentage of naïve CD8+ T-cells is typically lower and the percentage of effector CD8+ T-cells higher in CMV+ compared to CMV− healthy individuals (15–20). Also, in terms of absolute cell numbers, memory and effector CD8+ T-cells tend to be more frequent in CMV+ compared to CMV− healthy individuals (16–19, 21, 22). It is therefore important to carefully disentangle the influence of age and CMV (22, 23) when analyzing the changes in the CD8+ T-cell pools of LT-treated HIV-infected individuals.

Here, we studied the composition of the CD8+ T-cell compartment in LT cART-treated HIV-1-infected individuals who responded well to therapy, both in terms of control of virus load and CD4+ T-cell recovery and compared it to the changes in absolute numbers of naïve, central memory (CM), effector memory (EM), and effector CD8+ T-cells in CMV+ and CMV− individuals during healthy aging. We found that most changes observed in the CD8+ T-cell pools of HIV-infected individuals on LT successful cART were a direct reflection of their CMV+ status. Only the LT enlargement of the CM CD8+ T-cell pool in LT-treated HIV patients could not be attributed directly to CMV.

MATERIALS AND METHODS

Study Population

Thirty HIV-1-infected individuals of 18 years of age or older, who were under follow-up in the Department of Infectious Diseases of the University Medical Center Utrecht (UMCU Utrecht, The Netherlands), were included for cross-sectional analyses. At the moment of inclusion, they had been treated with cART for at least 7 years. In the last 5 years preceding study inclusion, they had undetectable HIV RNA plasma levels (<50 copies/ml) with no more than two isolated viral blips of HIV RNA (number of copies between 50 and 400/ml). Participants had to have CD4+ T-cell numbers above 500/μl of blood and express HLA-A2 and/or HLA-B8 alleles in order to measure their HIV-specific T-cell response using tetramers. Thirteen HIV-1-infected individuals (five from the original group and eight additional patients) were included in a longitudinal analysis. They were 18 years or older and had been treated with cART for at least 7 years, with undetectable HIV RNA plasma levels (<50 copies/ml) and CD4+ T-cell numbers above 400/μl of blood. In this group, a maximum of three viral blips above 50 copies/ml were allowed.

To study the effect of age and CMV in healthy individuals, we included 257 healthy donors, of which 119 adults (>18 years of age)—who were registered blood donors at the Dutch Blood Bank, or employees of the University Medical Center Utrecht—and 138 children (between 1 and 18 years of age) admitted to the UMCU to undergo elective urological, plastic, ophthalmologic or general surgery. To minimize interference on immunologic parameters, blood was drawn prior to or directly after the onset of anesthesia (24, 25). All participants were considered immunologically healthy, as they did not have any history of infectious diseases or hematological or immunological disorders, or showed any signs of acute infection at the time of venipuncture. CMV+ children had a mean age of 8 years and CMV− children of 7 years. Both CMV+ and CMV− adults had a mean age of 47 years. As a control group for the HIV-infected individuals, we used data from 107 (out of the 119) healthy adults so that the ages of the groups were matched. Basic characteristics of healthy and HIV-infected individuals are summarized in **Table 1**.

All patients or their legal guardians gave written informed consent in agreement with the Declaration of Helsinki (version: 59th WMA General Assembly, Seoul, October 2008). The protocols were approved by the Medical Ethical Committee of the UMC Utrecht. Blood from healthy adult (>18 years of age) volunteers was obtained under guidelines of the Medical Ethical Committee of the UMC Utrecht or under guidelines of Sanquin (Blood Bank, The Netherlands).

Flow Cytometry

Whole EDTA-anticoagulated or sodium heparine anticoagulated blood was obtained by venipuncture. PBMCs were obtained by Ficoll–Paque density gradient centrifugation directly analyzed or cryopreserved until further use. Absolute CD4+ and CD8+ T-cell

TABLE 1 | Basic characteristics of healthy and HIV-infected individuals.

Healthy children	CMV−	CMV+
CMV status (%, number)	71%, *N* = 96	29%, *N* = 42
Age in years[a]	7 (1–18)	8 (1–17)
CD4+ T-cell count/μl blood at study moment[a]	1,538 (442–7,644)	1,318 (528–3,404)
CD8+ T-cell count/μl blood at study moment[a]	750 (158–3,586)	928 (265–2,417)
CD4/CD8 ratio at study moment[a]	2.1 (0.8–5.4)	1.6 (0.7–4.1)
Healthy adults	**CMV−**	**CMV+**
CMV status (%, number)	61%, *N* = 74	39%, *N* = 45
Age in years[a]	47 (20–70)	47 (23–69)
CD4+ T-cell count/μl blood at study moment[a]	1,741 (114–8,811)	1,739 (249–7,939)
CD8+ T-cell count/μl blood at study moment[a]	652 (95–3,856)	911 (125–6,943)
CD4/CD8 ratio at study moment[a]	2.9 (0.8–10)	2.3 (0.6–5.3)
HIV-infected individuals with cross-sectional data only		**Based on**
CD4+ T-cell count/μl blood at start cART[a]	282 (11–558)	*N* = 25
Viral load in copies/ml at start cART[a]	2.2×10^5 (<400–7.5×10^5)	*N* = 21
Years on cART[a]	11.1 (7.0–14.2)	*N* = 25
Age at LT[b] cART in years[a]	48 (34–70)	*N* = 25
CD4+ T-cell count/μl blood at LT cART[a]	613 (214–1,173)	*N* = 25
CD8+ T-cell count/μl blood at LT cART[a]	750 (204–3,337)	*N* = 25
CD4/CD8 ratio at LT cART[a]	1.1 (0.1–4.2)	*N* = 25
HIV-infected individuals with longitudinal data		**Based on**
CD4+ T-cell count/μl blood at start cART[a]	275 (36–677)	*N* = 11
Viral load in copies/ml at start cART[a]	1.8×10^5 ($3.8.10^4$ to $>5 \times 10^5$)	*N* = 11
Years on cART[a]	8.1 (7.1–8.0)	*N* = 13
Age at LT cART in years[a]	47 (36–59)	*N* = 13
CD4+ T-cell count/μl blood at LT cART[a]	980 (500–1,470)	*N* = 13
CD8+ T-cell count/μl blood at LT cART[a]	800 (480–1,470)	*N* = 13
CD4/CD8 ratio at LT cART[a]	1.3 (0.5–2.2)	*N* = 13

[a]*Mean (range).*
[b]*LT cART = long-term cART.*

numbers were determined by dual-platform flow cytometry, using TruCount tubes (BD Biosciences) or were calculated by multiplying the percentage of the indicated subset as obtained by flow cytometry and the absolute lymphocyte number as determined using a Cell-Dyn Sapphire hematology Analyzer (Abbott Diagnostics). Naïve (CD27+CD45RO−), CM (CD27+CD45RO+), EM (CD27−CD45RO+), and effector (CD27− CD45RO−) CD8+ T-cells were assessed by flow cytometry. To this end, PBMCs were incubated with CD3 [PerCP or FITC (Biolegend)] or CD3-eFluor450 (eBioscience), CD8 [PerCP-Cy5.5, APC-Cy7, Amcyan or V500 (BD Biosciences)], CD27 [APC-Cy7 (BD Biosciences), APC, APC-AF750 (eBioscience) or FITC (Sanquin)] and CD45RO [PE or PE-Cy7 (BD Biosciences)] monoclonal antibodies (mAbs), in appropriate combinations. Within the subsets, characteristics of the cells were determined following standard staining protocols using CD28-FITC (BD Biosciences) and CD57-APC (Biolegend) mAbs for the level of senescence, AnnexinV-PE and 7AAD (BD Biosciences) mAbs for the level of apoptosis, and Ki67-FITC (DakoCytomation) mAbs as a measure of T-cell proliferation. HIV-specific CD8+ T-cells were detected using the following HLA-tetramers: HLA-B8-BFLKEKGGL, HLA-B8-EIYKRWII, and HLA-A2-SLYNTVATL, which were prepared as previously described (2). All experiments were analyzed on a FACS Canto II or FACS LSR II (BD Biosciences) with FACS Diva software (BD Biosciences).

Measuring HIV Viral Load

Human immunodeficiency virus viral load monitoring was performed on EDTA plasma samples using the Roche Cobas Taqman v2.0 or the Roche Cobas Amplicor v1.5 assay. The result of the viral load determination was reported as a specified load (copies/ml), RNA detected, or target not detected (i.e. no signal in the PCR, no viremia) with cut-off values depending on the sensitivity of the two assays (i.e. >20, 0-20, 0 and >50, 0-50, 0 copies/ml, respectively).

Analysis of CMV Serostatus

Analysis of CMV serostatus was performed in 30 HIV-infected individuals and 257 healthy individuals using the CMV IgG ELISA kit (IBL international) according to the manufacturer's instructions, using plasma. Individuals who were CMV seropositive or seronegative according to this assay are referred to as CMV+ and CMV−, respectively, throughout the article.

Statistical Analysis

Cross-sectional comparisons between HIV-infected individuals on LT cART and healthy individuals, and between CMV+ and CMV− individuals were based on Mann–Whitney *U*-tests. Wilcoxon matched-pair signed rank tests were used for longitudinal analyses. CD8+ T-cell changes over the age of CMV+ and CMV− individuals were studied using linear regression analysis; data from children and adults were analyzed separately. All statistical analyses were performed using the GraphPad Prism software (Graphpad Software, Inc.). To provide insight into the significance of observed differences, throughout the manuscript, we report exact *P*-values, without correction for multiple testing.

RESULTS

Cell Numbers in Most CD8+ T-Cell Subsets Remain Elevated Despite LT Successful cART

To study to what extent the different CD8+ T-cell subsets normalized on LT cART, we measured the total CD8+ T-cell numbers and the number of cells in different CD8+ T-cell subsets of 38 HIV-infected individuals on LT successful cART and 107 healthy, age-matched controls. All HIV-1-infected individuals had a CD4+ T-cell count of >400 cells/μl at the time of inclusion and had undetectable viral loads for at least the last 5 years preceding study inclusion, with maximally two isolated blips (viral load between 50 and 400 copies/ml). The mean time on cART was 10

(range 7–14) years. The mean age of the 107 age-matched healthy donors was 50 (range 27–70) years, which was not significantly different from that of the HIV-infected individuals, which was 48 (range 34–70) years.

In line with what has previously been reported (11–14), the total CD8+ T-cell numbers remained significantly elevated (median 638 vs. 449 CD8+ T-cells/μl blood in HIV-infected and healthy individuals, respectively, **Figure 1A**) despite LT successful cART. Based on the expression of the markers CD27 and CD45RO, we distinguished between naïve (CD27+CD45RO−), CM (CD27+CD45RO+), EM (CD27−CD45RO+), and effector (CD27−CD45RO−) CD8+ T-cells. Naïve CD8+ T-cell numbers of HIV-infected individuals on LT cART (median 231 cells/μl blood) had normalized to levels comparable to healthy individuals (median 205 cells/μl blood, **Figure 1B**). By contrast, CM, EM, and effector CD8+ T-cell numbers remained nearly twofold increased compared to healthy age-matched individuals (**Figures 1C–E**).

Memory CD8+ T-Cell Numbers Did Decline During cART

Since CM, EM, and effector CD8+ T-cell numbers had not normalized after at least 7 years of cART, we studied whether these subsets had been stably maintained during treatment or whether cART had decreased these cell numbers, albeit incompletely. To this end, we followed longitudinal changes in CD8+ T-cell numbers in 13 HIV-infected individuals, from pretreatment to at least 7 years of successful cART. Patients were selected to have CD4+ T-cell numbers of at least 400/μl blood. Two participants had one, one participant had two, and one participant had three occasional blips. Total CD8+ T-cell numbers remained relatively stable during LT treatment, at significantly higher levels than in healthy age-matched controls (**Figure 1A**). Nevertheless, there were substantial changes in the subset composition of the CD8+ T-cell pool on cART. Naïve CD8+ T-cell numbers, which were low at the start of cART, increased significantly over time and were no longer lower than in healthy controls (**Figure 1B**). By contrast, CM and EM CD8+ T-cell numbers decreased significantly during LT cART, but remained higher than in healthy controls, while effector CD8+ T-cell numbers showed a significant increase during LT treatment to significantly higher levels than in healthy controls (**Figure 1B**).

We next investigated whether the observed changes in the different CD8+ T-cell subsets during cART occurred gradually over time, or more abruptly at the start of cART. To this end, we compared CD8+ T-cell numbers pre-cART, 1 year after the start of cART and after LT cART in 9 of the 13 HIV-infected individuals (**Figure 2**). Naïve cell numbers changed quite gradually over time. By contrast, the dominant changes in CM and EM CD8+ T-cell numbers were observed during the first year of cART, after which CM numbers hardly changed and EM numbers declined more gradually. No clear pattern was observed for effector cells. Although we refrained from statistical analysis of these changes due to limited sample sizes, these data suggest that the successful suppression of HIV has a fast and large impact on memory CD8+ T-cell numbers.

Changes in the CD8+ T-Cell Pool in CMV+ and CMV- Healthy Individuals With Age

As CMV infection is known to increase CD8+ T-cell numbers in healthy individuals (16, 22), we studied whether the enlarged

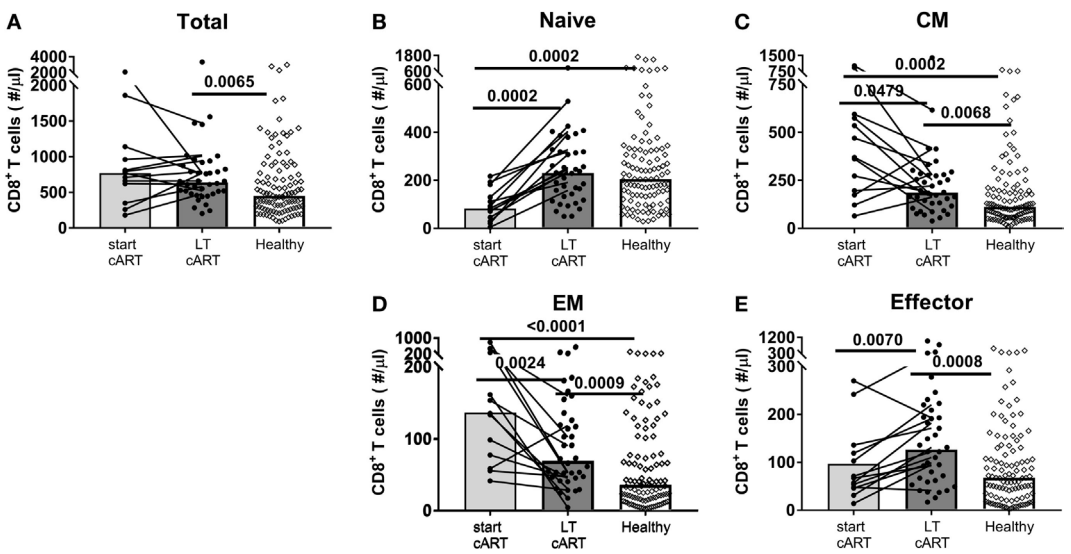

FIGURE 1 | Potential of the CD8+ T-cell pool to normalize on long-term (LT) combination antiretroviral treatment (cART). **(A)** Total CD8+ T-cell numbers and **(B)** naïve (CD27+CD45RO−), **(C)** central memory (CM) (CD27+CD45RO+), **(D)** effector memory (EM) (CD27−CD45RO+), and **(E)** effector (CD27−CD45RO+) CD8+ T-cell numbers of human immunodeficiency virus (HIV)-infected individuals at a moment just before the start of cART (*N* = 13), during LT (i.e., at least 7 years of) cART (*N* = 38), and of [a mixed group of cytomegalovirus (CMV)+ and CMV−] age-matched healthy controls (*N* = 107). Bars represent median values of all individuals in a group, and data from the same individual are connected by lines. Comparisons between cross-sectional data from HIV-infected individuals and healthy individuals were based on a Mann–Whitney *U*-test. A Wilcoxon matched-pair signed rank test was used to study the significance of longitudinal changes in cell numbers from HIV-infected individuals. All (uncorrected) *P*-values of <0.05 are provided in the figure.

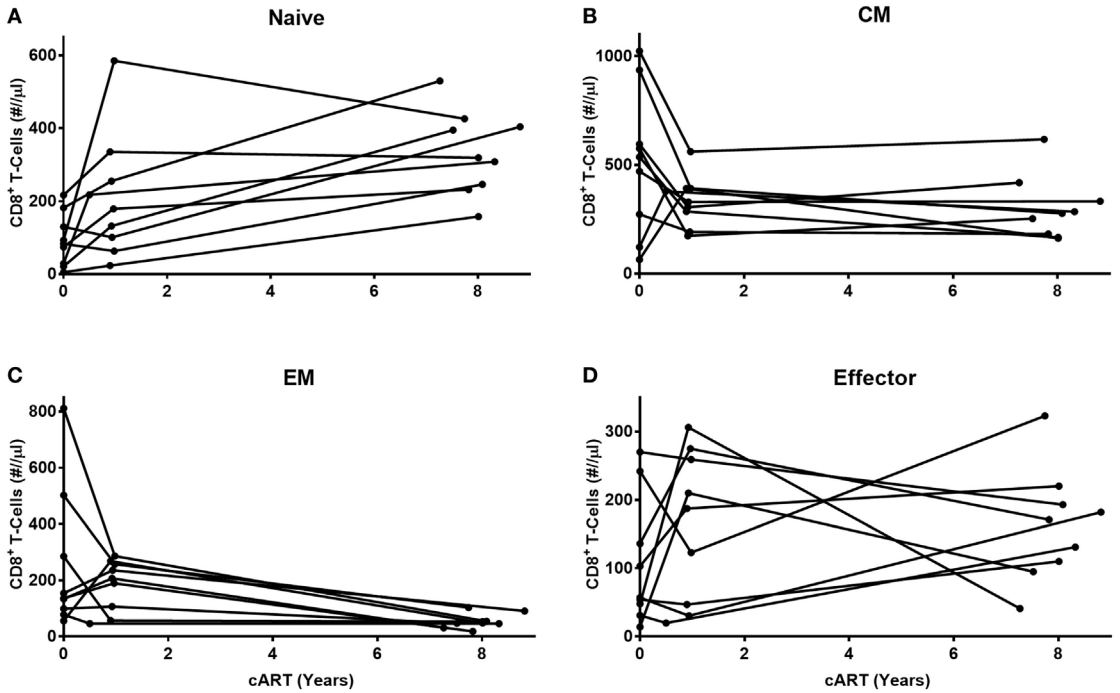

FIGURE 2 | Early and late effects of combination anti-retroviral treatment (cART) on CD8+ T-cell numbers. Longitudinal analysis of cell numbers in four CD8+ T-cell subsets [**(A)** naïve, **(B)** central memory (CM), **(C)** effector memory (EM), and **(D)** effector] of nine HIV-infected individuals, during the first year of cART until at least 7 years of cART. Because of low sample sizes, we refrained from testing these changes statistically.

memory and effector CD8+ T-cell pools observed in LT-treated patients could be a direct reflection of the increased CMV prevalence among HIV-infected individuals. Indeed, 93% ($N = 30$) of HIV-infected individuals in our study had a detectable anti-CMV IgG titer (results not shown). To test this hypothesis, we first followed the changes in absolute numbers of naïve, CM, EM, and effector CD8+ T-cells in 87 CMV+ and 170 CMV− healthy individuals of different ages, varying from 1 to 70 years.

Since T-cell numbers per ml blood in young children change considerably with age, possibly due to the growth of their blood volume (26), we separately analyzed the data obtained from children (<18 years of age) and adults (>18 years of age). In line with previous literature (22, 27), we observed a significant decline in naïve CD8+ T-cell numbers with age, both in CMV+ and in CMV− individuals (**Figure 3A**), with no significant difference between their rates of decline. When limiting our analyses to adults, no further decline in naïve CD8+ T-cell numbers was observed. CM CD8+ T-cell numbers did not differ significantly between CMV+ and CMV− healthy individuals and only showed a significant increase with age in CMV− adults (**Figure 3B**). By contrast, EM and effector CD8+ T-cell numbers all increased significantly—albeit only mildly—with age in CMV− adults but not in CMV+ adults (**Figures 3C,D**). When analyzed over the full age range, EM and effector CD8+ T-cell numbers were significantly elevated in CMV+ individuals, while naïve and CM CD8+ T-cell numbers were not. The fact that even in children, EM and effector CD8+ T-cell numbers were significantly higher in CMV+ compared to CMV− individuals suggests that the effects of CMV on these subsets occur rapidly and do not require years to accumulate. Summarizing, the most significant differences in absolute CD8+ T-cell numbers between CMV+ and CMV− healthy individuals were found in the EM and effector CD8+ T-cell compartments.

CD8+ T-Cell Expansions During LT cART Are Largely Explained by CMV

When comparing the sizes of the different CD8+ T-cell subsets in HIV-infected individuals on LT successful cART with those of CMV+ healthy age-matched individuals ($N = 39$), both EM and effector CD8+ T-cell numbers were no longer significantly increased (**Figure 4**). The relatively large sizes of the EM and effector CD8+ T-cell pools in LT-treated HIV-infected individuals may thus be a direct reflection of the increased prevalence of CMV among HIV-infected individuals. By contrast, CM CD8+ T-cell numbers were significantly higher ($P = 0.0063$) in LT-treated HIV patients, even when compared to those in CMV+ healthy age-matched controls. Their permanent increase despite years of successful cART could thus not be attributed directly to CMV.

Elevated CM CD8+ T-Cell Numbers Are Explained Neither by Low-Level Viremia Nor by HIV-Specific T-Cells

We next investigated whether low-level HIV viremia could explain why the CM CD8+ T-cell pool of LT-treated patients failed to normalize. Although all HIV-infected individuals had HIV RNA plasma loads below 50 copies/ml at the time of inclusion in this

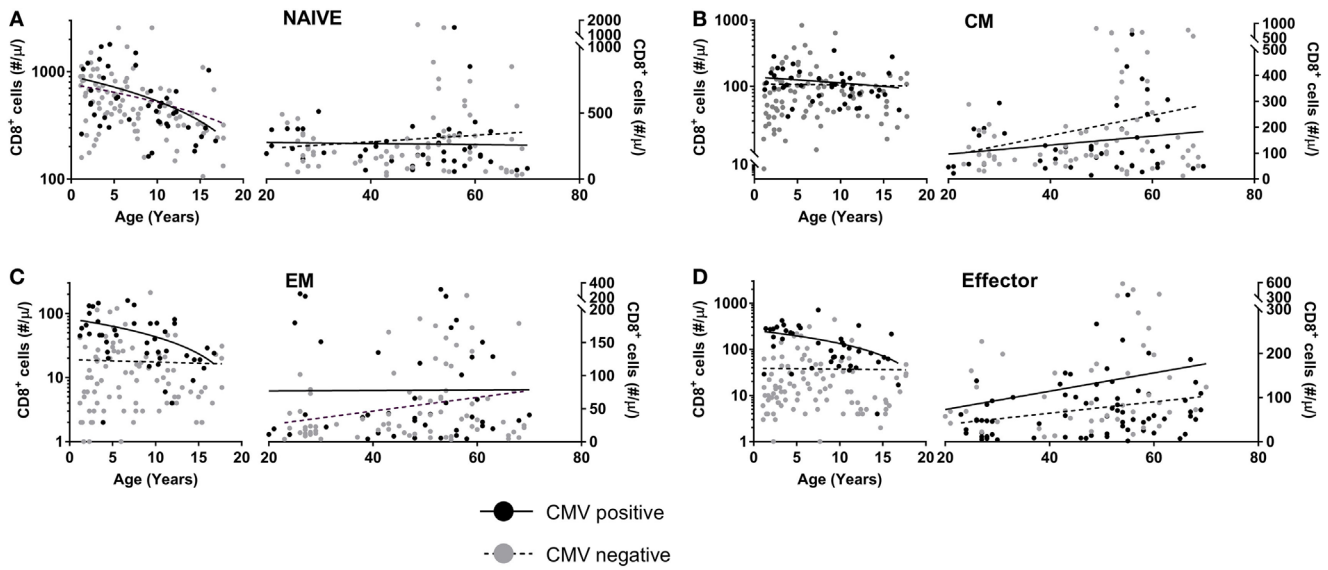

FIGURE 3 | CD8+ T-cell numbers in cytomegalovirus (CMV)+ and CMV− healthy individuals. Changes in absolute **(A)** naïve, **(B)** central memory (CM), **(C)** effector memory (EM), and **(D)** effector CD8+ T-cell numbers in CMV+ (black circles) and CMV− (grey circles) healthy individuals with age. Solid curves represent linear regression lines through the data from adults and children, which were analyzed separately. Naïve cell numbers declined significantly with age in both CMV+ and CMV− children, while EM and effector cell numbers declined significantly in CMV+ children only. CM, EM, and effector cell numbers increased significantly with age in CMV− adults only.

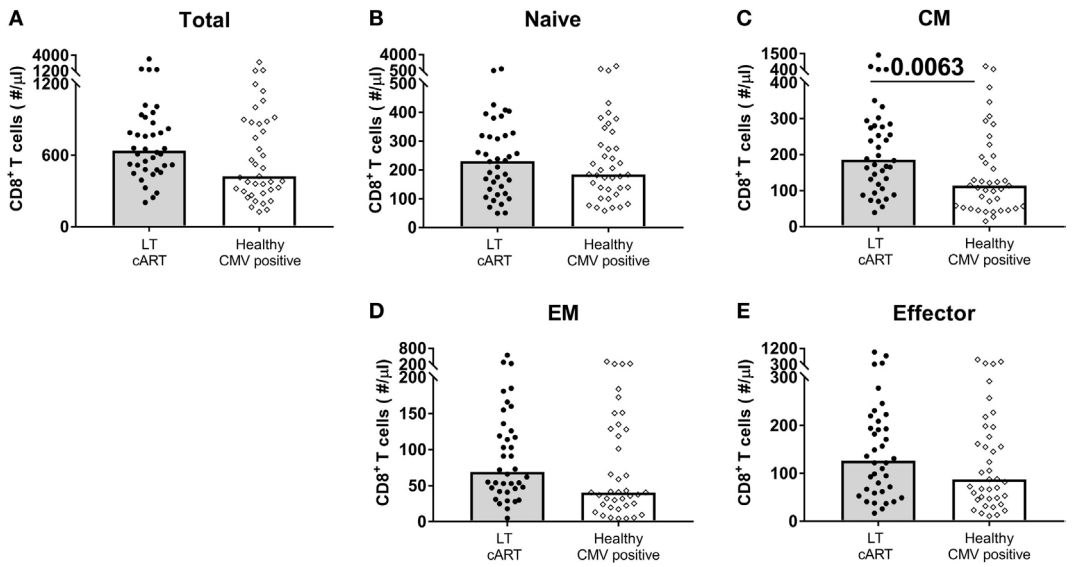

FIGURE 4 | CD8+ T-cell numbers in cytomegalovirus (CMV)+ healthy individuals and human immunodeficiency virus (HIV) patients on long-term (LT) combination anti-retroviral treatment (cART). Comparison of **(A)** total, **(B)** naïve, **(C)** central memory (CM), **(D)** effector memory (EM), and **(E)** effector CD8+ T-cell numbers between HIV-infected individuals on LT cART ($N = 38$) and CMV+ age-matched healthy controls ($N = 39$). Bars represent median values. Comparisons between data from HIV-infected individuals and healthy individuals were based on a Mann–Whitney U-test. All (uncorrected) P-values of <0.05 are provided in the figure.

study, we could not exclude the possibility that differences in viremia below 50 copies/ml caused the CM CD8+ T-cell pool to remain expanded. We therefore tested whether HIV plasma load below the commonly used detection limit of 50 HIV RNA copies/ml plasma correlated with the number of CM CD8+ T-cells after LT cART. We found plasma levels above 20 copies/ml in 20% and between 0 and 20 copies/ml in 40% of the HIV-infected individuals; nevertheless, individuals having these increased plasma levels did not have significantly higher CM CD8+ T-cell numbers after LT cART than individuals who had no detectable HIV load (**Figure 5A**).

Alternatively, increased CM CD8+ T-cell numbers on LT cART may reflect the presence/persistence of HIV-specific memory

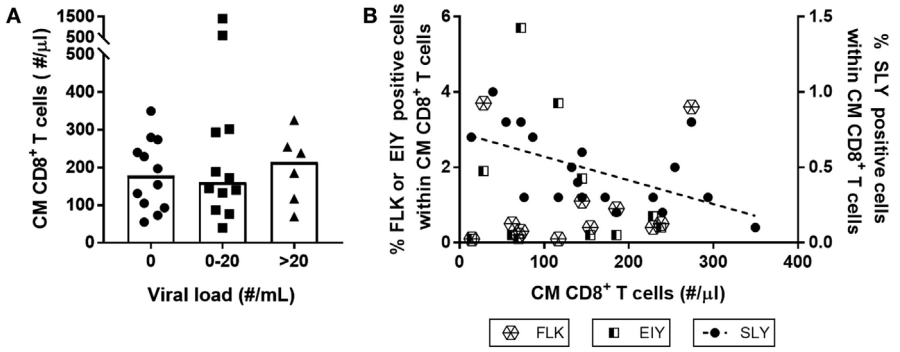

FIGURE 5 | Neither low-level human immunodeficiency virus (HIV) viremia nor the presence of HIV-specific T-cells explain increased central memory (CM) CD8+ T-cell numbers during long-term (LT) combination anti-retroviral treatment (cART). CM CD8+ T-cell numbers in HIV-infected individuals on LT cART were neither associated with **(A)** levels of plasma HIV RNA load (grouped by the number of viral RNA copies/ml plasma determined using the Roche Cobas Taqman v2.0 assay), nor by **(B)** the percentage of HIV-specific CM CD8+ T-cells, measured using tetramers of the three immuno-dominant HIV-1 peptides: HLA-A2-SLYNTVATL (SLY), HLA-B8-FLKEKGGL (FLK), and HLA-B8-EIYKRWII (EIY). Depicted are individual values (symbols), medians (bars in panel A), and a linear regression line for the results of HLA-A2-SLYNTVATL ($P = 0.01$).

CD8+ T-cells. We studied whether the presence of such responses correlated with absolute CM CD8+ T-cell numbers in patients on LT cART. To this end, we used tetramers of three immune-dominant HIV-1 peptides—the HLA-A2 restricted SLYNTVATL peptide and the HLA-B8 restricted FLKEKGGL and EIYKRWII peptides—to measure the frequencies of antigen-specific T-cells in HLA-A2 and/or HLA-B8 expressing individuals. We found no evidence for a positive correlation between the frequencies of HIV-specific CM CD8+ T-cell responses and numbers of CM CD8+ T-cells in LT-treated patients. The response to the HLA-A2 restricted SLYNTVATL peptide was even negatively correlated ($P = 0.01$) with the number of CM CD8+ T-cells (**Figure 5B**). Although HIV replication has been shown to be strongly correlated with increased HIV-specific CD8+ T-cell numbers (2, 28–30), we found no significant association between HIV plasma levels and frequencies of HIV-specific CM CD8+ T-cells (data not shown).

Taken together, these results suggest that neither HIV RNA plasma levels nor the LT maintenance of HIV-specific memory CD8+ T-cells could explain the lasting expansion of the CM CD8+ T-cell subset in LT-treated patients.

Dynamic Properties of CD8+ T-Cells in CMV and After LT cART

Finally, we investigated to what extent LT cART led to normalization of cellular dynamics, including the fraction of proliferating (Ki67+), senescent (CD28−CD57+), and apoptotic (AnnexinV+7AAD−) cells. For CM cells, we found no significant differences in these characteristics between HIV patients on LT successful cART and CMV+ healthy controls (**Figure 6**). The fraction of apoptotic EM cells and the fraction of senescent EM and effector cells were significantly increased in LT-treated patients, although the significance of these differences would be lost when correcting for multiple testing (e.g., using a Bonferroni correction). Taken together, we conclude that the increased CM CD8+ T-cell numbers in HIV-infected individuals on LT cART could not be explained by maintenance through an increased proliferation or resistance to apoptosis. Normalization of cell numbers in the other subsets tended to coincide with normalization of cell dynamics, although the percentage of senescent cells in the EM and effector subsets may be increased in LT cART-treated patients.

DISCUSSION

Changes in the CD8+ T-cell compartment of HIV-infected individuals are often compared to changes that occur during chronological aging, and HIV infection has therefore been described as a condition of accelerated immunological aging. In line with previous findings, we found that CD8+ T-cell numbers in LT-treated HIV patients remained significantly increased compared to (a mixture of CMV+ and CMV−) healthy age-matched controls, despite years of successful treatment. These persistent changes pertained to the CM, EM, and effector cell subsets, while the number of naïve CD8+ T-cells normalized to healthy age-matched levels. Our results suggest that the persistent expansions in the CD8+ T-cell pool of cART-treated individuals were a direct reflection of the increased prevalence of CMV among HIV-infected individuals. Indeed, the sizes of the EM and effector CD8+ T-cell pools of HIV patients on LT cART did not differ significantly from those of CMV+ healthy age-matched controls. We therefore conclude that the CD8+ T-cell pool of HIV-infected individuals—just like the CD4+ T-cell pool (10)—has the potential to normalize to a great extent on LT cART. Importantly, however, this normalization only becomes apparent when comparing to a healthy, age-matched, and CMV status-matched control group. Similarly, it was recently shown that the percentage of terminally differentiated T-cells in patients on LT cART was significantly higher than in unselected healthy blood bank donors, but similar to non-infected individuals matched for lifestyle and demographic factors with a higher prevalence of CMV (20). In our data, only the increased size of the CM CD8+ T-cell pool in HIV patients could not be explained directly by CMV.

Naïve CD8+ T-cell numbers tend to increase gradually during cART, and it was previously reported that 1.5 years of treatment is

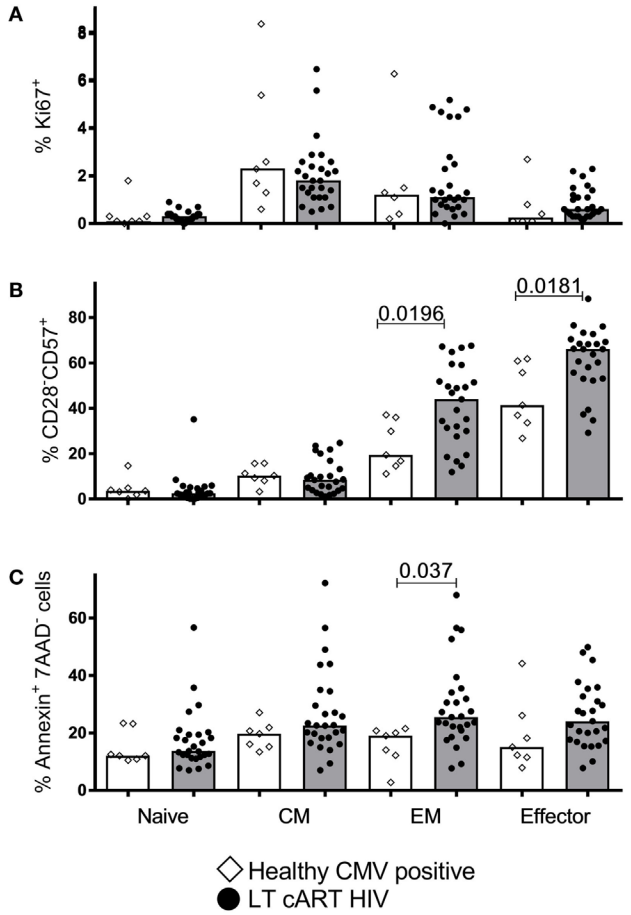

FIGURE 6 | T-cell proliferation, senescence, and apoptosis. Percentages of **(A)** proliferating (Ki67+), **(B)** senescent (CD28−CD57+), and **(C)** apoptotic (AnnexinV+7AAD−) CD8+ T-cells in the different subsets in human immunodeficiency virus (HIV)-infected individuals on long-term combination anti-retroviral treatment (cART) (filled circles) compared to cytomegalovirus (CMV)+ healthy age-matched controls (open diamonds). Bars depict medians. Comparisons between data from HIV-infected individuals and healthy individuals were based on a Mann–Whitney *U*-test. All (uncorrected) *P*-values of <0.05 are provided in the figure.

insufficient for complete normalization of the naïve CD8+ T-cell pool (29). We here found that 7 years of successful cART was sufficient for normalization of naïve CD8+ T-cell numbers, probably through a combination of low-level T-cell proliferation and *de novo* T-cell production by the thymus. In contrast to the gradual increase in cell numbers that we observed for naïve CD8+ T-cells, EM and CM CD8+ T-cell numbers underwent the largest changes during the first year of cART, after which cell numbers declined much more gradually or even remained constant. A similar biphasic pattern was observed for total CD8+ T-cell counts in a large-scale study among treated HIV-infected individuals (13). These changes match the changes in immune activation levels that are typically observed during cART, with a major decline in immune activation upon the initiation of cART and much more subtle changes in later years of treatment (31). Of the four CD8+ T-cell populations investigated, the effector population was the only population that increased during cART to levels higher than in healthy age-matched controls. A similar gradual accumulation of highly differentiated effector T-cells has been observed in healthy aging (32), as well as in untreated HIV infection (1). In accordance with the skewing of HIV-specific CD8+ T-cells toward a CM phenotype (3, 33), we found hardly any HIV-specific CD8+ T-cells in the effector compartment when staining with HIV tetramers (data not shown). The increased cell numbers in the effector compartment are thus not likely explained by the accumulation of HIV-specific T-cells. It was previously shown that the frequency of CMV-specific effector T-cells in HIV-infected individuals on cART (with undetectable viral load) was higher than in age-matched untreated HIV-infected individuals or healthy age-matched controls and was in fact comparable to that in the elderly (34). Since the prevalence of CMV in HIV-infected individuals was nearly 100%, it is plausible that infection with CMV is the driving force behind the increase in effector CD8+ T-cell numbers during cART, as it is in healthy individuals (16). The change that is perhaps least well understood is the persistent expansion of the CM CD8+ T-cell pool in patients on cART. Consistent with earlier findings on total CD8+ T-cell counts in treated HIV patients (13), increased CM T-cell numbers were neither related to residual HIV plasma load nor to the presence of HIV-specific T-cells. We also found no indications for increased levels of proliferation or apoptosis resistance of these cells.

We here show that also in terms of proliferation, senescence, and apoptosis, the CD8+ T-cell pool of HIV-infected individuals on LT successful cART tends to normalize to levels observed in CMV+ healthy age-matched controls, perhaps with the exception of increased senescence of EM and effector CD8+ T-cells. In a previous deuterium-labeling study in HIV-infected individuals who had been successfully treated with cART for at least 1 year, we observed that the turnover of the memory T-cell populations had already nearly normalized, while the turnover of naïve CD4+ and CD8+ T-cells had not yet normalized (35). Perhaps, it is not surprising that the naïve T-cell pool, which normalized most gradually in terms of cell numbers, also took more time to normalize in terms of cellular turnover. An earlier paper by Wittkop et al. (36) reported significantly increased levels of CD8+ T-cell activation after 5 years of cART. However, in contrast to our study, the study performed by Wittkop et al. (36) was not restricted to immunological responders, which might explain the discrepancy and suggests that in immunological non-responders, immune activation may persist.

In support of our interpretation that the increased EM and effector CD8+ T-cell numbers in patients on LT cART may be a direct reflection of the CMV+ status of these individuals, a previous study showed that CD8+ T-cell numbers in HIV patients on LT cART were significantly increased in CMV+ but not in CMV− individuals (37). In line with this, CD4/CD8 T-cell ratios were found to be significantly higher in CMV+ compared to CMV− cART-treated individuals with good CD4+ T-cell reconstitution (38). In our cohort, only 2 out of 30 HIV-infected individuals were CMV−, which hampered a direct comparison between CMV+ and CMV− HIV-infected individuals.

It has previously been reported that both age and CMV have a significant effect on CD8+ T-cell numbers (16, 22). In line

with previous literature (16–19, 22, 39), EM and effector CD8+ T-cell numbers were significantly higher in CMV+ compared to CMV− healthy individuals. This expansion may for a large part be composed of CMV-specific T-cells, since CD8+ T-cells specific for the major immediate early 1 protein (IE-1) or the structural phosphoprotein pp65 have been described to occupy up to 8% of the total CD8+ T-cell pool in adults (34, 40). Based on the combined responses against IE-1, pp65, and nonstructural phosphoprotein pp50, it has been estimated that up to 45% of total CD8+ T-cells may be CMV-specific in the elderly (41). In line with previous data showing that CMV-specific CD8+ T-cells reside mainly in the effector CD8+ T-cell pool and to a lesser extent in the EM subset (3), we observed a larger expansion of effector CD8+ T-cell numbers compared to EM CD8+ T-cell numbers. Interestingly, EM and effector CD8+ T-cell numbers in very young CMV+ individuals were also significantly elevated, suggesting that cell numbers in these subsets increase quickly after CMV infection and do not take years to accumulate.

A previous report by Wertheimer et al. (22), which beautifully disentangled the effects of age and CMV in a large cohort of healthy individuals, reported no significant increase in EM and CM CD8+ T-cell numbers with age in CMV− adults. We did observe a significant—albeit mild—increase in these subsets with age in CMV− adults. In line with Wertheimer et al. (22), we found no significant difference in naïve CD8+ T-cell numbers between CMV+ and CMV− adults. By contrast, a significant reduction in absolute naïve CD8+ T-cell numbers (based on the expression of LFA-1 and CD45RA) in CMV+ compared to CMV− adults was previously reported (16). These seemingly conflicting results may be due to the fact that inter-individual differences in T-cell counts tend to be larger than the effect of age on memory T-cell counts and the effect of CMV on naïve T-cell counts, respectively.

While our data suggest that most changes in the CD8+ T-cell pool of cART-treated individuals are a direct and natural result of CMV—as also observed in healthy CMV+ individuals—another study suggested that it is the *combination* of HIV and CMV that drives persistent CD8+ T-cell expansions (37). Indeed, increased CD8+ T-cell numbers in the latter study were only found in CMV+ and not in CMV− cART-treated patients. Whether directly or *via* interaction with HIV, both Freeman et al. (2016) (37) and our data suggest that CMV is a central player in the persistent changes in the CD8+ T-cell pool during cART. We can nevertheless not exclude the possibility that other factors play a role. In fact, our own comparison of CD8+ T-cell numbers in cART-treated and CMV+ healthy individuals (**Figure 4**) suggests that the expansions in cART may be larger (although not significantly) than in CMV+ healthy individuals. Residual immune activation, which itself may be related to CMV (37), may be another driver for CD8+ T-cell expansions in (treated) HIV (12, 38).

Taken together, our findings show that the CD8+ T-cell pool has great potential to normalize in HIV-infected individuals who respond well to treatment and underline the importance of matching not only for age but also for CMV serostatus (20). In this light, also previous interpretations of changes in the composition or dynamics of the T-cell pool in HIV-infected individuals may have to be reconsidered if they did not take the effects of CMV into consideration.

ETHICS STATEMENT

All patients or their legal guardians gave written informed consent in agreement with the Declaration of Helsinki (version: 59th WMA General Assembly, Seoul, October 2008). The protocols were approved by the Medical Ethical Committee of the University Medical Center (UMC) Utrecht. Blood from healthy adult (>18 years of age) volunteers was obtained under guidelines of the Medical Ethical Committee of the UMC Utrecht or under guidelines of Sanguin (Blood Bank, The Netherlands).

AUTHOR CONTRIBUTIONS

EV, JB, and KT designed the work. EV, LW, RG, BR, HR, LB, and SO acquired and analyzed the immunological data. AW acquired and analyzed the virological data. AH and TM analyzed patient characteristics, selected and included patients. EV, JB, and KT drafted the manuscript and all authors critically revised the intellectual content of the manuscript.

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Age and Age-Related Diseases: Role of Inflammation Triggers and Cytokines

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Cytokine dysregulation is believed to play a key role in the remodeling of the immune system at older age, with evidence pointing to an inability to fine-control systemic inflammation, which seems to be a marker of unsuccessful aging. This reshaping of cytokine expression pattern, with a progressive tendency toward a pro-inflammatory phenotype has been called "inflamm-aging." Despite research there is no clear understanding about the causes of "inflamm-aging" that underpin most major age-related diseases, including atherosclerosis, diabetes, Alzheimer's disease, rheumatoid arthritis, cancer, and aging itself. While inflammation is part of the normal repair response for healing, and essential in keeping us safe from bacterial and viral infections and noxious environmental agents, not all inflammation is good. When inflammation becomes prolonged and persists, it can become damaging and destructive. Several common molecular pathways have been identified that are associated with both aging and low-grade inflammation. The age-related change in redox balance, the increase in age-related senescent cells, the senescence-associated secretory phenotype (SASP) and the decline in effective autophagy that can trigger the inflammasome, suggest that it may be possible to delay age-related diseases and aging itself by suppressing pro-inflammatory molecular mechanisms or improving the timely resolution of inflammation. Conversely there may be learning from molecular or genetic pathways from long-lived cohorts who exemplify good quality aging. Here, we will discuss some of the current ideas and highlight molecular pathways that appear to contribute to the immune imbalance and the cytokine dysregulation, which is associated with "inflammageing" or parainflammation. Evidence of these findings will be drawn from research in cardiovascular disease, cancer, neurological inflammation and rheumatoid arthritis.

Keywords: aging, age-related diseases, inflamm-aging, redox, SASP, autophagy, cytokine dysregulation, inflammation resolution

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INTRODUCTION

The inflammatory response must be tightly regulated to ensure effective immune protection. It is a dynamic network that is continuously remodeling throughout each person's life as a result of the interaction between our genes, lifestyles, and environments (1–3). Infections and tissue damage from the external environment and our personal internal response to stress can act as triggers to initiate

the inflammatory defense response. While inflammation is part of the normal repair response for healing, and essential in keeping us safe from bacterial and viral infections and noxious environmental agents, not all inflammation is good. When inflammation becomes prolonged and persists, it can become damaging and destructive (4). It is essential that inflammation is tailored to the initiating stress and resolves in a timely and controlled way, to avoid pathology associated with chronicity.

The cytokine network is a highly complex system of immune molecular messengers, with multiple layers of activation and control mediated through soluble receptors, receptor antagonists, diverse serum mediators, as well as gene polymorphisms (5). Proteomic methods measuring cytokine production and expression have demonstrated further layers of complexity and control in cytokine production and expression involving long coding RNAs, siRNAs, and miRNAs, which make for challenging interpretation of cytokine production and control in the inflammatory process (6). Many cytokines are able to act in more than one-way or paradoxically at different times and many act in feedback loops with the ability to auto-control their own production (7). Cytokine expression is also influenced by local cellular microenvironments, suggesting that multiple pathways exist to achieve homeostatic immunologic control and effectiveness, or to conversely accentuate chronic immune activation. However, what seems clear is that mirroring other body systems, the homeostatic control, titration, and modulation of immune responsiveness becomes more fragile and less tightly focused with increasing age. This loosening of the cytokine balance between the pro-inflammatory and anti-inflammatory control or resolving mechanisms, or inflamm-aging (8, 9), is a characteristic feature of both aging and aging-related diseases. This kind of inflammation is similar to that originally described as "parainflammation" by Medzhitov (10).

Today there is increasing recognition that inflammation is a common molecular pathway that underlies in part, the pathogenesis of diverse human diseases ranging from infection, to immune-mediated disorders, cardiovascular pathology, diabetes, metabolic syndrome, neurodegeneration, and cancer, to aging itself (4, 11, 12). Although there is no exact understanding about the causes of "inflamm-aging", a common finding seems to involve a dysregulation of the cytokine network and its homeostasis. Several common molecular pathways have been identified that seem to be associated with both aging and low-grade inflammation. Excess oxidative stress and DNA damage trigger the inflammasome, stimulating NF-κB and the IL-1β-mediated inflammatory cascade. Autophagy, the cell machinery process that removes damaged proteins and large aggregates, is also slowed up at older age and in age-related disease, causing damaged material to accumulate and reduce cellular efficiency. Senescent cells increase with age and in age-related diseases, and the associated secretome or senescence-associated secretory phenotype (SASP) produces a self-perpetuating intracellular signaling loop and inflammatory cascade involving the NF-κB, IL-1α, TGF-β, IL-6 pathway that participates in the pro-inflammatory milieu. The molecular processes that damp down inflammation include the resolvin family of bioactive molecules, which have been much less evaluated in aging or age-related disease, but are important participants in effective and timely inflammation resolution.

Here, we will discuss some of the current ideas and highlight molecular pathways that appear to contribute to the immune imbalance and the cytokine dysregulation, which is associated with "inflamm-aging" or parainflammation. Evidence of these findings will be drawn from research in several age-related diseases, including cardiovascular and neurodegenerative disease, rheumatoid arthritis (RA), and cancers.

THE INFLAMMATION PATHWAY TO RESOLUTION

Inflammation is classically induced when innate cells detect infection or tissue injury. The pattern-recognition receptors (PRRs) on immune cells sense "danger" from protein-associated molecular patterns (PAMPs) associated with pathogens, or from danger-associated molecular patterns (DAMPs) triggered by a wide range of host-derived endogenous stress signals. DAMPs are molecules, such as ATP, the cytokine IL-1α, uric acid, and some cytoplasmic and nuclear proteins, which are released from damaged cells during necrosis and contribute to sterile inflammation (**Figure 1**). There have been suggestions that the extended IL-1 cytokine family (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ) might also act as DAMPs and stimulate necrosis-initiated sterile inflammation, as well as amplify inflammation in response to infection-associated tissue injury (13).

Members of the toll-like receptor (TLR) family are the major PRRs. They are expressed on monocytes, macrophages, neutrophils, and dendritic cells, and on some lymphocytes and they respond rapidly to the "danger" response. The cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) pathways of arachidonic acid (AA) metabolism (14, 15) produce highly pro-inflammatory lipid mediators responsible for the classical signs of inflammation—redness, heat, pain, swelling, and loss of function, with the aim of removing the injurious and noxious stimuli. A third pathway involves the cytochrome 450 pathway of AA metabolism and P450 epoxygenases and hydroxylases that produce both vasoconstrictor and vasodilatory effects in blood vessels and other tissues (**Figure 2**). The reactive biolipid molecules synthesized from AA are; the prostanoids—prostaglandins (PGs), prostacyclins, and thromboxanes produced by the action of COX 1 and 2 (COX 1 and 2); the leukotrienes (LTs), hydroxyeicosatetraenoids (HETEs), and lipoxins (LXs) produced by the action of the 5-, 12-, and 15-lypooxygenase (5/12/15-LOX) enzymes and; the P450 epoxygenase generates HETEs and depoxyeicosatrienoids (epoxides) (16). PGs act to amplify the inflammatory response through enhancing the inflammatory cytokine cascade, upregulating the innate response to DAMPs and PAMPs, activating subsets of T helper cells, recruiting macrophages associated with chronic inflammation, and increasing cytokine expression from cytokine inflammatory genes. Additional factors, such as histamine, pro-inflammatory cytokines, and chemokines amplify the response further and make the vascular endothelium increasingly leaky. The increase in vascular permeability combined with the expression of cellular adhesion molecules (i.e., selectins and integrins)

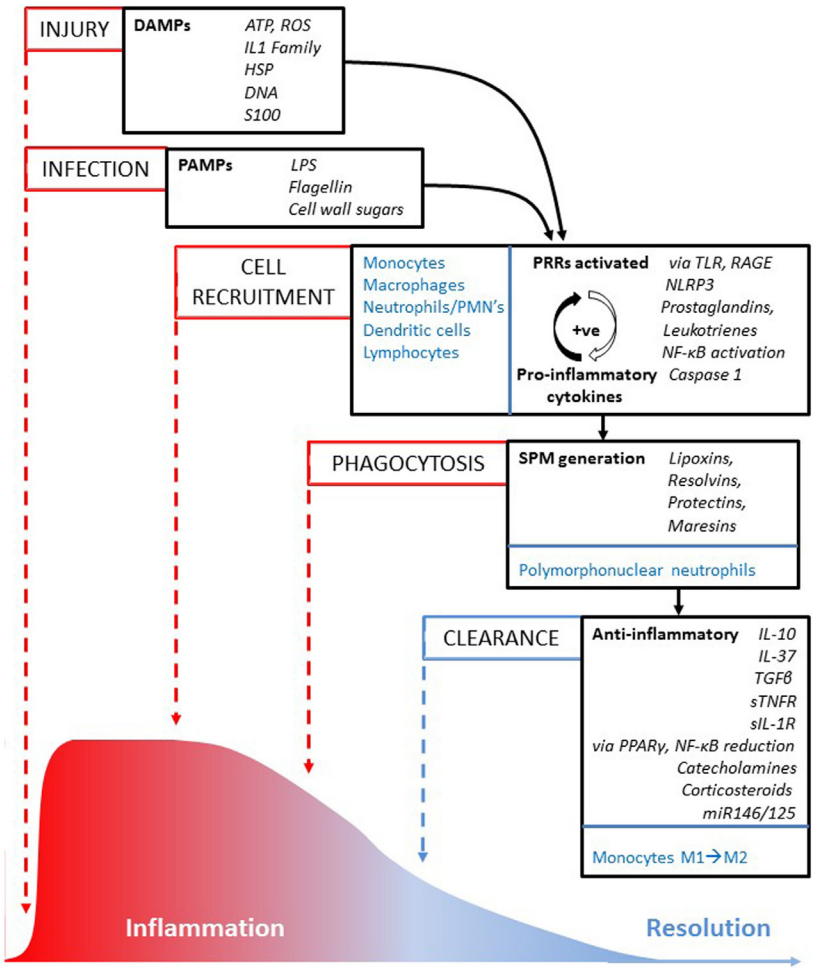

FIGURE 1 | Inflammation pathway to resolution. An illustration of the sequence of key processes (in capitalized text), cells and molecules involved in reaction to injury or infection, and how the inflammatory episode is resolved over time (from left to right). Cells from the innate and adaptive immune system that are involved in cell recruitment, phagocytosis, and clearance processes are highlighted in blue text; key molecules are in italic text.

allows neutrophils, the first responders, to transmigrate across post-capillary venules to the sites of injury or microbial invasion. Together this increases polymorphonuclear (PMN) neutrophil chemotaxis and allows PMNs to transmigrate along chemotactic gradients in order to maximize phagocytosis and killing of pathogens, and deal with the "danger" signal effectively.

As the acute inflammatory cascade develops to manage the "danger" signal, it is essential that a controlled resolution commences, so that immune homeostasis returns in an organized manner. If the inflammatory response does not shut down in a timely way, the inflammation cascade becomes chronic and smoldering. Lipid mediators derived from polyunsaturated fatty acids are now recognized to orchestrate the resolution of inflammation (17). At the peak of inflammation, the eicosanoids that initiated the inflammation undergo a class-switch so that they become the molecules that activate resolution, demonstrable through the clinical signs of removal of symptoms, relief of pain, restoration of function, regeneration of damaged tissues, and return to health. The so-called specialized pro-resolving mediators (SPMs) are key to resolving inflammation and include lipoxins derived from the 5-LOX arm of the AA pathway; the E-group of resolvins derived from dietary-derived eicosapentaenoic acid (EPA); the D-group of resolvins from dietary–derived docosahexaenoic acid (DHA); and protectins (PD), and maresins (MaR) (17–19) (**Figure 2**). The lipid class-switch starts early in inflammation and is initiated by lipoxins LXA4 and LXB4, and considered to be produced by platelets when they begin to aggregate with PMNs at the sites of inflammation (18).

After class-switching of the lipid molecules has occurred, SPMs are produced. Pro-resolving monocyte-derived macrophages begin to clear PMNs from the site of injury by a process called efferocytosis that removes apoptotic neutrophils, microbes, and necrotic debris. As resolution progresses, monocytes and macrophages, change from a pro-inflammatory (M1) to a pro-resolving phenotype (M2) by genetic and epigenetic reprogramming (20–22). Recent investigations suggest that SPMs, particularly the D-series resolvins (resolving D1 and resolving D2) and MaR 1 modulate adaptive immune responses in human peripheral blood

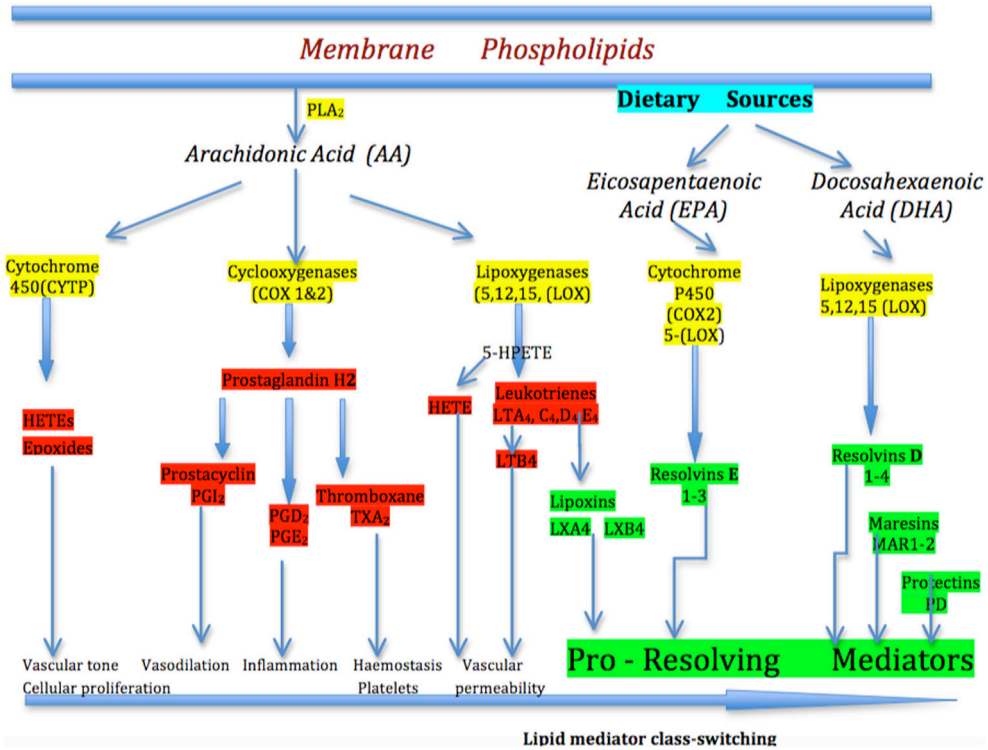

FIGURE 2 | The arachidonic acid (AA) pathway of inflammation mediators. In the simplified pathway for the eicosanoid metabolic pathway, AA is released from membrane stores by phospholipase 2 (PLA_2). AA is metabolized to biological mediators by three enzymatic pathways: cyclooxygenase, lipoxygenase, and cytochrome P450. Each pathway contains enzyme-specific steps that result in a wide variety of bioactive compounds that drive the pro-inflammatory (prostaglandins) response. After lipid mediator class-switching at the height of inflammation, the pro-resolving mediators-lipoxins begin to drive inflammation resolution. Eicosapentaenoic acid and docosahexaenoic acid-derived from dietary sources produce the E-series of resolvins and D-series of resolvins, maresins, and protectins, respectively, which are important pro-resolving mediators in progressing the resolution of inflammation.

lymphocytes. These lipid mediators reduce cytokine production by activated CD8+ T cells and CD4+ T helper 1 (TH1) and TH 17 cells, but do no modulate T cell inhibitory receptors or reduce their ability to proliferate (23, 24). Other reports show an increase in plasma cell differentiation and antibody production that supports the involvement of SPMs in the humoral response during late stages of inflammation and pathogen clearance (25). The anti-inflammatory cytokines interleukin 10 (IL-10), and IL-37 a member of the IL-1 family, together with TGF-β that is released from monocytes and platelets, are important contributors to damping down the inflammation. The soluble receptors, TNFR and IL-1 receptor (IL-1R) also limit inflammation in acting as decoy receptors, by binding to and neutralizing their respective cytokines, and inhibiting the biological activity. Additional anti-inflammatory mechanisms, include stress hormones, particularly corticosteroids and catecholamines and negative regulators, such as microRNAs—MiR-146 and MiR-125 (26).

The local environment and context also play an important role in the production and function of SPMs, which have both autocrine and paracrine actions. Inflammation resolution is likely to depend on prompt class-switching to pro-resolving lipid mediators, effective apoptosis, and efferocytic clearance of inflammatory cells and debris, timely damping down of pro-inflammatory signals and integrated repair of collateral damage. An imbalance between pro-inflammatory and pro-resolving mediators has been linked to a number of chronic inflammatory diseases (27).

In normal inflammation SPMs do not compromise host immune competence with examples of pro-resolving mediators increasing survival from infections in mouse models (28, 29). The common mechanism by which this occurs appears to be through suppression of the NF-κB activation in a partly PPAR-γ-dependent manner, with associated downstream signaling and alteration in transcriptomics pathways (30, 31). A maresin mediator has been shown to have potent anti-inflammatory and pro-resolving actions in a model of colitis, and attenuated inflammation in vascular smooth muscle and endothelial cells (32, 33). In human studies, the role of SPMs are being explored in chronic inflammatory diseases, such as RA (34), atherosclerosis (27), and cancer (35). In Alzheimer's disease, several SPMs promoted neuronal survival and β-amyloid uptake by microglia in "*in vitro*" models in Alzheimer's disease (36, 37). However, little is known about the pro-resolving mediators in aging itself. Studies are needed to assess whether pro-resolving molecules, such as E and D-resolvins, and maresins decrease or are less effective in damping down inflammation with increasing age and whether they could contribute to the pro-inflammatory phenotype associated with aging. Already synthetic analogs are in process of

development, and so the design of pharmacological mimetics of naturally occurring pro-resolving mediators and their receptors offers new potential targets for drug design and the opportunity to investigate the underpinning molecular mechanisms of inflammation resolution.

Could life-style factors play a role in the epidemic of non-communicable and age-related diseases and the associated pro-inflammatory phenotype? Evidence exists that suggests that the Mediterranean diet which includes olive oil and some omega-3 lipids, can ameliorate RA (38), may give some protection from atrial fibrillation and myocardial infarction (MI) (39), and improves diabetic control (40). Research has also demonstrated a protective role of the Mediterranean diet in gene/Mediterranean diet interactions for the risk TT allele of the TCF7L2-rs7903146 gene in stroke risk and mortality (41, 42). Improving knowledge about how inflammation shuts down in a timely way is crucial to the understanding of how chronic inflammation contributes to aging and age-related diseases. Further studies are likely to be needed to advise if dietary modifications with omega-3 lipids or whether synthetic resolving mimetics are part of the answer.

TRIGGERS OF THE INFLAMMATION PATHWAY

Several common molecular pathways have been identified that seem to be associated with both aging and low-grade inflammation. These pathways trigger the inflammasome, stimulating NF-κB, and the IL-1β-mediated inflammatory cascade.

Age-Related Redox Imbalance

A redox imbalance has long been associated with aging and led to the development of the redox stress hypothesis of aging (43). Redox stress is caused by an imbalance between unregulated and overproduced reactive oxygen species (ROS) that are produced secondary to mitochondrial energy production, active immunological phagocytic processes, and the prostaglandin pathway through COX enzyme production. While ROS are important molecules regulating numerous physiological and pathological processes in the cell, there is now clear evidence that overproduction of ROS is involved in the development of a number of diseases, such as Alzheimer's disease, rheumatoid, and cardiovascular diseases. Increasing evidence supports the notion that low concentrations of ROS or "primary ROS" are involved in well controlled processes (44), where their effect on reactive target molecules can be reversible, suggesting that "primary" ROS acts as an important intracellular signaling molecule (45). In contrast, the very active OH ROS is less effectively controlled and forms the main damaging type of ROS that is able to react with many macromolecules, such as lipids, proteins, and nucleic acids. This results in DNA oxidation and cell membrane damage, which contributes to the burden of damaged molecules related to aging and age-related diseases.

Mitochondrial ROS

Mitochondria are highly efficient producers of energy, but in doing so they produce ROS. It is estimated that about 90% of intracellular ROS is generated in the mitochondria through the mitochondrial transport chain. The chain of electron flow is considered to leak prematurely between complexes 1, 11, and 111 leading to the formation of damaging oxidants like O_2^-. This ROS has been considered to cause damaging mutations in the mitochondrial genes with increasing age (43). With increasing age, mitochondrial function becomes sluggish and this compromises energy production, which in turn further contributes to mitochondrial dysfunction (46). A vicious cycle develops with age-reduced physical activity producing muscles that become weaker, are infiltrated with fat cells, and show less efficient mitochondria energy production (47). Ischemia and apoptosis can trigger O_2^-, and mitochondria themselves can be damaged by ROS production. Mitophagy, the removal of damaged mitochondria is also reduced as age increases (48). A reduced age-related capacity of the body's anti-oxidative defense systems to mop up free radicals also plays an important role in maintaining the inflammatory background of chronic inflammation (49).

The Nicotamide Adenine Dinucleotide Phosphate (NADPH) Pathway of ROS

One of the other main producers of ROS is the specialized enzyme group of the NADPH oxidases of the NOX family—(NOX1, NOX2, NOX3 NOX4, NOX5, DUOX1, and DUOX2). The NOX family or NADPH oxidases' generate O_2^- or H_2O_2 radicals by transferring electrons from cytoplasmic NADPH or the "NOX" catalytic subunit to molecular oxygen (50). The ROS produced by these enzymes has an essential function in neutrophils and macrophages as a mechanism for effective bacterial killing and host defense (51, 52). When the phagocytes sense an endogenous or exogenous danger signal, the NADPH-oxidase unit translocates to fuse with the plasma membrane to form the phagosome. This generates large amounts of highly reactive ROS called the phagocytic burst that is very effective in killing microbes, though phagosomal pH and ion concentration are also likely to be contributors.

Although NOX family of isoenzymes was initially associated with the ROS produced in phagocytes, other members of the NOX family are now known to be involved in a wide range of regulatory functions in many tissues and seem likely to play a role in aging and age-related diseases. Studies in the human vascular system suggest that NOX1, NOX2, and NOX5 promote endothelial dysfunction, inflammation, and apoptosis in the vessel walls, whereas NOX4 by contrast is vasoprotective, by increasing nitric oxide bioavailability (53). NOX enzymes, therefore, appear to play a role in vascular pathology as well as in the maintenance of normal physiological vascular function. Activation of NOX2 and NOX4 occurs in humans with atrial fibrillation and inhibition of NOX by angiotension converting enzyme inhibitor drugs or statins has proved helpful in preventing post-operative atrial fibrillation (54).

COX Pathways of ROS

The biolipids are highly reactive substances that contribute to both inflammation and healing and their pathways produce and use ROS signaling. The reaction that converts AA through COX2 into

prostaglandin H2 (PGH_2) by a two-stage free radical mechanism (55) involves superoxide and can contribute to cellular oxidative stress as well as signaling. Other enzymes that generate ROS during AA metabolism include the arachidonic 12-lipoxygenase (LOX-12 or ALOX12) and arachidonic 5-lipoxygenase (LOX5 or ALOX5), both of which also activate and induce NADPH-oxidases (56).

While mitochondrial ROS are traditionally seen as the main source of intracellular ROS and, therefore, major mediators of ROS-induced damage, the relative contribution of mitochondrial and non-mitochondrial sources of ROS to induction of cellular senescence remain unclear. Both mitochondrial ROS and NADPH-produced ROS appear to be able to cross signal between each other and mitochondria have significant antioxidant capacity, which may act as a cellular redox buffer for NADPH-produced ROS, suggesting there is tight control and integration of ROS signaling within the cell.

The cellular systems that protect against ROS, include the antioxidative defense enzymes, superoxidase dismutase, glutathione peroxidase, and catalase (57), oxidant scavengers (vitamin E, vitamin C, carotenoids, uric acid, and polyphenols), and mechanisms to repair oxidant damage to lipids, proteins, or DNA. Despite these protective mechanisms, uncontrolled ROS can overwhelm the antioxidant capacity of the cell causing mitochondrial dysfunction (49). Increased ROS production from the various cellular sources stimulates intracellular danger-sensing multi-protein platforms called inflammasomes (58–60). Through the inflammasome, the ROS activates NF-κB which sets in motion the transcription of a cascade of pro-inflammatory cytokines—tumor necrosis factor-alpha (TNF-α), IL-1β, IL-2, and IL-6, chemokines—IL-8 and RANTES, and adhesion molecules, such as ICAM-1, VCAM, and E-selectin, that are central mediators in the inflammatory response.

Autophagy Slowing and Aging

Approximately a third of all newly synthesized proteins are formed in the endoplasmic reticulum (ER), where they are folded, modified, sorted, and transported to sites where they perform specialized roles. Stressors, such as low glucose as in fasting, alterations in calcium levels, low oxygen states, viruses, cytokines, and nutrient excess or deficiency can trigger the autophagy pathway with the aim of returning normal homeostasis to the cell.

Autophagy is a cellular process whereby cellular waste, such as modified proteins, protein aggregates, and damaged organelles are removed from the cell. It is a tightly controlled process that plays a role in growth and development and maintains a balance between the synthesis, degradation, and subsequent recycling of cellular products. Autophagy can be considered a protein and organelle quality control mechanism that maintains normal cellular homeostasis.

Two major pathways degrade cellular proteins. The ubiquitin-proteasome system (UPS) degrades 80–90% of denatured and damaged proteins. In the ATP-dependent UPS, damaged or misfolded proteins are tagged with a small protein called ubiquitin. Three different sets of enzymes—E1, E2, and E3, identify and categorize proteins in order to link ubiquitin or ubiquitin complexes to the damaged proteins. The ubiquitin-protein complexes pass through the proteasome, where they are degraded and discharged as free amino acids into the cytoplasm (**Figure 3A**).

The other main pathway is the autophagy system that degrades cystolic components, including larger aggregated proteins and cellular organelles, such as mitochondria, peroxisomes, and infectious organisms (61). This process involves membrane formation, fusion, and degradation (**Figure 3B**). When autophagy is induced, a small separate membrane structure called a phagophore arises in the cytoplasm, which gradually expands to form the autophagosome. The outer membrane of the autophagosome fuses with the lysosome and the autophagosome contents are degraded by lysosomal hydrolases (62). Like the proteasome, the macroautophagy system is stimulated by intracellular and extracellular stress-related signals, including oxidative stress. Both proteasome and autophagy produce small polypeptides that help maintain a pool of amino acids and control energy balance in starvation, since recycling amino acids is more energy efficient than *de novo* amino acid synthesis.

In aging and age-related disease there are gradual reductions of cellular repair mechanisms that lead to the accumulation of damaged molecules, proteins, DNA, and lipids leading to loss of efficient cellular function. The cell's capacity for autophagic degradation also declines with age and this in itself may contribute to the aging process (63). While both major systems for intracellular protein degradation are slowed up with increasing age, a physical reduction of autophagy-related proteins also contributes to the accumulation of misfolded proteins and damaged macromolecules in the cell. Diseases associated with increased oxidative stress, such as cardiovascular and Crohn's disease and obesity also slow up cellular clearing and reduce autophagy, further contributing to disease (64–66).

The lysosome–autophagy system carries out a wide range of non-specific intracellular degradation and cleaning processes, which include managing pathogens, damaged intra-cellular macromolecules, and surface receptors (67–69). Lysosomal dysfunction is associated with age-related pathology that reduce lifespan, such as Parkinson's and Alzheimer's diseases (70, 71). Senescent cells accumulate abnormal protein aggregates in the cytoplasm, and contribute to neurodegenerative disease (72).

The dysregulation in autophagy has important effects in the innate immune response, in aging and age-related diseases by influencing inflammasome activity, cytokine secretion, antigen presentation, and lymphocyte function (73, 74). Under normal circumstances the nod-like receptor 3 (NLRP3) inflammasome fine-tunes the progression of the innate immune response that it has initiated, by upregulating autophagy activity so that the removal of immune mediators is expedited (74). In aging and age-related diseases, the autophagy response becomes blunted, the immune mediators remain active and prolong the inflammatory response (75).

The UPS and autophagy act synergistically and cooperatively to maintain cellular homeostasis (76). Effective autophagic uptake of dysfunctional mitochondria and efficient lysosomal degradation of damaged aggregated proteins and macromolecules are crucial elements in maintaining tissue homeostasis and good health (77). The decline in the autophagy capacity, that impairs cellular housekeeping in aging, seems to be an

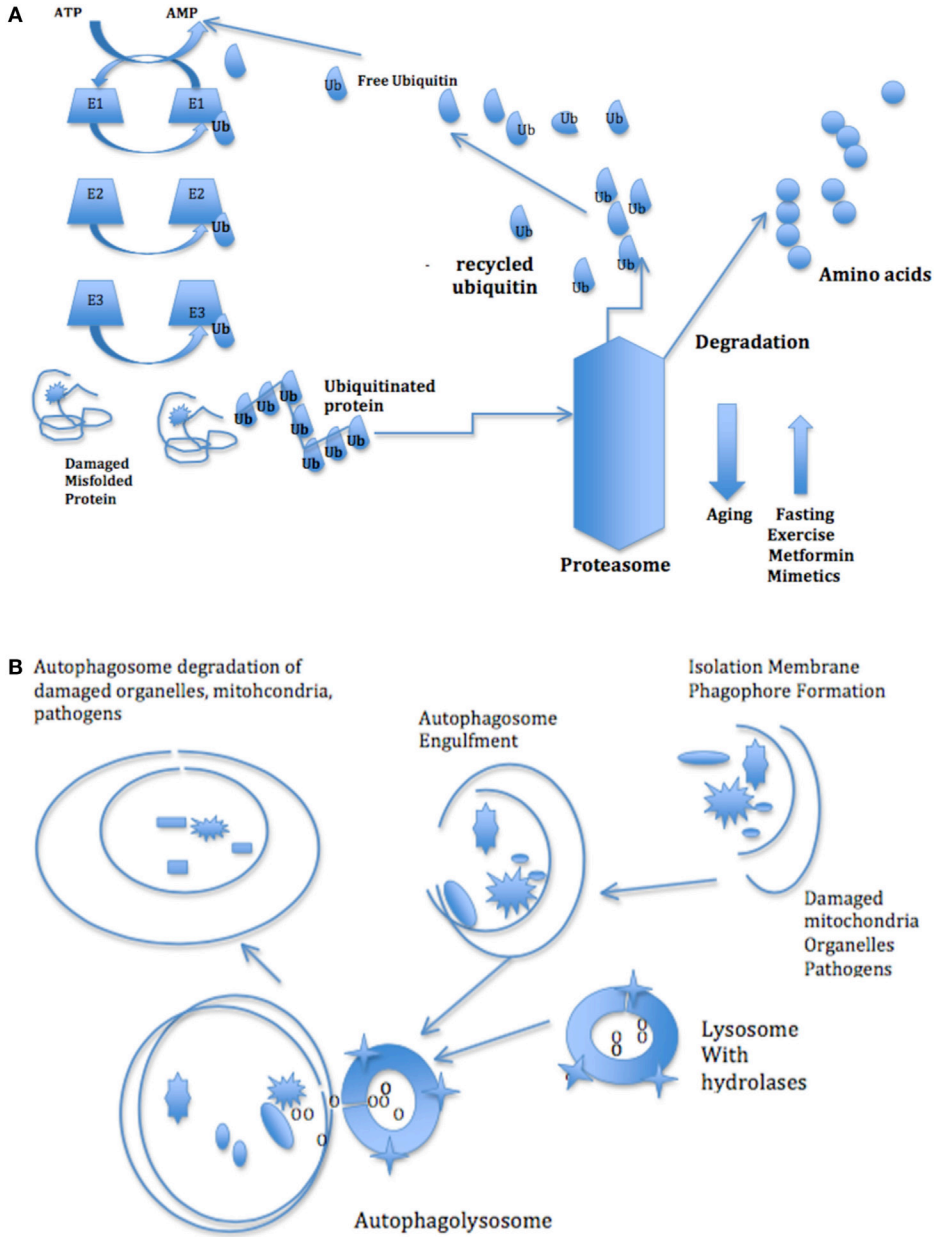

FIGURE 3 | **(A)** The ubiquitin–proteasome pathway of protein degradation. Three different sets of enzymes—E1, E2, and E3, identify and categorize proteins in order to link ubiquitin or ubiquitin complexes to the damaged proteins. The ubiquitin-protein complexes pass through the proteasome where they are degraded and discharged as free amino acids into the cytoplasm. **(B)** The autophagy pathway of degradation of damaged organelles and pathogens. The autophagy system degrades larger aggregated proteins and cellular organelles, such as mitochondria, peroxisomes, and infectious organisms. The process involves membrane formation, fusion, and degradation. A small separate membrane called a phagophore forms and then forms the autophagosome that fuses with the lysosome. The autophagosome contents are degraded by lysosomal hydrolases.

attractive molecular pathway to target to improve the quality of aging.

Two groups of drugs, the mammalian target of rapamycin (mTOR) inhibitors and AMP-activated protein kinase (AMPK) activators are promising pharmacological agents which stimulate autophagic degradation (78–80). Other drugs, such as the diabetic drug metformin and the oncology agent 5-aminimidazole-4-carboxamide ribonucleoside are pharmacological activators of AMPK, which are soon planned for clinical studies in relation to aging (81–83). A number of substances, such as curcumin, berberine, and quercetin, regularly available in normal diets, appear able to mimic the action of AMPK and upregulate autophagy. The action of AMPK has important anti-inflammatory and immunosuppressive effects (83). By upregulating autophagic activity, AMPK promotes effective clearing of DAMPs and by preventing the activation of the inflammasome, it reduces

the triggering of the inflammatory cascade. Further evidence of the anti-inflammatory role comes from research with the AMPK agonist A-769662 that mimics AMPK activity (84). This AMPK mimetic has been shown to suppress inflammatory arthritis in mice and reduce IL-6 expression in serum and arthritic joints, suggesting that targeted AMPK activation could be an effective therapeutic strategy for IL-6-dependent inflammatory arthritis (85).

Non-pharmacological life-style changes also upregulate autophagy. One of the best researched is the effect of exercise which improves mitochondrial mitogenesis and stimulates mitogeny, so improving the quality of muscle function and exercise performance, with improvement in the quality of aging (86, 87). Furthermore in animal model studies, both modulated caloric restriction and exercise increase autophagy, downregulate endotoxin-induced IL-1β production, improve the aging-related pro-inflammatory profile, and reduce disease symptoms (78, 88).

Further understanding of molecular pathways of the signaling networks underpinning autophagy should help to identify other novel drug targets. Important research areas include those that could improve the sensitivity of degradation inhibitors useful to improve anticancer treatment, or new drugs to upregulate autophagy to maintain good cellular housekeeping, with the potential for improving the quality of aging and the management of age-related degenerative diseases.

Senescent Cells

Senescent cells increase with age and are considered important contributors to the pro-inflammatory phenotype (89). The two major hallmarks of cellular senescence are an irreversible arrest of cell proliferation and production of the pro-inflammatory secretome, called the SASP. When replicative senescence was first identified in serial cell passage studies (90), telomere attrition was considered to cause the cellular growth arrest that acted as a mechanism to stop damaged or transformed cells from proliferation and transiting to tumor initiation. Today senescence is considered to have much broader role as both a contributor to damage protection and in the control of cellular growth, or as both a "friend and foe" depending on the cellular context. Senescence together with apoptosis is recognized to play an important physiological role in normal embryonic development, in ongoing tissue homeostasis throughout life (91, 92), but is increasingly considered to have a role in causing or exacerbating aging and age-related diseases (91, 93–95).

Senescence is a stress response triggered not only by telomere attrition as originally described (90, 96), but also by stress insults, such as genomic instability, DNA damage, protein misfolding and/or aggregation, and ROS. There is also an association between senescent cells and the dysregulated mitochondrial network and associated metabolic dysfunction that is seen with increasing age (97). Through the SASP, the senescent cell has an important influence on the extrinsic microenvironment, which suggests a link between senescence and alterations in intracellular and intercellular communications (93).

Cells that express senescence markers accumulate with age in some tissues in studies in mice and man (98–100). Senescent cells are found in association with age-related diseases, such as atherosclerosis, RA, neurodegenerative diseases, and cancer (101–104). In RA patients T-cells are described as showing a pre-aged phenotype with apparent loss of CD28 expression that reduces T-cell activation and this in association with reduced RA-related NK surveillance, could allow senescent cells and the associated SASP to persist. In cancer, SASP factors promote angiogenesis, cell proliferation, and cancer invasiveness. Cells attracted by SASP influence the local microenvironment with the potential to promote tumor invasion and cancer progression (105). Senescent cells have been seen in atherosclerotic plaques (101). Recent data from several laboratories has suggested that both aging and age-related neurodegenerative diseases show an increase in SASP-expressing senescent cells of non-neuronal origin in the brain, which correlated with changes in neurodegeneration (103).

The SASP consists of a complex combination of growth factors, proteases, chemokines, matrix metalloproteinases, and is particularly enriched in pro-inflammatory cytokines, especially IL-6 (106–108). The SASP-secreting cells respond by switching on a self-perpetuating intracellular pro-inflammatory signaling loop, centered around the NF-κB, TGF-β, IL-1α, IL-6 pathway (109–111), with suggested mechanisms related to higher basal phosphorylation and altered threshold signaling (112) or alternative splicing (113). Senescent cells influence other cells by paracrine and bye-stander effects (114). There appears to be multi-level control of senescence and the SASP secretome, which includes the tumor suppressor pathways involved in the cell cycle arrest and the NF-κB and persistent damage response (DAMP) pathway, involved in triggering transcription of the SASP-related factors (115). Several pathways of investigation suggest that senescent primary human CD8+ T cells use anaerobic glycolysis to generate energy for effector functions and that p38 mitogen-activated protein kinase (p38 MAPK) blockade may reverse senescence *via* the mTOR-independent pathway (116). Low doses of glucocorticoid suppress elements of the SASP in patients with RA and improve clinical symptoms (117). Senescent cells effectively recruit the immune system to organize their removal, but with increasing age, removal becomes sluggish or otherwise impaired (118, 119).

It can be argued that the increase in senescent cells with aging reflects either an increase in their rate of generation or a decrease in their rate of clearance because the immune response is attenuated or weakened with aging and less capable of clearing senescent cells (120–122). Senescent cells express ligands for cytotoxic immune cells, such as natural killer (NK) cells, and have been shown to be able to be specifically eliminated by the immune system (123, 124). Through a proteomics analysis of senescent cell chromatin, the NF-κB pathway appeared to act as a master regulator of the SASP, with NF-κB suppression causing escape from immune recognition by NK cells (125). Other studies show that processes which eliminate senescent cells with p16(Ink4a)-positive markers, delay age-related pathologies in the mouse model of aging though side-effects can be problematical (126, 127). Therapies that specifically recognize and trigger the elimination of senescent cells would seem important to enhance the immune system in older people. New methods are in the process of being developed to enhance the immune clearance and autophagy of the increased senescent cell burden in aging and age-related disease (128).

Inflammasome NLRP3

The inflammasomes, intra-cellular multiprotein sensors that recognize danger signals, are likely key players in initiating and maintaining the pro-inflammatory phenotype found associated with aging. The NLRP3 is a major inflammasome sensor for intracellular stress molecules called DAMPs, which together with damaged aggregated proteins that are released from destabilized lysosomes and damaged mitochondria contribute to the cellular stress (ROS) and trigger NLRP3 activation (129). Once activated, the NLRP3 inflammasome initiates the inflammatory response cascade by stimulating caspase-1 (casp-1) that acts to induce the active precursors of pro-inflammatory cytokines, such as IL-1β, IL-1α, and IL-18, and on-going interaction with NF-κB (130, 131) (**Figure 4**). Although the baseline activity of NLRP3 is low, the initiation process of the inflammatory cascade requires a complex oligomerization-priming phase that includes association with NF-κB and so contributes several layers of regulatory control.

Nod-like receptor 3 has been shown to be able to activate NF-κB and induce cytokines in response to sterile signals, such as monosodium urate crystals and aluminum adjuvant, suggesting that NLRP3 could initiate NF-κB activation to both pathogen-induced and sterile inflammation (132). Conversely NF-κB, which primes the NLPR3 inflammasome for activation also prevents excessive inflammation and restrains NLRP3 activation by enhancing the NF-κB-p62 mitophagy pathway. By self-limiting the host response, the NF-κB-p62 mitophagy pathway maintains homeostasis which under normal conditions leads to tissue repair (75). It is, however, unclear if this layer of control of NF-κB function remains as tightly controlled in aging and age-related disease.

The NLRP3 inflammasome is a key component of the innate inflammatory response to pathogenic infection and tissue damage. It responds to a wide range of cellular stress and is considered to contribute to the aging process and to age-related diseases (133). Zhou and colleagues identified that mitochondrial ROS was involved in the activation of NLRP3 (58). This study emphasized the important role of mitochondria in maintaining a correct balance between cellular energy production and ROS production and that effective clearance of damaged mitochondria through autophagy was an important regulatory activity. Damaged mitochondria increase with aging and age-related diseases (134). Mitochondrial dysfunction drives mitochondrial mutagenesis, affecting respiratory chain genes, and compromising the efficiency of oxidative phosphorylation, which may lead to further mt-DNA mutations and more cell damage. The subsequent mitochondrial impairment leads to more ROS that further reduce ATP generation and increases the chance of cell death. Mitochondria have been identified as a key source of DAMPs, the so-called mito-DAMPs, which have been considered to play a role in DAMPS-modulated inflammation in diseases, such as RA, cancer, and heart disease (135–138) as well as in the aging process (139). Degraded mt-DNA has also been reported in neuroinflammation (140). Dysfunctional mitochondria seem to be able to initiate an auto-feedback loop to increase autophagy, so that damaged mitochondria or misfolded proteins are degraded which reduces inflammasome activation and risk of further tissue injury, though this system is less efficient in aging (141).

Lyosomal destabilization is also associated with NLRP3 activation and can be induced by a number of molecules, including cholesterol crystals in macrophages linking atherosclerosis progression with inflammation (142). There is deposition of other harmful intra- and extracellular material in several age-related diseases. The aggregates compromise cellular homeostasis and can provoke the activation of the NLRP3 inflammasome. Research has shown that amyloid fibrils and Alzheimer's amyloid-β can trigger NLRP3 inflammasomes and in that way stimulate inflammation and enhance pathogenesis and association between type 2 diabetes and Alzheimer's disease, respectively (143). Palmitate, a saturated fatty acid has been shown to activate NLPR3, whereas oleic acid did not initiate the same inflammatory response (144). The inflammasome has been implicated in the development of the metabolic syndrome through impairment of adipose tissue sensitivity. Evidence showed that obesity triggered NLRP3 activation, and that the secreted IL-1β impaired insulin signaling which promoted insulin resistance in mice (145). Other research has shown that obesity was associated with the activation of the NLRP3 in adipose tissues (146, 147).

A number of intracellular processes seem likely to work together to stimulate and augment the inflammasome pathway and contribute to pro-inflammatory cytokine upregulation associated with increased age and age-related diseases. Both the redox-sensitive inflammatory pathway and the senescent cell-related SASP activate the inflammasome through the NF-κB and IL-α cascade, causing persistence of the inflammatory response that delays resolution and healing (125, 138). Similarly, reduced autophagy processes allow the accumulation of damaged intracellular proteins and senescent cells that further perpetuate and amplify the pro-inflammatory milieu that is found with increased age and is associated with age-related diseases.

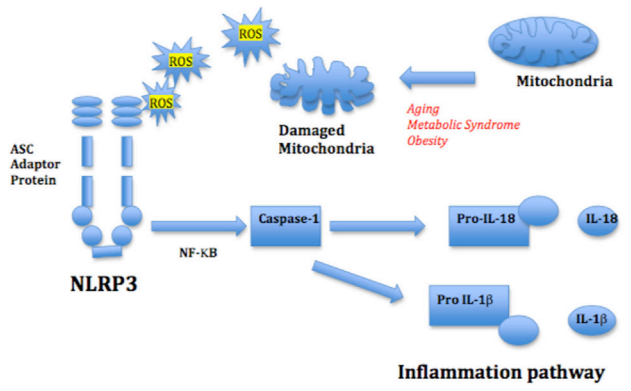

FIGURE 4 | Mitochondrial reactive oxygen species (ROS) and nod-like receptor 3 (NLRP3) activation of inflammation pathway. Mitochondrial ROS from damaged mitochondria triggers the inflammasome NLRP3, stimulating NF-κB and the IL-1β and IL-18-mediated inflammatory cascade. The adapter protein ASC mediates innate signaling by bridging the interaction between the damage recognition receptor and the NF-κB caspase-1 inflammasome complex.

PRO-INFLAMMATORY AND ANTI-INFLAMMATORY CYTOKINE DYSREGULATION

Pro-Inflammatory Cytokines in Aging and Age-Related Disease

Various biomarkers and biochemical indices are used in medicine and age-related diseases as a way of improving diagnosis, beyond the well-recognized clinical signs. Modest increases in concentration of C-reactive protein, a circulating marker of inflammation, have been widely reported to be associated with a large number of age-related conditions and lifestyles felt to be associated with poor health; these conditions represent or reflect minor metabolic stresses. Alongside C-reactive proteins, cytokines have come under investigation as the molecular processes and pathways underpinning inflammation have become better identified. A common finding in aging and age-related diseases is "inflamm-aging," a dysregulation of the cytokine network and its homeostasis. Downstream from NF-κB signaling, the pro-inflammatory cytokines play a central role in the remodeling of the immune system with age (**Figure 5**).

The major pro-inflammatory cytokines, such as IL-6, TNF-α, and IL-1α contribute significantly to the phenomenon of inflamm-aging in healthy elderly individuals (8), while also playing a major role in many age-related diseases (11, 27, 148–151). The key to healthy aging must lie in the ability to maintain a balanced response to these immune messengers and a prompt and integrated return to inflammation resolution and immune homeostasis (17). A summary of the changes that have been described in pro-inflammatory and anti-inflammatory cytokines in aging and some age-related diseases are outlined in this section.

Interleukin-1 (IL-1) Family

IL-1α and IL-1β, known as IL-1, and IL-18 are important cytokine initiators of the stress-induced inflammatory cascade (152). IL-1β and IL-18 are cleaved to active forms by Casp-1, whereas IL-1α is activated by calpain protease. All bind to and activate the IL-1R that is downregulated by the receptor anatagonist IL-1Rα, which blocks IL-1-mediated signal transduction.

Studies in elderly people, including centenarians have reported an age-related rise in the IL-1R antagonist, (IL-1Rα), whereas IL-1β showed no detectable age-related trend. The age-related rise is associated with increased co-morbidity, age-related disease, and mortality (153–156).

Certain IL-1 haplotype-carriers produce increased IL-1β, and IL-1 gene variations associate with earlier onset or more severe progression of cardiovascular and Alzheimer's disease, but not with osteoporosis (157–161). In centenarians, no single IL-1 gene polymorphism showed a survival advantage, but in Swedish elderly males an IL-1 gene polymorphism shortened life expectancy (153, 162, 163). IL-1 gene variants appear to increase the risk of age-related diseases and recombinant drugs, such as IL-1Rα-blockers may have a role in the clinical control of inflammation (164).

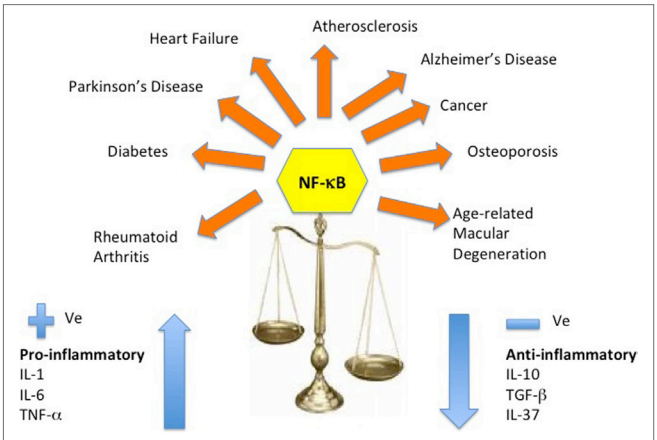

FIGURE 5 | Cytokine dysregulation and NF-κB inflammation pathway. This reshaping of cytokine expression pattern with a progressive tendency toward a pro-inflammatory phenotype has been called "inflamm-aging" and is found associated with age-related diseases. Several molecular pathways have been identified that trigger the inflammasome and stimulate the NF-κB and the IL-1β-mediated inflammatory cascade of cytokines.

Interleukin-18 (IL-18)

Interleukin-18, a linked IL-1 pro-inflammatory cytokine, signals in a complex with IL-18 receptors α (Rα) and β (Rβ) chains and induces IFN-γ that is essential for defense against infections (165). IL-18's multiple pro-inflammatory effects are modulated through IL-18 binding protein (166).

Higher levels of IL-18 have been found in centenarians, associated with heart failure, ischemic heart disease, and type 1 diabetes in patients, and in the Alzheimer's disease brain (167–172). IL-18 levels associate with physical functioning and with a frailty index in the English longitudinal study of aging, where carriers of IL-18 gene polymorphism that reduced IL-18 levels, showed improved walking speed (173–175). Evidence consistently shows that IL-1 and IL-18 are mediators of inflammation and associated with the aging process (168). Drugs blocking binding between IL-18 and the receptors are currently in development and may provide benefit in the treatment in diabetes, macular degeneration, and autoimmune disease (176).

Interleukin-6 (IL-6)

Interleukin-6 has been long recognized as important in aging and age-related disease and has been called the "gerontologist's cytokine" (177, 178). IL-6 plays a key role in the acute phase response, in the transition from innate to acquired immunity, in metabolic control, and in the pathogenesis many chronic diseases (11, 148–151, 179). It has both pro- and anti-inflammatory activities, and modulates the acute inflammatory response by producing IL-1 Rα and soluble tumor necrosis factor receptor p55 (sTNF-R55), which suppresses TNF-α and IL-1.

Interleukin-6 is normally present in low levels in the blood, but is increased in aging and in subjects with markers of frailty and chronic disease, where it tracks with mortality (180–183). IL-6 is a risk factor associated with cardiovascular disease and is associated with sarcopenia and muscle loss (184, 185).

The G allele of IL-6-174C/G polymorphism shows higher IL-6 levels and associates with cognitive decline and mortality in age-related vascular disease, whereas CC allele carriers show decreased

Alzheimer's risk (186–191). In a meta-analysis of longevity in a large cohort of European nonagenarians and centenarians there was longevity benefit for carriers of the lower cytokine producing IL-6 allele, with similar supporting findings for this IL-6 allele in a case control study (192, 193). IL-6 or IL-6 receptor blockers are already used successfully in the treatment of RA, and are proof of concept that damping down IL-6, a product of the NF-κB pro-inflammatory cascade, can improve clinical symptoms. Studies are either in progress or planned to assess the outcome of blocking IL-6-related inflammation in other age-related diseases with the potential for contributing to more successful aging (194, 195).

Tumor Necrosis Factor Alpha

Another major player in the immune response is the pro-inflammatory cytokine TNF-α, which increases with age and is associated with age-related disease (196). It is a pro-inflammatory mediator that can be beneficial when it acts locally in the tissues, but can be highly harmful when released systemically.

Tumor necrosis factor-α has been reported to be increased in intracellular aging studies in elderly people, in centenarians and octogenarians with atherosclerosis, and associated with mortality (197–202). In post-MI patients, a rise in TNF-α increased risk of recurrent cardiac events and in renal patients TNF-α receptors predicted cardiovascular disease (203–205). In genetic studies, the A allele of TNF-α 308 G/A gene associated with risk for MI, whereas TNF-α polymorphisms and TNF-α itself, have been variably associated with increased Alzheimer's disease risk (206–210). TNF-α mediates metabolic changes and increased TNF-α was found in type 2 diabetes mellitus and was associated with lower muscle mass and strength in older groups (211).

In studies in nonagenarian/centenarian groups from three European countries, there was no attrition of the TNF-α-308 A/G polymorphism in centenarians (162, 212, 213). With increasing evidence of an association between increases in TNF-α and age-related diseases, research re-purposing anti-inflammatory drugs are under development. Research has demonstrated that TNF-α inhibitors may have possible prophylactic or ameliorating roles in cardiovascular and Alzheimer's disease in animal models (214, 215).

Other Pro-Inflammatory Cytokines

Other pro-inflammatory cytokines are increasingly being recognized as dysregulated in association with aging and age-related disease.

Interleukin-2

Interleukin-2 plays a pivotal role in the immune response. It is a growth factor that promotes NK cell activity and the differentiation of naïve T cells into Th1 and Th2 cells (216). Conversely, IL-2, acting *via* STAT5 pathway negatively regulates interleukin 17 (IL-17) production (217). Most studies show that lymphocytes in elderly people produce significantly less IL-2, compared to young people (218–220). Intracellular cytokine studies have shown variable results for IL-2, whereas mitogen-induced stimulation of mononuclear cells from elderly subjects showed significant decreases in IL-2 and IFNγ production (197, 221).

The IL-7/IL-7R

The IL-7/IL-7R network is essential at various stages in T-cell development and survival (222). It has an important role in the maintenance of a vigorous health span and higher IL7R gene expression is associated with long life (223–225). Serum IL-7 is increased in some age-related diseases, including osteoarthritis and genetic variation in the IL7RA/IL7 pathway increased susceptibility to multiple sclerosis (226, 227). Research has suggested that silencing of the IL-7R gene may be an important mechanism underpinning an aging-related loss of binding to NK-κB (228), linking IL-7R gene to the NF-κB pathway and inflammation control.

Interleukin-12

Interleukin-12, a pro-inflammatory member of the IL-6 family has an active role in the development of cardiovascular diseases, such as atherosclerosis, MI, and stroke (229). Patients with cardiovascular disease show increased levels of IL-12, 23, and 27 with higher IL-12 predicting poorer long-term outcome after acute MI (230). Other research shows variable results for IL-12 and its receptor antagonist, with increased IL-12 (total) and IL-12p40 in apparently healthy nonagenarians, lower IL-12p70 and IL-23 production in association with frailty and IL-12/23p40 ameliorating Alzheimer's disease in animal models (231–233).

Interleukin 17

Interleukin 17 is a key pro-inflammatory cytokine that belongs to a family of six cytokine members (A–F). IL-17A (referred to as IL-17) plays a central role in host defense against invading pathogens and is produced by a subset of CD4+ cells (234, 235). Elderly people (age ≥65) have shown a decreased frequency of IL-17-producing cells in memory subset of CD4+ T cells compared to healthy younger people (236). IL-17 enhances production of IL-6, TNF-α, the acute phase reactants, C-reactive protein, and serum amyloid A and activates the induction of IL-6, IL-8, and G-CSF in non-immune cells, such as fibroblasts and epithelial cells, in part through activation of the NF-κB transcription factor (237). IL-17 promotes inflammation and is overexpressed in many autoimmune diseases, such as RA, systemic lupus erythematosus, inflammatory bowel disease, and psoriasis and its effects are stabilized by IL-23 (238–241). An IL-17 expressing CD8+ T subset of cells has also been reported to be involved in psoriatic arthritis and some other autoimmune diseases (242, 243).

Interleukin-8

Interleukin-8 (or CXCL8) is a chemokine secreted by monocyte/macrophages whose key role in the inflammation process is the recruitment and activation of neutrophils. IL-8 has been implicated in a number of inflammatory conditions, such as cystic fibrosis, asthma, chronic pulmonary disease, inflammatory bowel disease, and some autoimmune diseases, including RA and psoriasis.

Increased levels of IL-8 have been detected after LPS-stimulation of leukocytes from elderly individuals (244). In one small study of centenarians, IL-8 was proposed as a possible longevity factor (245). A single study of IL-8 polymorphisms found no significant difference in IL-8 -251 A/T polymorphisms in nonagenarians compared to young controls (212). IL-8 signaling occurs *via* the MAPK and PI3K pathways, by binding to the IL-8 receptors-CXCR1/2. Several agents that block IL-8-CXCR1/2 signaling have been developed in an attempt to target inflammatory pathways in cancer, asthma, chronic obstructive pulmonary disease, psoriasis, and RA (246).

Anti-Inflammatory Cytokines in Aging and Age-Related Disease

The anti-inflammatory cytokines play a key role in balancing the immune response, and in preventing the tipping of the steady state of immune homeostasis across into inflamm-aging and a disease-inducing state. Anti-inflammatory cytokines are an important arm of inflammation resolution. They block or modulate the synthesis of IL-1α, TNF, and other major pro-inflammatory cytokines and damp down the inflammatory response, so that inflammation resolution can begin. Specific cytokine receptors for IL-1, TNF-α, and IL-18, together with soluble receptor antagonists, chemokines, microRNA, siRNAs, also function as inhibitors for pro-inflammatory cytokines. The anti-inflammatory cytokines and families of soluble receptor antagonists work within a complex network of control of immune regulation. They are critical for balancing the inflammatory outcome and together with pro-resolving lipoxins are critical to resolving inflammation in an integrated and organized manner.

As age increases and in age-related diseases, a chronic inflammatory state predominates, which is not properly contained or resolved and the anti-inflammatory side of the immune system seems to be similarly dysregulated, and unable to damp down the inflammatory episode in a timely effective manner. The following cytokines are the major players in the anti-inflammatory pathway of the control of inflammation and changes in their production and expression have been quite widely reported in aging and age-related disease. Where increases in anti-inflammatory cytokines have been reported, one interpretation would be that increases might reflect the immune system's attempt to suppress the persistent pro-inflammatory response and support a return to immune homeostasis.

IL-10 Family

Interleukin 10 is one of the key anti-inflammatory cytokines, which suppresses the actions of IL-6, TNF-α, and IL-8 (247, 248). Higher IL-10 serum levels and production by both lymphocytes and monocytes have been reported in elderly people (155, 244, 249). Conversely an age and gender-related decline in cellular stimulation studies has been reported (250).

In age-related disease, IL-10 has been reported to be associated with vascular protection in atherosclerosis and improved endothelial dysfunction (251–253). However, at variance, the authors from the ERA (254) and PROSPER (255) studies, concluded that elevated IL-10 increased cardiovascular risk among elderly groups, and suggested that IL-10 blockers merited investigation. In male Sicilian centenarians, male carriers of the high producing GG 1,082 allele of the IL-10 promoter polymorphism showed a survival advantage, suggesting that IL-10 anti-inflammatory activities might be a marker for male longevity (213). This result was not replicated in Sardinian, Irish, or Finnish nonagenarian/centenarians (162, 212, 256). It has been argued that an enhanced anti-inflammatory phenotype could be beneficial and contribute to longevity by controlling the pro-inflammatory milieu that predominates in later life and contributes to increased morbidity and mortality (9, 11, 257).

TGF-β

TGF-β, another important anti-inflammatory cytokine limits both the acute phase response, and is involved in tissue repair post-damage or infection (258). Several authors have reported that TGF-β was increased in octogenarians and centenarians (148, 259). It is also involved in aging-related disease, such as in obesity, in vascular wall integrity, in muscle loss and sarcopenia, in osteoarthritis, and with frailty in the Newcastle longitudinal study (260–264). In stroke, TGF-β signaling was increased in microglia and macrophages suggesting that increased TGF-β likely regulated glial scar formation (265). Reports have linked TGF-β or its polymorphisms with atherosclerosis and Alzheimer's disease (266–268). Other research found TGF-β genotypes associated with longevity in Italian centenarians, a finding not replicated in BELFAST nonagenarians (212, 269). Context-specific environmental factors, epigenetic regulation, and non-coding RNAs are suggested to play a role in TGF-β's paradoxical pro-and anti-inflammatory functions (7, 270, 271), but important uses have been found for TGF-β in fibrosis management and oncology (272).

Interleukin-37

Interleukin-37, formerly an IL-1 cytokine, limits innate inflammation *via* suppression of pro-inflammatory cytokine production (273). Carriage of an IL-37 haplotype that decreases IL-37 levels contributes to increased inflammation. Research demonstrates that IL-37 reduces TNF-α and IL-1β cytokine production from human macrophages, is increased in chronic heart failure patients and attenuated the production of inflammatory cytokines in serum or synovial joints in RA, suggesting IL-37 may have a role in clinical disease (274–276).

AGE-RELATED DISEASES

Cancer

Cancer increases with aging, with one in two people likely to develop malignant tumors in their lifetime. Probable reasons for this age-related increase include exposure to environmental toxins, declining immune surveillance, and increasingly ineffective DNA repair mechanisms. Inflammation is involved at different stages of tumor development, at initiation, promotion, malignant conversion, invasion, and metastasis, has a paracrine bystander role and is an essential part of the tumor micro-environment. Inflammation also affects immune surveillance and responses to

therapy (277). Thus, malignancy is a major threat to successful aging.

While inflammatory pathways are vital to promote immune homeostasis, over-activation or dysregulation can be pathological and lead to malignant progression. Prolonged inflammation, either as a result of chronic infections, or reduced homeostasis in the inflammatory response, plays a role through the production of pro-inflammatory cytokines that may be directly or indirectly implicated in the oncogenesis (278, 279). More recent investigations have focused on the role of the inflammasone pathway, whose biochemical function is to activate casp-1, which leads to the activation of the IL-1β and IL-18 pathways and induction of pyroptosis, a form of cell death. Although inflammasomes have an important role in inhibiting cancer, through the triggering of the programmed-death pathway, they both initiate and maintain carcinogenesis, dependent on tumor type and the tumor environment (280, 281).

Bacterial and viral infections are associated with malignancies. For example, *Helicobacter pylori* (*H. pylori*) infection of the gut is associated with both gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma (282). Epstein–Barr virus (EBV) is a causative agent in Hodgkin's disease (HD), where chronic inflammation is considered a major contributory factor (283), human papilloma virus is implicated in most cases of cervical cancer (284), while human T-lymphotrophic virus 1 (HTLV-1) is a causative agent in adult T-cell leukemia lymphoma (285). A common factor is the association of infection with oncogenesis, with chronic inflammation a contributory factor.

In *H. pylori* chronic infection, elevated levels of IL-1β are detected and recognized as important in the development of gastric carcinoma. Normally gastric acid in the stomach does not permit bacterial survival, but in circumstances of low stomach acidity, *H. pylori* grow vigorously in the mucosa and induces caspase-mediated cleavage of pro-IL-1β and pro-IL-18 in association with the NLRP3 inflammasome. The overexpression of IL-1β induces NF-κB activation and the transcription and expression of IL-6, TNF-α, and IL-10. The proinflammatory cytokine milieu increases the risk for developing both gastric carcinoma and MALT lymphoma (286). Persistently high levels of IL-1β and IL-18 suppress acid secretion, allow hypoacidity in the stomach, loss of parietal cells, gastric atrophy, metaplasia, and eventually gastric cancer. In addition, IL-1β inhibits gastric acid secretion and carriers of IL-1β polymorphisms producing higher IL-1β carry increased gastric cancer risk (287, 288). *H. pylori* infection of gastric mucosa can cause a monoclonal B cell proliferation, with a histological diagnosis of MALT lymphoma. This tumor-like proliferation of gastric mucosal cells and clonal B cells can regress after eradication of the *H. pylori* infection with combined antibiotic therapy and proton pump inhibitor treatment (289).

Viral infections strongly stimulate inflammatory responses and may lead to malignant transformation of the host cell (290). Although the activation of the inflammasome benefits the clearance of viruses and the regression of cancer, there are several examples of viruses, such as EBV and HTLV-1 developing strategies to evade detection, triggering the inflammasome, and high-jacking the inflammatory cascade to induce, and amplify the cancer spread. For example, when EBV infects B-lymphocytes and nasopharyngeal cells through its receptor CD21 (291), this leads to a proliferation of infected B cells, followed by an increase in CD8+ T cells, that controls the infected cells by lysis. However, where the normal infection-limiting response is "exhausted" or dysregulated, B cell proliferation continues unabated leading to chromosomal damage, which drives cell proliferation outside normal control mechanisms and may result in an aggressive non-Hodgkin's or Burkitt's lymphoma (292). NLRP3 activation has been demonstrated in EBV-associated cancerous tissues (293). Furthermore, EBV has been shown to be able to overcome the immune response by means of EBV miRNA binding to the 3′-untranslated region of NLRP3 (294), so preventing effective immune activation and control mechanisms.

Retro-viruses stimulate inflammatory responses and are associated with malignant transformation of host cells. They reverse transcribe their RNA into the host cell's DNA, leading to dysregulation of cellular proliferation and programmed cell death responses, and elicit a pro-inflammatory response. HTLV-1 causes adult T-cell leukemia by targeting CD4+ T cells that express CD25 (IL-2Rα) and FoxP3, similar to Tregs (295, 296). The persistent activation of the NF-κB pathway in HTLV-1-infected T cells and the associated NF-κB oncoprotein Tax contribute to the oncogenic transformation (297). The resulting hijacking of the NF-κB pathway, allows uncontrolled upregulation of cellular genes that govern growth-signal transduction, amplify the pro-inflammatory cytokines (IL-2, IL-6, IL-15, TNF), together with increasing expression of proto-oncogenes (c-Myc), and antiapoptotic proteins (bcl-xl) Hiscott Rayet (298, 299). Inter-individual susceptibility to HTLV-1 infection has been associated with allele carrier status of the NLRP3 gene (300).

In summary, the interaction of infective agents, host cells, adaptive immune cells, cytokine production, and the inflammasome response is complex and incompletely understood. Many cancers arise from sites of infection, chronic irritation, and inflammation, which although sometimes reversible in the pre-malignant phase by eradicating the causative virus or bacterium, often treatments are too delayed to prevent the cancer development. There needs to be improved understanding about the roles of inflammation, the inflammatory cells, and the paracrine effects that allow tumor cell proliferation, survival, and migration. Does the pro-inflammatory environment found in aging enhance and facilitate cancer cell proliferation or does it alternatively represent an upregulated immune surveillance mechanism to deal with increased damaged and dangerous cancer cells? Improved understanding of the pathways involved should begin to provide insights that could contribute to new anticancer and anti-inflammatory therapeutic approaches through manipulation of autophagy for cancer treatment regimes or conversely tagging cancer cells for destruction through proteasome or autophagy upregulation (301).

Rheumatoid Arthritis

Chronic tissue inflammation has an important role in the etiology and immunopathogenesis of RA (302), with genetic and environmental factors contributing to a predilection to develop the disease. In the *pre-clinical* asymptomatic phase of RA disease, the immune system is characterized by reduced self-tolerance and production of autoantibodies, whereas in the *clinical* phase (303)

innate and adaptive immune cells infiltrate the synovial joints and produce symptoms of joint pain and stiffness (304, 305). As RA progresses, immune cells and synovial fibroblasts produce a pro-inflammatory environment in the joint (306, 307) leading to joint destruction (302). Cell-specific cytokines, include TNF-α, IL-1, and IL-6 from macrophages, IL-6, IL-7, and IL-15 from memory T-cells, IL-1 and IL-17 from helper T-cells, and IL-1, IL-6, IL-18, GM-CSF, and TGF-β from synovial fibroblasts (303, 308). This complex cytokine milieu attracts further immune cells, promotes abnormal angiogenesis and osteoclastogenesis, poorly formed leaky vasculature and leads to systemic effects (309).

There is evidence to suggest that activation of the NLRP3-inflammasome contributes to the inflammatory processes in RA. Active RA subjects have increased expression of NLRP3 and NLRP3-mediated IL-18 secretion in whole blood upon stimulation *via* TLR3 and TLR4, but not TLR2 receptors (310, 311). Functional polymorphisms in the genes coding for NLRP3 and its component parts, including CARD8 has been shown to contribute to higher disease activity at diagnosis and for response in the early months of treatment (312, 313).

Patients with RA show premature immune aging and accumulation of $CD28^-$ pre-aged effector T cells that associate with disease activation and prognosis (314, 315). A novel T-cell subset $CD28^-$ Treg-like cell has been described that produce pro-inflammatory cytokines, mirroring the SASP associated with senescent cells (316). RA patients who show $CD28^-$ senescent Treg-like cells in blood seem to demonstrate earlier and more severe osteoporosis (317).

Limiting inflammation before damage occurs is central to successful RA management and the use of specific monoclonal antibodies has been a key therapeutic strategy. The central roles of TNF and IL-6 in RA have been corroborated by clinical trials of biologic drugs, which can specifically target and neutralize these cytokines. Evidence from RA clinical subgroups stratified by responses to specific biologic drugs strongly suggest that for a particular individual, inflammation is coordinated by a predominant cytokine pathway, such as TNF or IL-6 (318).

Anti-TNF biologics, such as adalimumab, etanercept, and infliximab reduce inflammation, pain, neovascularization, lymphocyte infiltration, and increase macrophage apoptosis (318–321). Anti-IL-6R biologics, such as tociluzimab and anti-IL-6, such as sirukumab, strongly reduce disease activity and erosive progression (322, 323). Evidence suggests that the predominant cell cytokines seen in synovial histopathology may act as prognostic biomarkers for stratification of RA patients (324–326).

Studies of TNF and IL-6 gene polymorphisms further support their role in RA risk and severity. SNPs in IL-6 and IL-6R genes associate with increased RA risk and joint damage (327–329), and the TNF 308 G gene polymorphism with RA disease severity and poor response to anti-TNF treatment (330–334). In the elderly person with RA, there is difficulty in distinguishing whether chronic inflammation or genetic "predisposition" initiates disease or if late-onset RA is hastened by the pro-inflammatory phenotype associated with aging. TNF-α inhibitors used as disease-modifying agents in RA improve not only the clinical symptoms of RA, but also decrease the associated vascular risk (335), suggesting that a stratified biologic approach may be of use to therapeutically dampen chronic systemic inflammation related to aging and other age-related diseases.

Like other age-related diseases and aging itself, there is evidence for dysregulation in both the autophagy–lysosomal and the ubiquitin–proteasomal systems in RA (102). Autophagy seems to be activated in RA in a TNFα-dependent manner and regulates osteoclast differentiation and bone resorption, emphasizing a central role for autophagy in joint destruction (336). Gene and allele frequency population differences seem also to contribute to how effectively cellular autophagy processes work within the cell in removing damaged proteins and other necrotic cellular debris. Polymorphisms of the ubiquitn E3 ligase gene that directly influence autophagy have also been identified and have been associated with the etiology and response to drug treatment in RA (337, 338). Both are likely important contributors to the action and effectiveness of disease modifying and monoclonal biological drugs used in RA treatment. The role of the NLPR3 inflammasome may give opportunities for developing other disease-modifying drugs by targeting upstream triggers of the NLPR3 pathway.

Atherosclerosis

Atherosclerosis is recognized as a chronic inflammatory condition (339) and atherosclerotic plaques show cellular senescence (340, 341). Cytokines are involved in all stages of the pathogenesis of atherosclerosis, having both pro- or anti-atherogenic effects (342, 343). In response to increased low-density lipoprotein (LDL), hypertension, and subsequent shear stress, cytokines modulate endothelial cell permeability and recruit monocytes and T-lymphocytes (344, 345). The continuous monocyte recruitment, foam cell and fatty steak formation eventually result in unstable plaque development, thrombosis, and a cardiac event (345, 346).

Chronic unresolved inflammation is a key feature in atherosclerosis and the levels of SPMs, particularly resolvin D1, and the ratio of SPMs to pro-inflammatory leukotriene B_4 (LTB_4), are significantly decreased in the vulnerable plaque regions (27). Vulnerable atherosclerotic plaques are recognized as having distinct features; increased inflammation; oxidative stress; areas of necrosis overlain by a thin protective layer of collagen (fibrous cap). In advanced atherosclerotic plaques, macrophages have more abundant nuclear 5-LOX, which is suggested to lead to conversion of AA to proinflammatory LTs, with the potential to contribute to plaque rupture (27).

The NLRP3 inflammasome, a central regulator of inflammation (58), is activated by cholesterol crystals and oxidized LDL (347, 348) that drives the IL-1β inflammation pathway. Recent research targeting IL-1β inflammation in atherosclerosis using cannakinumab, a therapeutic monoclonal antibody, has shown up to 15% lower rates of recurrent cardiovascular events, which was independent of lipid lowering (349). As well as playing a major role in chronic inflammation, NLRP3 is also upregulated during endothelial cell senescence (350) *via* ROS, and is negatively regulated by autophagy (351, 352). The NLRP3 inflammasome, therefore, appears to warrant further investigation as a potential target for inflamm-aging related to atherosclerosis given that such mechanisms are now of well known importance in atherosclerosis (353).

The gut microbiome has been implicated in age-related inflammation (354) with numerous studies reporting bacterial organisms in arterial plaque (355–357). Emerging research reports bacterial DNA in blood associated with a personal microbiota fingerprint as a predictor of cardiovascular events and stool microbiome as a signature of cardiovascular disease (358, 359). Similarly, bacterial DNA has been noted in cell-free plasma in cardiovascular and chronic renal disease patients (360, 361). Altered gut microbiota composition or dysbiosis is also seen in elderly people, and is associated with inflammatory markers (354). Aging leads to changes in intestinal permeability in gut bacterial milieu (362), and the increased circulatory bacterial DNA observed associated with atherosclerosis support further investigation of the microbiome as a contributory factor to age-related inflammation and atherosclerosis.

Neuroinflammation and Neurodegenerative Disease

Inflammation has been well established as a major component of neurodegenerative disorders, but it has never been clear if this was a direct cause of the disease or a consequence of the progressive degenerative process that was occurring (363, 364). The central role of cytokines in regulating the immune response has been implicated in neurodegeneration, but over the past decade, there has been a revolution in our understanding of how cytokines contribute to the etiology of the leading neurodegenerative disorders, including Alzheimer's (AD) and Parkinson's disease (PD).

In AD, central events seem to include the inflammasome, the NF-κB pathway, and the activation of microglia by a variety of factors, including beta amyloid and pro-inflammatory cytokines (172). Microglia, the primary components of the CNS innate immune system (365), produce cytokines and monitor the integrity of CNS. Together with astrocytes, microglia are the primary effectors of neuroinflammation and express PPRs that allow early recognition of PAMPs and DAMPs. When the NLRP3 inflammasome is activated, the inflammation cascade begins with casp-1 that facilitates the processing of IL-1β and IL18. These proinflammatory cytokines drive the inflammatory cascade through downstream signaling pathways and lead to neuronal damage and death (366). The activated microglia release proinflammatory cytokines, such as IL-1β, IL-6, TNF-α, and IL-18, that contributes to neuronal death and dysfunction.

There is interest in the role of sphingolipid metabolites, such as ceramide and sphingosine-1-phosphate, which regulate a diverse range of cellular processes that are important in immunity, inflammation, and inflammatory diseases (367). Growing evidence suggests that ceramide may play a critical role in NLRP3 inflammasome assembly in neuroinflammation. Research has shown that microglia treated with sodium palmitate (PA) induce *de novo* ceramide synthesis, triggering the expression of NLRP3 inflammasome assembly and resulting in release of IL-1β (368), linking neuroinflammation with dietary lipids. Recent insights into the molecular mechanisms of action of sphingolipid metabolites suggest roles in altering membrane composition, with effects on cellular interactions and signaling pathways with potential causal relationships to neuroinflammatory disease.

Dysregulated autophagy has been considered to play a role in neurodegenerative diseases, particularly AD, and is felt to be a key regulator of Aβ abnormal protein generation and clearance (369). In AD the maturation of autophagolysosomes (i.e., autophagosomes that have undergone fusion with lysosomes) and their clearance are hindered. Evidence suggests that Aβ peptides are released from neurons in an autophagy-dependent manner and that the accumulation of intracellular Aβ plaques is toxic to brain cells leading to AD pathology (370). Furthermore, lysosomal and autophagocytic dysfunction has been associated with both Alzheimer's and Parkinson's diseases (71, 72). Senescent cells too, accumulate abnormal protein aggregates in the cytoplasm that contribute to neurodegenerative disease (72). Cellular senescence has been reported in the aging brain with an increase in SASP-expressing senescent cells of non-neurological origin that are likely to contribute to the pro-inflammatory background (103, 371).

In AD and PD, the application of genome-wide association studies (GWAS) has demonstrated a number of key genes, relating to immunity, including the human leukocyte antigen (HLA) complex on chromosome six that regulates the immune and inflammatory response (372, 373). In the most recent Parkinson's disease GWAS a locus containing the IL-1R2 gene was identified as significantly associated with disease risk and awaits further investigation (372). There is some evidence that carriage of certain pro-inflammatory cytokine gene alleles may confer increased Alzheimer's disease risk. Single studies have reported that carriers of the A allele of the TNF-α 308 G/A gene were variably associated with increased risk of Alzheimer's disease (207–210) and that carriage the higher IL-6 producing allele of IL-6 (174 G/C) may confer increased risk (186, 190, 191). Animal studies have provided some clearer understanding of the role of TNF-α in Alzheimer's disease with evidence of disease modulation with the use of anti-TNF agents (215). Three studies, published in 2013, confirmed a role for the immune response in AD identifying the microglia-related gene TREM2 as harboring an intermediate effect size variant in risk of AD that has also been implicated in other related neurodegenerative diseases (374–376). A recent study of rare variants has also implicated a role for microglial-mediated innate immunity in AD (377).

A better understanding of the molecular pathways involved in the use of established drugs, such as non-steroidal anti-inflammatory or statin drugs in risk and progression of neurological disorders may provide further opportunities to treat earlier or prevent disease onset (378–380). It has been considered that downregulation of the type and magnitude of the pro-inflammatory immune response in neurodegeneration might be a key to earlier and more successful targeting of these pathways. However results, to date, have been disappointing and anti-TNF-α therapies and targeted treatment of TNF-α levels that are elevated in cerebrospinal fluid and in patients' serum, have produced, at best, modest results (381). Multiple sclerosis patients have benefited from treatment with fingolimod (FTY720) that has been reported to attenuate neuroinflammation, by regulating the activation and neuroprotective effects of microglia, by modulating the sphingosine-1-phosphate receptor (S1P receptor) (382). Given the success of FTY720 for treatment of multiple sclerosis, it is hoped that next-generation

S1PR1 modulators will find wider therapeutic uses in other inflammatory disorders. Fingolimod is now under a phase 2 clinical trials for acute stroke and phase 4 for neurodegeneration (383).

FUTURE CONSIDERATIONS

Aging is heterogeneous among people and highly variable between different organs and tissues. Our genes, our lifestyles, and our response to stress are infinitely individual and variable, so that the immunobiography of each life tells a different story of how each will respond to the internal and external environmental stressors (1–3, 384). But evidence is accumulating that the aging process may be malleable.

Because aging is the major risk factor for age-related diseases, understanding age better and maintaining the health of older people and societies is highly important personally and for societies and governments. Knowledge about the underlying molecular pathways and the genetic and life-style processes associated with age-related disease and aging itself is increasing. Evidence from centenarian and nonagenarian studies suggests that these oldest members of populations have had the ability to delay aging and age-related disease (385, 386). Other studies suggest that centenarians may demonstrate optimized cardiovascular risk factors (387, 388), or have either intuitively or through social example, adopted lifestyles which have interacted with their genes to facilitate a successful aging phenotype (3, 389, 390).

Population studies across the world show that the age-specific incidence of cardiovascular disease, stroke, and dementia is decreasing (391–395). This suggests that better blood pressure and diabetic control and statin use may directly or indirectly link into and downregulate molecular pathways associated with inflammation (396–399). Research into how carriage of certain gene alleles, such as TCF7L2 or IL-6 can increase inflammation or stroke risk, respectively, and can be ameliorated by following a Mediterranean-type diet (42, 400, 401), or how gene splicing and features of senescence may be modulated by resveratrol in food (402), herald research into how gene, diet, and lifestyles can interact, with positive or negative effects on the immune system and health. Increased knowledge is emerging as to how epigenetic modulation can affect cytokine genes with reports linking cytokine epigenetic change to neuroinflammation (403–405). Obesity, smoking, and malnutrition have been shown to have next generational epigenetic effects, and seem likely to contribute to the predilection of offspring developing age-related disease or conversely the longevity phenotype (406–409).

Other strategies should be adopted which link with public health messages and encourage people to adopt behavioral changes in lifestyles. Modifications should include: changes in diets to include more omega-3 containing foods or fruits and vegetables as in the Mediterranean diet (410–413); engagement in regular moderated exercise routines (414–417); continued engagement with social connections and intellectual activities in daily lives (418–420); or best of all a combination of life-style factors (3, 421, 422), all of which have been shown to reduce the inflammatory profile and improve the quality of aging. Although the role of diet on human health and connections through nutrition, inflammation, and cancer are not as linear as those between tobacco, smoking, and lung cancer, obesity is linked to chronic inflammation through several mechanisms, including the dysregulation of autophagy, whereas fasting has anti-inflammatory effects, similar to the effect of exercise (423–426), and may downregulate inflammatory biomarkers (427–429). There is, therefore, considerable interest in the role of the intestinal microbiota and health and the so-called immune-relevant microbiome (324, 354), with important correlations between inflammation and neurodegenerative disease (430), bacterial β-hydroxybutyrate metabolites (431), and the role of vagal stimulation (432).

Increasing evidence shows that many signaling pathways are activated in a stress-type-dependent fashion, and all appear to converge with nuclear factor (NF)-κB signaling, which is a central controller of the immune response, and inflammatory cascade (110, 433–436). With increasing age, immune homeostasis loosens, NF-κB signaling becomes less tightly controlled or is more readily triggered, cytokine dysregulation occurs, and a pro-inflammatory phenotype predominates that underpins most major age-related diseases from atherosclerosis to cancer, and aging itself (**Figure 5**). Understanding how different factors trigger the NF-κB cascade is an important pathway of research (434). In animal models, miRNA-based regulatory networks involving miR-155 and miR-146a, finely regulate NF-κB activity, with miR-146a downregulating and miR-155 upregulating NF-κB expression (435). There is an important temporal separation of miR-155 and miR-146a cellular expression that allows finely controlled NK-κB signaling and enables a precise macrophage inflammatory response, which merits further research.

Therapeutic opportunities may arise through better understanding of the molecular mechanisms that induce senescent cells and SASP in the cellular environments of chronic disease or whether senescent cells can be removed by upregulating autophagy and using sophisticated tagging mechanisms (110). There will be increased opportunities to use the knowledge gained from clinical studies in autoimmune disease, about the roles and actions of monoclonal antibodies in modulating inflammation, which may be able to be utilized in treatments for other age-related diseases involving inflammation (436). The formulations of new and more specific drugs are likely to become available as the modes of action of kinases, such a AMPK and mTOR which control the senescence and inflammation pathways, become better understood (81, 84, 437). Old drugs, such as metformin, still used in diabetes control, are being repurposed and have been shown to have exciting new uses through their ability to modify epigenetic gene expression. Clinical studies are underway to assess any modulating effect of metformin in aging and age-related diseases (81). The use of histone deacetylating drugs is likely to increase as the clinical use of deacetylation and methylation agents is evaluated in cancer with improved knowledge of their effects and safety criteria (438). The current interest in diet and modified diets will encourage further studies assessing how nutrachemicals modify gene expression, for example, through the regulation of intracellular receptors that bind the promoters of certain genes, and may help to design more specific drugs to modify metabolism and benefit health (439).

Turning research to focus on improved understanding of the mechanisms of inflammation resolution in aging and age-related disease, should also be prioritized, since it is an under researched area. Developing synthetic resolvins for use in inflammation resolution may have advantages over the use of single biological

anti-inflammatory blockers in autoimmune disease clinical management, since cytokine networks are highly interactive and complex (440), with many auto-regulatory feedback loops. All these molecular pathways are, or have the potential for being developed as drug targets toward clinical interventions useful in damping down and modulating inflammation (441, 442) and may have a role in delaying the onset or treatment of age-related diseases.

Evidence from on-going global studies of the oldest members of our societies, such as centenarians and nonagenarians (443–454) suggests that it may be possible to delay age-related diseases and that aging may be a potentially modifiable risk factor (455). Further investigation has shown that centenarians and super-centenarians also have an enhanced pro-inflammatory background (9, 456, 457), which at first seems surprising, given their long lives. However, studies have demonstrated that the pro-inflammatory background is accompanied and perhaps modulated, by an enhanced anti-inflammatory status in some centenarians. Some have argued that an enhanced anti-inflammatory phenotype could be beneficial as a contributor to longevity by effectively controlling the pro-inflammatory background (9, 11, 257). Others suggest that some inflammation is good, in the same way as hormetic stress triggers systems, and upgrades them but does not overwhelm them (458). Regular exposure to pro-inflammatory stressors could train the immune system to upregulate and fine-tune its cellular processes, so that it responds better and provides better outcomes, when faced with real life-threatening pathogenic threats.

Genome-wide association studies have proved a powerful methodology to assess the influence of common variation in AD and PD disease susceptibility, but by their nature have reflected low effect size variants that likely have a cumulative effect on risk (459). As next-generation sequencing technology becomes more cost-effective, the ability to identify variants that are less common (<1% minor allele frequency) will become more achievable. These unbiased approaches should aid the identification of key players in the inflamm-aging pathway and will play a critical role in the development of therapeutic intervention strategies in neurodegenerative and age-related diseases.

There is the increasing opportunity to link large global datasets with the technologies of genomics, transcriptomics, and proteomics through bioinformatics and artificial intelligence methods to unlock the physiological, genetic, and molecular pathways that underpin the pro-inflammatory aging-phenotype. Using systems biology methods has the potential to lead to the generation of novel therapeutic approaches for old diseases and modern health challenges. Improving knowledge about how to delay or modify the pro-inflammatory aging-phenotype, the hallmark of aging and age-related disease, will give hope of a better quality aging and the longevity dividend for all.

AUTHOR CONTRIBUTIONS

IR conceived and designed the outline of the manuscript. All authors IR, DG, VM, SM, DA, and OR contributed to the manuscript draft. All authors contributed to the drafting and revising of the manuscript and approved the manuscript prior to submission.

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REVIEW
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Debunking the Myth of Exercise-Induced Immune Suppression: Redefining the Impact of Exercise on Immunological Health Across the Lifespan

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Epidemiological evidence indicates that regular physical activity and/or frequent structured exercise reduces the incidence of many chronic diseases in older age, including communicable diseases such as viral and bacterial infections, as well as non-communicable diseases such as cancer and chronic inflammatory disorders. Despite the apparent health benefits achieved by leading an active lifestyle, which imply that regular physical activity and frequent exercise enhance immune competency and regulation, the effect of a single bout of exercise on immune function remains a controversial topic. Indeed, to this day, it is perceived by many that a vigorous bout of exercise can temporarily suppress immune function. In the first part of this review, we deconstruct the key pillars which lay the foundation to this theory—referred to as the "open window" hypothesis—and highlight that: (i) limited reliable evidence exists to support the claim that vigorous exercise heightens risk of opportunistic infections; (ii) purported changes to mucosal immunity, namely salivary IgA levels, after exercise do not signpost a period of immune suppression; and (iii) the dramatic reductions to lymphocyte numbers and function 1–2 h after exercise reflects a transient and time-dependent redistribution of immune cells to peripheral tissues, resulting in a heightened state of immune surveillance and immune regulation, as opposed to immune suppression. In the second part of this review, we provide evidence that frequent exercise enhances—rather than suppresses—immune competency, and highlight key findings from human vaccination studies which show heightened responses to bacterial and viral antigens following bouts of exercise. Finally, in the third part of this review, we highlight that regular physical activity and frequent exercise might limit or delay aging of the immune system, providing further evidence that exercise is beneficial for immunological health. In summary, the over-arching aim of this review is to rebalance opinion over the perceived relationships between exercise and immune function. We emphasize that it is a misconception to label any form of acute exercise as immunosuppressive, and, instead, exercise most likely improves immune competency across the lifespan.

Keywords: exercise, physical activity, upper respiratory tract infections, open window hypothesis, infection susceptibility, ageing, immunosenescence, immune competency

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INTRODUCTION

Lifelong physical activity[1] is a potent means of reducing the risk of non-communicable diseases including cancer, cardiovascular disease, and other chronic inflammatory disorders (1). Evidence also shows that a physically active lifestyle diminishes the risk of contracting a range of communicable diseases including viral and bacterial infections (2–6). In contrast to the widely accepted long-term health benefits that are achieved by regular physical activity, which imply that immune competency and regulation are improved by frequent exercise bouts, the effect of a single bout of exercise on immune function remains hotly disputed. Undeniably, acute vigorous exercise has a profound effect on the phenotypic makeup and functional capacity of the immune system. Indeed, the behavior of almost all immune cell populations in the bloodstream is altered in some way during and after exercise (7, 8). However, for decades, it has been widely accepted that these changes result in a temporary decline in immune competency in the hours following exercise. In the first part of this review, we re-interpret these data and strive to dispel the misconception that an acute bout of exercise is detrimental to immunological health. In the second part of this article, we demonstrate that rather than suppressing immunity, contemporary evidence shows that an acute bout of exercise improves immune surveillance, for example leading to enhanced antibacterial and antiviral immunity. Finally, in the third part of this article, we summarize recent data suggesting that regular physical activity and frequent exercise, which reduces systemic inflammatory activity and improves aspects of immune function, also leads to alterations in classical biomarkers of an aging immune system. These changes could be interpreted as limiting or delaying immunological aging (9–13).

PART A: IS IT TIME TO CLOSE THE SHUTTERS ON THE "OPEN-WINDOW" HYPOTHESIS? A BOUT OF EXERCISE DOES NOT SUPPRESS IMMUNE COMPETENCY

A prevailing myth has formed in the literature that participating in an acute bout of aerobic exercise, particularly if it is vigorous and prolonged, can be detrimental to immune competency. The foundations of this belief lay in research publications emanating from the 1980s and 1990s, reviewed extensively elsewhere (7, 8, 14). Findings from these early studies led to three principles of exercise immunology being formed, which have, until now, generally been unchallenged in the literature: (i) infection risk is increased after an acute bout of prolonged and vigorous aerobic exercise; (ii) acute bouts of vigorous exercise can lead to a temporary reduction to salivary IgA levels culminating in a higher risk of opportunistic infections; and (iii) transient decreases in the number of peripheral blood immune cells, which occurs in the hours following vigorous exercise, represents a period of immune suppression. Over the years, these collective observations have coalesced and led to the creation of the so-called "open-window" hypothesis, which purports that the immune system is compromised in the hours after vigorous exercise, leading to an increased risk of opportunistic infections in the days thereafter. To this day, the "open window" hypothesis continues to be discussed (15), despite the existence of contradictory evidence. Here, in the first part of this review, we aim to dispel the "open-window" hypothesis by revisiting key research studies and highlighting that limited evidence exists to support each of the three pillars that lay its foundation.

Exercise and Opportunistic Infections

It has been speculated for over a century that participation in physical activity heightens the risk of opportunistic infections (16). However, deeper investigation into the relationship between exercise and infection susceptibility did not take place until the end of the twentieth century. At this juncture, the principle focus of many of these studies in the late 1980s and early 1990s was to determine whether infection incidence was increased in elite and recreational athletes in the weeks following mass participation distance running events. One of the first studies from this era found that one third of 150 runners participating in the 1982 Two Oceans 56 km ultramarathon in Cape Town South Africa self-reported symptoms of upper respiratory tract infections (URTIs; symptoms = runny nose, sore throat, sneezing) within 2 weeks of the race (17). The control group, who were age matched and shared a home with another of the race competitors, reported only half the amount of URTIs in the same period (17). Similar observations were made in an often-cited larger study of the 1987 Los Angeles Marathon (18). Of 2,311 respondents who had completed the marathon and whom did not report an infection in the week prior to the race, 12.9% reported an infection in the week after the race compared to only 2.2% of individuals who withdrew from the race for reasons other than illness (odds ratio of infection in runners versus non-runners = 5.9). A separate study conducted around the same time found that shorter duration running events, such as 5, 10 km, and half-marathons (21 km) did not appear to elicit an increased incidence of self-reported URTIs (19), thereby suggesting that URTI symptoms are increased only when the duration of the exercise is long.

A fundamental limitation of each of the aforementioned studies is that none of the self-reported infections were clinically confirmed by laboratory analyses (e.g., molecular or microbiological techniques, such as polymerase chain reaction or bacterial cultures). As a consequence, it was questioned whether the

[1] "Physical activity" refers to activities undertaken during leisure time, at home, as part of employment, or for transport purposes. "Exercise" is a component of physical activity within the leisure time domain and refers to physical activities that are planned, structured, repetitive, and undertaken for the purpose of improving or maintaining components of physical fitness and/or sporting performance. When individuals are referred to as being "active" or "inactive," the description infers that these people undertake (or fail to undertake) a defined level of exercise or physical activity (e.g., age-specific physical activity recommendations, such as those published by the World Health Organisation). In this review, the term "exercise" will generally be used to describe the effects that active behaviours have on immune competency. Individuals described as being "sedentary" accumulate prolonged periods of behaviour eliciting low energy expenditure (e.g. sitting and lying).

self-reported URTIs in these studies represented genuine infections. Clarifying this issue, a study employing nasopharyngeal and throat swabs in athletes who reported URTI symptoms over a 5-month period—including periods of competition—found that few of the self-reported infections were of bacterial, viral, chlamydial, or mycoplasmal nature (20). Indeed, of 37 episodes of URTI reported by athletes in this study, only 11 of these (30%) had a positive laboratory diagnosis. These findings place the previously discussed marathon studies in a different light (17, 18) and open the possibility that many of the URTIs reported were a symptom of other non-infectious causes. Indeed, of the of the non-infectious "URTIs" reported by Spence et al. (20), and likely captured elsewhere (17, 18), it is proposed that these symptoms are a result of other causes, including allergy and asthma, non-specific mucosal inflammation, or airway epithelial cell trauma due to increased ventilation or exposure to cold air (21). In the few cases of clinically confirmed URTIs, these appear to be from viruses—in particular rhinoviruses (i.e., the "common cold")—rather than bacterial infection (20, 22), which is in line with the typical incidence and etiology of infections at the population level (23).

In the previously discussed clinical laboratory study of infection incidence in athletes (20), it is notable that the proportion of athletes with a confirmed infection during the 5-month period was as follows: 2/20 controls (10%), 3/31 (10%) recreational athletes, and 6/32 (19%) elite athletes. Although this study was small, these data appear to align with observations from earlier self-report studies which found that the incidence of URTI symptoms was higher in those with the fastest race time and those who had completed a greater training volume pre-race (17, 18). These early observations contributed to the formulation of the "J-shaped curve" (24). This hypothesis infers that those who undertake an excessive volume of exercise, over a period of weeks and months, sometimes referred to as "over-training" or "intensified training" (25, 26), are at a greater risk of infections (24). In this scenario, other factors present *prior* to an acute bout of exercise, such as psychological stress and anxiety (27–29), or nutritional deficiencies (30) which are known to impact immune regulation, are likely to impact immune competency and contribute to the risk of genuine URTIs, rather than the acute and transient immune changes that arise *after* the acute bout of exercise itself; these acute immunological changes arising after acute exercise are discussed later in this article (see Part A: "Is it Time to Close the Shutters on the "Open-Window" Hypothesis? A Bout of Exercise Does Not Suppress Immune Competency"; and see "Exercise and Salivary IgA and Changes to Lymphocyte Frequency and Functional Capacity in the Hours After Acute Exercise").

Moreover, we contend that attendance at any mass participation event—whether it is a marathon or otherwise—is likely to increase the risk of acquiring novel infectious pathogens, which are in abundance due to the mass gathering of people. For example, it has been shown that around 40% of individuals attending the Hajj—a crowded religious event in Saudi Arabia—self-report an URTI (31). In this study, there was a greater risk of infection among those with the longest exposure to crowds (31). Thus, it is important to consider that other underlying factors, often not measured in the context of exercise and illness studies, likely play a greater role in infection risk than exercise participation *per se*. For example, recently, it has been demonstrated that air travel is a significant predictor of illness symptoms in athletes (32). Infections linked to air travel are exacerbated by long-haul flights crossing multiple time zones, implicating many other factors known to influence immune function, including exposure to hypobaric hypoxia, radiation, temperature changes, sleep disruption, fatigue, altered or inadequate diet, dehydration, and psychological stress (32–34).

Contrary to the aforementioned reports that exercise heightens infection incidence, it is often overlooked that other studies indicate that exercise participation may in fact *reduce* the incidence of infections. For example, a recent prospective cohort study of 1,509 Swedish men and women aged 20–60 years found that higher physical activity levels were associated with a lower incidence of self-reported URTIs (35). A much smaller but very detailed analysis of illness records kept by 11 elite endurance athletes over a period of 3–16 years showed that the total number of training hours per year was inversely correlated with sickness days reported (36). Similarly, another study of swimmers monitored for 4 years found that national level athletes had higher incidence of infections than more elite international level athletes (37). Finally, studies of ultramarathon runners, who undertake the largest volume of exercise among athletes, have shown that these individuals report fewer days missed from school or work due to illness compared to the general population. For example, the mean number of sickness days reported over 12 months was 1.5 days in a study of 1,212 ultramarathon runners and 2.8 days in a study of 489 ultramarathon runners (38, 39). These studies compared their findings to data from the United States Department of Health and Human Services report in 2009, showing that the general population report on average 4.4 illness days each year. Thus, a number of studies challenge the "J-shaped curve," indicating that athletes undertaking the largest training loads, become ill less frequently than athletes competing at, and training at, a lower level. These findings have previously been conceptualized by extending the "J-shaped curve" into an "S-shaped curve," thereby suggesting that very elite athletes are better adapted to the demands of their training (40). Given the nature of their design, very few of these reports—akin to many of the aforementioned studies showing increased infection risk among athletes following mass participation endurance events—used appropriate laboratory diagnostics to confirm an infection. However, despite their limitations, it is important to highlight that there are as many epidemiological studies showing that regular exercise *reduces* infections as there are studies showing exercise *increases* infections, and that these studies are often overlooked in the exercise immunology literature.

It should also be considered whether the commonly reported "increased frequency" of illness symptoms among athletic populations or those taking part in sporting events is indeed more frequent than among the general population. For example, large studies have reported that approximately 7–10% of athletes competing in the Olympic Games report symptoms of illness during the competition weeks (41, 42). However, accumulating evidence suggests the incidence of infection among athletes is

not substantially different to other populations. For example, in a telephone survey of 2011 adults considered to represent the general population in the USA, 24% experienced a cold during a 4-week period, which is a similar timeframe to many international sporting competitions (43). In another telephone survey of 4,051 adults in the USA, 72% experienced at least 1 non-influenza related URTI over 12 months, and on average, experienced 2 infections annually (44). In a year-long Internet-based monitoring study of 627 individuals over 14 years of age in Germany, weekly acute respiratory illness rates were 2.7–8.2%, manifesting in 1.3–3.2 episodes annually (45). Thus, evidence suggests that the frequency of illness episodes in athletic communities is similar to the general population annually.

In the context of exercise participation in older age, it would appear to be counterintuitive that the incidence of URTI symptoms appears to be inversely correlated with age: it has been shown that URTI symptoms are more common in younger rather than older runners (18). While, once more, this aforementioned study did not confirm infections by laboratory analyses, it is notable that if acute exercise does suppress immune competency, it might be expected that older adults—whom typically have inferior immune function—would be at greatest risk of exercise-induced immune suppression. Rather than exercise *per se,* again, a more likely explanation for differences in illness symptoms between groups of athletes are other factors present before competing, such as fatigue, nutritional deficiency, psychological stress, or environmental exposures. On the other hand, proponents of the "open-window" hypothesis could portend that experienced athletes have higher tolerance of the symptoms associated with URTI, and/or have developed coping methods or strategies to reduce symptoms. Alternatively, experienced athletes may have evolved strategies to reduce infection risk by adopting good practice (e.g., sleep, diet, hygiene) before, during and following attendance at a mass participation event.

Separately, it has also been questioned whether illness symptoms—even if confirmed to be infectious—are a result of encountering a novel pathogen. For example, over a decade ago, some studies suggested that reactivation of latent viruses—such as *Epstein Barr Virus* which had most likely infected the host during childhood—was responsible for illness symptoms after exercise (46, 47). Although these studies suggest new pathogens were not to blame, it was interpreted that exercise-induced immune suppression had resulted in loss of viral control. However, herpes viruses can reactivate even with a fully functioning immune system, for example, in response to adrenergic activity, reactive oxygen species and inflammatory cytokines (48–50), all of which increase during exercise. Moreover, recent evidence appears to discount herpes virus involvement in causing symptoms of illness, by showing that individuals previously infected with *Cytomegalovirus*, or those infected with both *Epstein Barr Virus* and *Cytomegalovirus*, exhibited a lower incidence of illness symptoms than individuals not latently infected (51).

Taken together, evidence that participation in an acute bout of vigorous exercise leads to heightened infection incidence remains spurious. If symptoms of URTI are observed after a bout of vigorous exercise, the cause is unlikely to be infectious. However, if infection or immune impairment is confirmed, their trigger is more likely to be the physical, nutritional, and psychological wellbeing of the individual prior to undertaking the single bout of acute vigorous exercise. In the context of mass participation sporting events, it is likely that increased exposure to pathogens, or the influence of environmental factors that can affect immune function (e.g., travel, sleep disruption) most likely explain genuine infections. Thus, we conclude that it is unlikely that vigorous and prolonged exercise heighten the risk of infections and should not be considered a deterrent to those seeking to become more physically active.

Exercise and Salivary IgA

A second mainstay of exercise immunology that has received considerable attention over the last three decades is the assessment of exercise-induced changes to mucosal immunity, principally *via* measurement of IgA levels in saliva (52). Given that IgA is the most abundant immunoglobulin in mucosal secretions and that its principle role is the inhibition of invading pathogens, isolated changes to salivary IgA following exercise has been considered of some importance in light of the purportedly higher risk of infections among athletes (7, 8).

One of the earliest and most cited papers in this research area found that IgA was reduced by 20% after 2–3 h of cross-country skiing (53). Another study found that this effect is transient, whereby salivary IgA concentrations decreased immediately after 2 h of intensive cycling exercise, and remained low in samples collected 1 h post-exercise, but returned to normal levels within 24 h (54). A criticism of these studies at that time was that the absolute IgA levels reported did not adequately control for the amount of saliva produced, and thus these results may misrepresent IgA secretion. Although some studies measuring IgA secretory rate (IgA protein concentration multiplied by saliva flow rate) support the early findings with IgA concentration, others have shown profoundly contradictory results. For example, in alignment with prior observations, it was found in trained runners that IgA secretion rate decreased by 25% from pre-marathon to 90 min post-marathon (55). Likewise, in a separate study, a 20% reduction in IgA secretion rate was observed in elite athletes after a 2-h rowing exercise session (56). Several other studies of similar design reported analogous findings (57–59); however, contradictory findings are also in abundance but are much less cited in the literature. Indeed, an elegant study exploring the effects of different exercise intensities, including moderate- and high-intensity exercise to exhaustion, found that although saliva flow rate decreased, IgA secretion rate actually increased in response to both of the exercise bouts. In the words of the authors, exercise to exhaustion has an "*effect on the quantity of saliva, but not the quality of saliva*" (60). Many other studies have also reported that exercise does not elicit a decrease in IgA secretion rates following exercise (61–66).

Any subtle isolated changes to IgA that occur after exercise appear to be clinically insignificant as it would appear that the increased incidence of URTI symptoms that has been purported following vigorous and prolonged exercise is unrelated to salivary immunoglobulin status. A longitudinal field study of participants in the Comrades Marathon (86.5 km ultramarathon) in South

Africa found that salivary IgA levels in the 4 weeks prior to the race, and 2 weeks following the race, were unrelated to the incidence of self-reported URTI (67). In the aforementioned study, it was found that symptoms of URTI were highest 4 weeks prior to the marathon, and URTI symptoms seemed to re-appear within many of the same athletes 1 or 2 weeks post-marathon. This study did not confirm the re-emergence of infections by laboratory testing, but if this was indeed demonstrated in future studies, it again shows that acute exercise participation *per se* does not heighten risk of opportunistic infections. In this case, an underlying infection, not resolved prior to exercise participation, or some other idiosyncrasy, is perhaps to blame. Such conclusions appear to be supported by evidence from athletes who report the most frequent illness symptoms. For example, these individuals exhibit mostly anti-inflammatory cytokine responses when whole blood, collected at rest, is cultured *ex vivo* with antigens from diphtheria, tetanus, acellular pertussis, poliomyelitis, and hemophilus influenza type b (68, 69). These findings suggest the immune system may be functionally altered by underlying illness, or is already different in these "illness-prone" athletes prior to infection, rather than exercise affecting immune function *per se*.

A flaw in studies investigating the link between mucosal immunity and purported exercise-induced infection risk is that oral health status is rarely adequately evaluated. Salivary IgA is heavily involved in host-bacterial ecology and mucosal homeostasis (70, 71). As optimal oral health is rare in adults—with nearly all exhibiting caries, gingivitis or periodontitis—profound between-person IgA variation has been reported, which is dependent on oral health status (71). Moreover, periodontal diseases are complex and multifactorial, and as a result, studies report large fluctuations in IgA levels relative to disease status between persons, probably due to the bespoke ecological makeup of different host mucosa (71). In addition, oral disease is a common problem in athlete populations (72), which is likely to be caused by high volume and frequent carbohydrate consumption, and in some, a neglect of oral hygiene, perhaps due to practical constraints. Thus, changes to oral inflammatory status has not been adequately considered, and emerging salivary biomarkers of oral inflammation, such as immunoglobulin-free light chains (73), may offer a means of controlling for this confounder. As highlighted elsewhere (70), salivary IgA is also highly vulnerable to short-term variation, in particular, due to circadian rhythms, typically peaking in the morning, and falling thereafter (74). As salivary IgA secretion is controlled by the parasympathetic and sympathetic nervous system, psychological stress also plays a powerful role in regulating IgA levels (75). Animal models suggest that salivary IgA levels could vary up to 27-fold within the same host over a short period of time (76). Salivary IgA levels are also affected by factors such as sex differences (77), diet, ethnicity, disease, medications, tobacco, and phase of the menstrual cycle, as reviewed elsewhere (71). To overcome some of these variations in salivary IgA, it is often the case that studies evaluate only secretory IgA (sIgA; i.e., IgA containing the secretory component) as this represents IgA produced by local mucosal plasma cells, and not IgA from the bloodstream transported *via* crevicular fluid. While this approach may reduce some confounding error, most IgA in saliva contains the secretory component and is, itself, subject to large variation; extensively reviewed elsewhere (78). Given these many considerations, we propose that longitudinal measurement of salivary IgA, as an isolated measure of immune competency within a single host, and even more so between persons, depicts too confusing a picture, and it is ambitious to say that any subtle changes to salivary IgA following exercise reflects immune suppression and a heightened risk of opportunistic infections. Given the limitations of salivary IgA measurement, research is being undertaken to explore mucosal IgA in other biofluids, and a recent study has shown links between reduced tear IgA levels and infection incidence (79). Others have moved toward more comprehensive oral immunity panels (80), and such strategies could benefit further from an integrative approach that, in addition to immune parameters, incorporates full dental examination, oral inflammation biomarkers, and host mucosal ecology.

Changes to Lymphocyte Frequency and Functional Capacity in the Hours After Acute Exercise

One of the most reproduced findings in human exercise physiology is the profound and transient time-dependent change that arises to the phenotypic composition and functional capacity of lymphocytes in the peripheral bloodstream in response to a single bout of exercise (8). During vigorous aerobic exercise, it is commonly observed that peripheral blood lymphocyte frequency—and, concomitantly, the functional capacity of the lymphocyte pool—is dramatically increased, leading to the concept that, during exercise, exercise appears to "stimulate" the immune system. On the other hand, in the hours following exercise, it is typically observed that total peripheral blood lymphocyte frequency—and the functional capacity of the lymphocyte pool—is decreased below pre-exercise levels, leading some to propose that exercise induces a short-term window of immune suppression (termed the "open-window" hypothesis). The purpose of this part of our review is to outline that it is a misconception to state that the "reductions" to lymphocyte frequency and function, that arise in the hours following acute exercise, reflects immune suppression, and instead we emphasize that during this post-exercise period, the immune system is in a heightened state of immune surveillance and regulation.

Transient Changes to Blood Lymphocyte Frequency in the Hours Following Exercise

The classic biphasic response of lymphocytes to acute steady state vigorous exercise lasting for around at least 45–60 minutes, is first characterized by a dramatic lymphocytosis. This response is typified by a dramatic influx of natural killer cells, which rise by up to 10-fold, and $CD8^+$ T cells which increase to a lesser—but still profound—extent by approximately 2.5-fold (81). This exercise intensity-dependent mobilization is driven in part by increased shear forces and blood pressure during exercise causing a non-specific flushing of the marginal pools (82) but, moreover, is principally governed by adrenergic stimulation of beta-2-adrenergic receptors on the surface of lymphocytes, arising from adrenaline released during exercise, causing endothelial detachment and subsequent recirculation

of lymphocytes into the bloodstream (83–85). Indeed, the lymphocyte mobilization response observed during exercise appears to broadly mirror the differential expression of beta-2 adrenergic receptors on lymphocytes: natural killer cells > $CD8^+$ T cells > B cells > $CD4^+$ T cells, including regulatory T cells (81, 86–88). Upon exercise cessation, the classic biphasic exercise response is next characterized by a dramatic decrease in the frequency of lymphocytes in the bloodstream. This nadir is typically observed approximately 1–2 h post-exercise when the lymphocyte numerical count is lower than pre-exercise levels; lymphocyte frequency normally returns to pre-exercise levels within 24 h (87, 89, 90). The lymphopenia that occurs 1–2 h later is exercise intensity dependent and the most profound reductions during this period are typically observed among natural killer cells and $CD8^+$ T cells (90). Akin to purported reductions to salivary IgA, discussed earlier, it was perceived that the numerical decline of blood lymphocytes that arises during this time represented "double jeopardy" (89), temporarily exposing an individual to impaired immune competency and concomitantly providing an "open-window" for opportunistic infections (91, 92).

Rather than suppressing immune competency however, a more contemporary viewpoint is that this acute and transient lymphopenia 1–2 h after exercise is beneficial to immune surveillance and regulation. Indeed, in what appears to be a highly specialized and systematic response, it is widely proposed that exercise redeploys immune cells to peripheral tissues (e.g., mucosal surfaces) to conduct immune surveillance. Here, these immune cells are thought to identify and eradicate other cells infected with pathogens, or those that have become damaged or malignant, termed the acute stress/exercise immune-enhancement hypothesis (93). A seminal study by Kruger and colleagues, using fluorescent cell tracking in rodents, found that T cells are redeployed in large numbers to peripheral tissues including the gut and lungs, and to the bone marrow following exercise (84, 94). In line with Dhabhar's theory, it is hypothesized that this redistribution reflects heightened immune surveillance in sites where pathogens are likely to be encountered during and after exercise (i.e., lungs, gut). This response has also been proposed to maintain immune homeostasis *via* augmented regulatory activities (12). In this context, evidence implies that bone marrow homing and subsequent apoptosis of senescent T cells stimulates the production or mobilization of new progenitor cells into the periphery (95), which has been hypothesized as an exercise-induced means of maintaining a younger immune system (12), discussed later in Part C "Does Exercise and Regular Physical Activity Influence Immunological Ageing." Links between exercise-induced apoptosis and lymphopenia have in the past been interpreted as detrimental, with speculation that apoptosis could be responsible for the fall in lymphocyte number in the hours after exercise (96, 97). Other studies have reported increased lymphocyte apoptosis immediately after exercise (i.e., as a result of the large mobilization of cells) but not in the hours following exercise during lymphopenia (98–100). Although the magnitude of lymphocyte apoptosis reported in studies is dependent on the measurement technique, typically <10% of lymphocytes undergo post-exercise apoptosis (96, 97). Given the 30–60% decrease in lymphocyte numbers post-exercise (89, 101, 102), apoptosis could be a small contributor to exercise-induced lymphopenia, but this process of cell death is likely to be beneficial given the stimulation of progenitor cells from the bone marrow (95).

While it has not been shown in humans that exercise—in line with rodent models—causes the redistribution of immune cells to peripheral tissues, further support for a coordinated, exercise-induced immune surveillance response elicited by lymphopenia, is revealed by studying the phenotypic characteristics of the cells that preferentially mobilize and subsequently extravasate out of the circulation after exercise. With regard to natural killer cells—the most exercise-responsive lymphocyte subset—$CD56^{dim}$ cells are preferentially redeployed rather than their $CD56^{bright}$ counterparts (81). $CD56^{dim}$ cells are a mature subset of natural killer cells which have exclusive migratory potential for non-lymphoid tissue and potent effector capabilities, including the capacity to produce high amounts of perforin and granzyme, whereas $CD56^{bright}$ cells are a more immature regulatory cell subset (103) and reside in secondary lymphoid organs, typified by their cell-surface expression of CD62L and CCR7 (104). $CD56^{dim}$ natural killer cells can be further dissected, into cells with highly potent effector function based on loss of NKG2A and expression of killer immunoglobulin-like receptors and CD57 (105, 106). In a recent study, it was shown that these natural killer cells, which are capable of rapid effector functions, are preferentially redistributed after exercise (107, 108). Synergistically, T cells also appear to exert heterogeneous but highly coordinated responses to acute exercise. Indeed, it is consistently observed that discrete populations of $CD8^+$ but not $CD4^+$ T cell subsets are redeployed by exercise. For some time, there was confusion pertaining to the exact behavior of $CD8^+$ T cells in response to exercise. Rather than losing or gaining markers of adhesion or activation—as evaluated elsewhere (8)—these changes represent a uniform redeployment of a preferentially mobilized group of memory cells. In a flurry of studies about a decade ago, it was shown that exercise selectively mobilizes memory $CD8^+$ T cells with a phenotypic propensity for homing to peripheral tissues—typified for example by CD11, and not CCR7 or CD62L expression—and the distinctive capacity to mount rapid effector functions (81, 109–111). This response presumably facilitates the detection and elimination of neoplastic, stressed or infected cells in synergy with natural killer cells, as proposed elsewhere (112). Aligned with the immune surveillance theory of Burnet and Thomas, reviewed elsewhere (113), these results imply that sentinel cells of the immune system are redeployed by exercise-induced perturbations to stress hormones, to exert effector functions against neoplastic, stressed, or infected cells in the hours following exercise. This process, which occurs daily in a natural diurnal process (114), orchestrated subtly by stress hormones (115, 116), appears to be primed in response to exercise, leading to enhanced immune surveillance (117). Principles of the acute stress/exercise immune-enhancement hypothesis continue to be investigated. For example, it has recently been shown that acute exercise does not preferentially mobilize $CD8^+$ T cells and natural killer cells with the capacity

for skin-homing (118). However, skin-homing in this context is a role that may be fulfilled by exercise-responsive mucosal-associated invariant T cells (119); but further work is needed in this area.

A key example illustrating how exercise-induced immune cell redistribution is beneficial to host health can be found in the rapidly emerging field of exercise oncology. Indeed, a recent seminal study demonstrated inhibition of tumor onset and disease progression across a range of tumor models in voluntarily active rodents (112). In this work, natural killer cell infiltration was significantly increased in tumors from active versus inactive rodents, leading to the conclusion that the presence of natural killer cells (but perhaps also T cells) in tumor sites, redeployed by adrenaline during exercise stress, "provides a spark" for tumor elimination, in what could be considered a form of "exercise immunotherapy" (112, 120). Importantly, administration of propranolol—a beta 2 adrenergic blocker—abolished the adrenaline-induced redistribution of immune cells, and nullified the anti-tumor effect of exercise on neoplastic growth (112). While these studies are limited to rodents, there is growing evidence that exercise may promote anti-cancer effects in humans. For example, in a key study recently conducted in humans, it was shown that natural killer cells with a highly mature effector phenotype are preferentially redistributed after exercise, and have the capacity to exert augmented cytotoxicity against myeloma and lymphoma cells *in vitro* (107, 108). In light of these results, research is now being conducted to harness the beneficial impact of acute exercise on lymphocyte kinetics for the purposes of cancer immunotherapy (121). It is beyond the scope of this review to discuss other findings in this exciting field and we briefly conclude that the aforementioned studies imply that exercise-induced lymphocytosis, and the lymphopenia that follows, is beneficial to the immune system's capacity to identify and neutralize damaged and neoplastic cells in peripheral tissues. Furthermore, in the context of neoplastic growth, this process may be directly responsible for reduced incidence of cancer among physically active people across the lifespan (122). Further comprehensive discussion of the role of exercise and lymphocyte kinetics in anti-tumor responses can be found elsewhere (117). Clearly more research is needed in this area, and a shift in focus toward investigating the benefits—rather than purported detrimental effects—of exercise on health, is no doubt underway and will be a key focus for exercise immunologists in the coming years.

Transient Changes to Blood Lymphocyte Function in the Hours Following Exercise

A common misinterpretation, brought about by the exercise-induced reductions to blood lymphocyte frequency in the hours following exercise, is the observation that the functional capacity of immune cells in the peripheral blood is reduced in the hours following vigorous exercise. As measurements of cell function in peripheral blood are entirely dependent on the cells present at the time of sampling, a change to the composition of the cells in blood in the hours after exercise—as outlined in the section; "Transient Changes to Blood Lymphocyte Frequency in the Hours Following Exercise"—will consequently lead to parallel changes in overall cell function, indicated by the performance of the sampled cells being assayed. For example, among $CD8^+$ T cells, subsets that exhibit strong effector function (e.g., CD45 $RA^+CD27^-CD28^-CCR7^-CD62L^-CD57^+$), substantially increase during exercise (81, 123). Thus, during exercise, blood is predominantly occupied by cells capable of responding strongly (i.e., IFN-gamma production) to *in vitro* stimuli, and therefore, many studies have shown an increase in IFN-gamma production by cells isolated close to the exercise stimulus. In the hours following exercise, the same effector $CD8^+$ T cells are subsequently redeployed to peripheral tissues, and, as such, this results in the blood having fewer cells capable of responding strongly to *in vitro* stimuli, thus explaining the commonly reported decrease in cellular function post-exercise. These effects have been neatly demonstrated approximately two decades ago when it was shown that IFN-gamma production by stimulated $CD8^+$ T cells is reduced 2 h after completing a prolonged 2.5 h bout of cycling (124). Importantly, it was shown that this reduced capacity to produce IFN-gamma was due to a reduced number of IFN-gamma-positive $CD8^+$ T cells in peripheral blood at the same time-point (124, 125).

The same principles apply to other cell functions, such as *in vitro* proliferation in response to mitogenic stimuli. However, with this measurement in particular, the commonly reported increase in T cell proliferation immediately after acute bouts of exercise is also strongly influenced by laboratory processes and *in vitro* assay conditions (e.g., blood processing, temperature, mitogen selection) (126). A recent meta-analysis of 24 studies concluded that lymphocyte proliferation is suppressed following acute bouts of exercise, and that a greater magnitude of suppression is caused by exercise lasting longer than 1 h, regardless of exercise intensity (127). However, this meta-analysis did not examine the most important determinant of cell function following exercise: the time-dependent changes in the cellular composition of the samples assayed. Thus, findings such as these should be interpreted with caution if analyses did not differentiate between studies collecting samples immediately after exercise or in the hours following.

As with research focusing on T cells, a similar group of studies citing reductions to natural killer cell cytotoxicity following acute exercise, reviewed elsewhere (128), did not always take into account dramatic shifts in the constitutional makeup of the natural killer cell compartment, which is known to change in response to exercise (81). Once more, changes to the functional capacity of the total natural killer cell pool are likely to have been misrepresented, given that many of these cells, with potent effector functions, are redistributed to peripheral tissues following exercise cessation. The principles discussed herein regarding lymphocyte function are also broadly applicable to the assessment of function in other cells, such as neutrophils and monocytes; the response of these cells to exercise is beyond the scope of this article and is reviewed elsewhere (8).

Taken together, it is important to emphasize that statements such as "*acute intensive exercise elicits a depression of several aspects of acquired immune function*" and "*prolonged bouts of heavy exertion reduce the normal functioning of all major immune cell subtypes*" mentioned elsewhere (8, 15) should be interpreted

with caution. We conclude that the results of studies exploring the effects of acute exercise on cell function must be considered very carefully in light of the time-dependent changes in the cellular composition of blood that typically arise following a vigorous bout.

Summary: Exercise Induces Lymphocyte Immune Surveillance Not Immune Suppression

In summary, strong evidence implies that a reduction in the frequency and function of lymphocytes (and other immune cells) in peripheral blood in the hours following vigorous and prolonged exercise does not reflect immune suppression. Instead, the observed lymphopenia represents a heightened state of immune surveillance and immune regulation driven by a preferential mobilization of cells to peripheral tissues. As such, nutritional interventions, which have been employed to dampen the magnitude of exercise lymphopenia (124, 129) are unlikely to reduce the incidence of infections, but interventions that augment exercise-induced lymphocyte trafficking may provide benefits (130).

PART B: REGULAR PHYSICAL ACTIVITY AND FREQUENT EXERCISE AUGMENT ASPECTS OF IMMUNE COMPETENCY ACROSS THE LIFESPAN

Contrary to a commonly held belief—outlined in Part A "Is it Time to Close the Shutters on the "Open Window" Hypothesis? A Bout of Exercise Does Not Suppress Immune Competency?"—that acute vigorous exercise can suppress aspects of immune function, an increasingly large body of research indicates that both single bouts of exercise, or frequent participation in regular exercise, can act as an adjuvant to stimulate the immune system. Numerous methods exist to assess the effects of behavioral interventions on immunity (131) but arguably the optimal means of evaluating global immune competency at a systems level is *via* assessment of the immune response to *in vivo* challenge, ideally with a novel and clinically recognized pathogen, for example *via* vaccination (132). Thus, here, in the first section of Part B "Regular Physical Activity and Frequent Exercise Augment Aspects of Immune Competency Across the Lifespan," we focus on the influence that exercise has on immune responses to antigenic challenge, which requires a coordinated response from most, if not all, innate and adaptive immune system components. In light of the age-associated decline in immune competence with aging—caused in part by underlying changes to the numerical, phenotypic, and functional capacity of almost all innate and adaptive immune cells—the second section of Part B "Regular Physical Activity and Frequent Exercise Augment Aspects of Immune Competency Across the Lifespan," will evaluate the impact aging has on the immune benefits that can be attained from exercise throughout the lifespan. A detailed discussion of immunological aging processes and the influence that an active lifestyle has on established biomarkers of an aging immune system is covered in Part C "Does exercise and Regular Physical Activity Influence Immunological Ageing?" of this article.

Exercise and Immune Responses to Experimental *In Vivo* Challenge Across the Lifespan

In a research context, the most clinically relevant model to assess *in vivo* challenge in a controlled manner is *via* vaccination. Vaccine administration assesses the integrated capacity of the immune system to recognize and process antigen, leading to antigen neutralization. In a clinical research context, vaccination responses are principally quantified clinically in two ways, either *via* antibody production from antigen-specific plasma cells or *via* cytokine responses—typically IFN-gamma production—from T cells stimulated with cognate antigen.

Vaccination: Effects of a Single Exercise Bout

Evidence from an array of studies, evaluated recently in a comprehensive review elsewhere (9), indicates that a single acute bout of exercise appears to enhance immune responses to vaccination in both younger and older individuals. The majority of studies to date have examined muscle-damaging upper arm resistance exercise performed close to the time of vaccination which is administered shortly after the regimen into exercised muscle. However, other modes of exercise, including acute bouts of whole body aerobic activity, have also been investigated. Six of the eight trials identified in the aforementioned review indicated a statistically significant exercise-induced enhancement of immune responses against constituent antigens contained within the vaccine administered (133–138). It is notable that in five of these studies, statistically significant benefits were found where the vaccine strains appeared to have lower immunogenicity (9). For example, it was shown in a trial of 133 young adults, approximately 20 years of age, receiving either a full- or half-dose Pneumovax-23 (a pneumococcal vaccine), that those who exercised at the time of receiving the half-dose vaccine had heightened responses to five of the eleven pneumococcal strains contained in the vaccine, whereas no differences were observed for the other six strains; and no benefits of exercise were observed for the full-dose vaccine (137). As such, given the potential effectiveness of exercise as an adjuvant in situations where vaccine immunogenicity is low, studying the effects of exercise on antibody responses in older adults—whom typically exhibit impaired responses—has received considerable attention. In one recent study, it was found that antibody responses in women 55–75 years of age, were significantly improved when moderate-intensity aerobic exercise was performed immediately prior to vaccination; however, no beneficial effects were found in men (138). Another trial reported no benefits of a single 45-min bout of moderate-intensity walking exercise on the immune response to influenza and pneumococcal vaccination in women around 47 years of age (139). Finally, a very recent study found no effect of a bout of resistance exercise on antibody responses to influenza vaccination in adults approximately 73 years of age (140). It is possible that a number of factors including immunological aging, biological sex and variations in sex hormone levels, and perhaps latent infections (e.g., herpes viruses) (141–143) limit the immunostimulatory effects of exercise, and many studies have not adequately controlled for these factors. Alternatively, in light of the mechanisms

proposed in the acute stress/exercise immune-enhancement hypothesis (93), it is plausible that more intensive exercise may be needed to elicit enhanced vaccine responses. Despite null findings, it is important to point out that few, if any, studies investigating the effects of acute exercise on vaccination responses have reported exercise-induced *impairment* to immune responses, and rather, these studies report that exercise has no effect, or in some cases a beneficial effect, on the immune response to vaccination in older adults.

Vaccination: Effects of Frequent Exercise Bouts

Data from vaccine studies exploring the effects of regular physical activity or frequent exercise training on the immune response to vaccination provides robust support for the argument that exercise enhances, rather than suppresses immunity. Indeed, at least eight studies have demonstrated heightened vaccination responses in older adults, typically over 60 years of age, who are physically active (144–151). For example, an early study categorized adults aged 62 years or older, into one of three groups: active (undertaking at least 20 min of vigorous exercise three or more times per week), moderately active (undertaking regular exercise but with lower intensity, frequency, and/or duration), or sedentary (non-exercisers). Two weeks after influenza vaccination, it was shown that serum anti-influenza IgG and IgM titers were higher in active versus sedentary adults, and so too were peripheral blood mononuclear cell responses to antigen-specific stimulation (144). In addition, a recent study has shown that men aged 65–85 years, who regularly undertook moderate or vigorous exercise training, exhibited higher antibody responses compared to inactive controls in response to an influenza vaccine (151). Data linking habitual levels of physical activity to enhanced immune competency in humans are supported by evidence from animal studies, and show that the immunological benefits of exercise may be particularly beneficial in enhancing otherwise poor responses in older age (152).

Interpreting Data From Vaccine Trials

A major criticism of vaccine and exercise trials conducted in humans is that many solely focus on the maximal antibody titer following vaccination, and it is not practical to follow-up with investigations into infection incidence as a gauge of protection status following vaccination (153). As such, it is unknown whether differences observed with absolute antibody titers, or the amount of IFN-gamma produced from stimulated T cells, between exercise and control groups, is representative of clinically meaningful benefits in terms of protection from infections. Three elegant studies in rodents imply that benefits, beyond antibody titer, may be brought about by acute exercise. In one of these studies, it was found that antibody responses to influenza exposure were *lower* in rodents that exercised around the time of exposure, compared to those that did not exercise, yet, exercised mice were still protected and did not exhibit any signs of infection upon re-exposure to the virus (154). Moreover, in an earlier study by the same authors, it was found that mice undertaking an acute bout of exercise before being expose to influenza exhibited a lower severity of infection and had enhanced viral clearance and lower inflammation in the lungs in the days following (155). Thus, it may be the case that exercise enhances immune responses, beyond those captured using maximal antibody titer as an endpoint. In accordance with this view, an elegant rodent study conducted by an independent group (156) found that mice exercised for 20–30 min at moderate intensity 4 h after intranasal influenza exposure had a substantially higher survival rate (18 of 22 survived; 82%) when compared to mice that did not exercise after influenza exposure (10 of 23 survived; 43%).

Contact Sensitivity Reactions and Acute Exercise Bouts

More recent studies have examined the effects of acute exercise on immune competency using other *in vivo* models of immune challenge that, in principle, also assess the coordinated efforts of immune system components. These studies have employed contact-sensitivity reactions by topically applying to skin, the dendritic cell and T cell stimulant (or attractant) diphenylcyclopropenone (DPCP), and the non-specific inflammatory stimulant, croton oil (157, 158). For example, in studies of young adults (approximately 20–30 years of age), by applying a primary sensitizing dose of DPCP 20 min after 2 h of moderate-intensity treadmill running, and assessing recall challenge 4 weeks later, it has been concluded that this form of exercise impairs both the induction of T cell immunity and the memory response (159, 160). Thirty minutes of moderate- or vigorous-intensity running had no effect, and no forms of exercise modulated the non-specific inflammatory challenge in response to croton oil (159, 160). Although these findings are biologically interesting, the clinical relevance of exercise-induced change is unclear, in part, because the process of DPCP-induced immune modulation is not well defined (158) unlike the immune response to antigen administration by vaccination.

Summary: Exercise Enhances Immune Responses to *In Vivo* Antigenic Challenge

We conclude that there is growing evidence from a powerful array of studies in humans and rodents, indicating that exercise enhances, or at least does not suppress immune responses to *in vivo* challenge in younger and older individuals. These observations—which contradict those predicated by the "open-window" hypothesis—support the contention that an acute bout of exercise has no detrimental immune consequences for health. Thus, exercise should be encouraged, particularly for older adults who are at greatest risk of infections and who may obtain the greatest exercise-induced benefits to immune competency; an overview of the impact of aging on the immunological benefits that can be attained from exercise is described next.

Does Aging Influence the Immunological Benefits of Exercise and Regular Physical Activity?

Effect of a Single Exercise Bout

Research investigating the effects of exercise on immune function has sought to establish whether the observed benefits, as outlined earlier in young adults, such as exercise-induced immune cell mobilisation, that has been implicated in protection against cancer, is also applicable to older adults. For example, it has been reported that the magnitude of T cell mobilization

in response to acute vigorous exercise is smaller in older (65 ± 1 years) compared to younger (22 ± 1 years) adults (161, 162). However, in this study, it was also shown that following exercise, the magnitude of T cell proliferation in response to mitogens was smaller in young adults, whereas a similar exercise-induced stimulation of natural killer cell cytotoxicity was observed for both groups (161, 162). It is beyond the scope of this review to fully critique investigations examining the influence of single exercise bouts on the function of different immune cells, with comparisons made between younger and older individuals across the lifespan; we refer the reader to comprehensive reviews on this topic (163–165). Nevertheless, it is important to point out that many studies in this area are difficult to interpret: at the time of their publication, the influence of *Cytomegalovirus* infection on the magnitude of exercise-induced immune responses was not known and was therefore not considered (123, 166). Moreover, while the magnitude of change to lymphocyte kinetics is likely to be important for detecting and eliminating viral and bacterial infections and neoplastic cells, this process is complex to study, and comparisons between younger and older people is difficult, partly due to other age-associated changes that influence the physiological response to exercise, such as the decline in fitness (e.g., sarcopenia, cardiorespiratory fitness) with age. It is likely that some, or all of these factors impact upon on the efficacy of exercise as an adjuvant to vaccination responses in older adults, outlined earlier in the section "Exercise and Immune Responses to Experimental *In Vivo* Challenge Across the Lifespan." In light of the challenge interpreting the clinical relevance of the aforementioned lymphocyte kinetics studies, from here onward, we briefly evaluate the impact of regular physical activity or frequent exercise bouts on immune competency across the lifespan, using measurements in samples collected from participants at rest.

Frequent Bouts of Exercise

Immune competency at rest has been assessed in cross-sectional studies, comparing elderly individuals differentiated by physical activity level or cardiorespiratory fitness, or by examining immune function before and after structured exercise training interventions. For example, it has commonly been reported among the elderly, that the most active participants, compared to those who are least active, show the highest T cell proliferation and cytokine production in response to mitogens (163–165). Fewer studies have assessed innate immune competency, but higher natural killer cell cytotoxicity has been consistently shown among the elderly who are active compared to less active age-matched controls (163–165). Recent studies have expanded measurements into other innate immune cells such as neutrophils. For example, a recent cross-sectional study of 211 elderly adults, showed that neutrophils from the 20 most active participants, compared to the 20 least active participants, migrated toward interleukin (IL)-8 with greater chemotactic accuracy, but there were no differences in chemotactic speed (167). In addition, a recent exercise training study has shown in both young and middle-aged adults, that 10 weeks of moderate-intensity continuous cycling training, or high-intensity interval cycling training, improve neutrophil and monocyte phagocytosis and oxidative burst irrespective of age (168). Improvements in these common measurements of immune competency, however, are not always consistent in longitudinal studies employing exercise training interventions, with around half of studies reporting improvements, and half reporting no change (163–165). One reason for this could be because the dramatic effects of *Cytomegalovirus* on driving immunological aging was not considered by most of these studies, and it is feasible that results would be different when examining individuals who are latently infected compared to those who are seronegative. Importantly however, no studies report impaired immune competency from increased participation in structured exercise. Altogether, we conclude that despite declines in fitness and immune competency, aging does not appear to negate the immunological benefits that can be attained from exercise, and indeed, frequent participation in exercise across the lifespan may lead to immune benefits, even in older age.

PART C: DOES EXERCISE AND REGULAR PHYSICAL ACTIVITY INFLUENCE IMMUNOLOGICAL AGEING?

Since the first exercise immunology research in the early 1900s, and the substantial increase in scientific interest from the late 1980s and early 1990s (169), studies examining interaction between immune function and lifestyle factors such as exercise and physical activity have become common. Although a few exercise studies published in the last 10–15 years have investigated some immunological processes relevant to aging, health, and disease, the theme of exercise-induced "immune suppression" continues to influence the design of new studies or at least features in the interpretation of findings and is often justified or contextualized with relevance to self-reported illness symptoms among athletes. As outlined in Part A "Is it Time to Close the Shutters on the "Open-Window" Hypothesis? A Bout of Exercise Does Not Suppress Immune Competency," there is limited reliable evidence to show that exercise heightens risk of opportunistic infections, but there is, however, a growing body of evidence to show that exercise enhances, rather than suppresses, immune competency, as summarized in Part B: "Regular Physical Activity and Frequent Exercise Augment Aspects of Immune Competency Across the Lifespan." The beneficial effects of exercise on immune function are likely to be greatest for elderly people exhibiting the age-associated deterioration of immune competency, also referred to as immunosenescence. Moreover, preliminary evidence suggests that physical activity and regular structured exercise may even limit or delay immunological aging. Here, in Part C: "Does Exercise and Regular Physical Activity Influence Immunological Ageing?" we evaluate whether an active lifestyle influences immunosenescence.

The Aging Immune System

Aging is associated with profound changes to the numerical, phenotypic, and functional capacity of almost all innate and adaptive immune cells, resulting in altered immune responses. Some innate immune cells exhibit numerical, phenotypic, and functional alterations with aging, whereas others appear to be less affected (170).

For example, the numbers and functions of eosinophils, basophils, and mast cells appear to be largely unchanged with aging, or at least, there is not a clear effect of age based on the limited current literature (170). Neutrophil numbers often increase with aging, but these cells exhibit diminished phagocytosis and impaired chemotaxis, although chemokinesis is maintained (170). Natural killer cells increase with aging, driven by an accumulation of cytotoxic $CD56^{dim}$ cells but a decline in regulatory $CD56^{bright}$ cells, and overall, cytokine production and cytotoxicity are less on a per cell basis (170). Other innate lymphocytes, such as invariant CD1d-restricted natural killer T cells (iNKT cells), which represent <1% of the T cell pool, decline in number but age-associated changes to their function have not been established (171). Monocyte numbers are stable with aging, but classical cells ($CD14^{++}CD16^{-}$) decline, and intermediate ($CD14^{+}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) cells increase, but overall, monocyte cytokine production is impaired (170, 172). These changes with blood monocytes are thought to be mirrored by tissue-resident macrophages, whereby classically activated M1 cells decline, and alternatively activated M2 cells accumulate (173). However, alterations in tissue-resident cells with advancing age are very likely to be a result of adipose tissue accumulation and dysfunction that also occurs in parallel with aging (174, 175). Indeed, inflamed adipose tissue attracts macrophages with cell-surface characteristics similar to M2 alternatively activated cells—often assumed to be anti-inflammatory. However, despite their cell-surface phenotype, these cells are potent producers of inflammatory cytokines in adipose tissue, and likely drive age- and obesity-associated inflammation (176–179). Thus, the M1/M2 paradigm for macrophages is likely to be an over-simplification (180, 181). It is unknown if other, primarily tissue-resident cells, are affected by adipose tissue dysfunction, but with aging, the number and function of dendritic cells have been reported to decrease in the skin and mucosal membranes (170). There is also an age-associated increase in myeloid-derived suppressor cells—a heterogeneous population of granulocytes, macrophages, and dendritic cells—that may impair aspects of immune function by producing reactive oxygen species and inhibitory cytokines (182).

Within the adaptive immune system, there are substantial changes to the numbers, function, and phenotype of T cells with aging. Among the broad population of $CD4^{+}$ T-helper cells, aging is associated with a predominance of Th2 (i.e., IL-4 and IL-10), and Th17-producing cells (i.e., IL-17-producing cells that are associated with autoimmune disease), whereas there is a decline in cells with a Th1 profile [i.e., IFN-gamma- and tumor necrosis factor-alpha (TNF-alpha)-producing cells] (183, 184). With aging, the numbers and proportions of antigen-inexperienced $CD4^{+}$ and $CD8^{+}$ T cells decreases (e.g., $CD27^{+}CD28^{+}CD45RA^{+}CD57^{-}CD62L^{+}CCR7^{+}KLRG1^{-}$ naïve cells) (185, 186). In parallel, the numbers and proportions of antigen-experienced $CD4^{+}$ and $CD8^{+}$ T cells increases (e.g., $CD27^{-}CD28^{-}CD45RA^{+}CD57^{+}CD62L^{-}CCR7^{-}KLRG1^{+}$ memory cells), and these cells are potent producers of inflammatory cytokines (185, 186). These changes are driven by lower hematopoietic stem cell numbers, thymic involution resulting in reduced output of antigen-naïve T cells, and infection with latent viruses, in particular *Cytomegalovirus* (185, 187). With aging, T cells that express natural killer cell-associated cell-surface proteins (NKT-like cells) also accumulate, exhibiting similar changes to their phenotype, functional properties, and specificities as with the broader population of $CD4^{+}$ and $CD8^{+}$ T cells (171). There is an age-associated decline in the total number of γδ T cells; however age *per se*, in the absence of chronic infections, is associated with a decline in Vδ2 cells (60–80% of γδ T cells), whereas Vδ1 (15–20% γδ T cells) remain stable (188). Some evidence suggests that γδ T cell proliferative responses are impaired with aging, perhaps due to increased susceptibility of Vδ2 cells to apoptosis (189, 190). Natural regulatory T cells increase with aging whereas inducible regulatory T cells decrease, but it is unclear if their function is affected (191). As with T cells, aging is associated with a decline in the numbers and proportions of naïve B cells, an accumulation of memory B cells with limited specificities, and impaired plasma cell antibody production (192).

Several robust and accepted hallmarks of immunosenescence have been established, especially within the adaptive immune system. For example, low numbers and proportions of naïve T cells (in particular $CD8^{+}$ T cells) and high numbers and proportions of memory T cells (especially late-stage differentiated $CD8^{+}$ T cells) are well established biomarkers (185, 186, 193, 194). In addition, a cluster of parameters, revealed in longitudinal studies of octogenarians and nonagenarians from an isolated population in Sweden, have been referred to as the immune risk profile (195–197). Biomarkers included low numbers and proportions of B cells, high numbers and proportions of late-stage differentiated $CD8^{+}$ T cells (i.e., $CD27^{-}CD28^{-}$), poor T cell proliferation in response to mitogens, a CD4:CD8 ratio of <1.0, infection with *Cytomegalovirus* and high plasma IL-6, which together, predicted greater all-cause mortality at 2-, 4-, and 6-year follow-up (195, 197–200). Indeed, the age-associated increase in systemic inflammation, referred to as "inflammageing" is another principle observation among aging and longevity studies (201, 202). Subsequently, high levels of IL-6, TNF-alpha, and C-reactive protein, have been associated with shorter survival (203–205). Overall, it is well established that elderly individuals exhibit impaired immune responses to *in vivo* challenge with novel antigens (143, 206, 207) and these individuals are subsequently thought to be at increased risk of infection. Encouragingly however, as outlined in Part B: "Regular Physical Activity and Frequent Exercise Augment Aspects of Immune Competency Across the Lifespan" exercise can be a potent stimulus of immune function, including the response to vaccination, and some evidence suggests that exercise might delay or limit the age-associated decline in immune competency.

Relationships Between Exercise and Regular Physical Activity With Hallmarks of an Aging Adaptive Immune System

As summarized in Part B: "Regular Physical Activity and Frequent Exercise Augment Aspects of Immune Competency Across the Lifespan" many studies have examined the influence of regular physical activity or frequent structured exercise on the function of the adaptive immune system with aging (163–165). Here, we focus on recent studies that have examined relationships between exercise, physical activity or cardiorespiratory fitness, and the numbers and proportions of $CD4^{+}$ and $CD8^{+}$ naïve and memory

T cells, as hallmarks of immunosenescence. Indeed, a small number of studies have investigated whether the characteristics of the T cell pool are influenced by an active lifestyle. There is a larger body of evidence in young adults, typically between 18 and 30 years, compared to older adults, hereafter considered as being over 40 years of age due to the characteristics of studies published to date. If an active lifestyle can be linked with a smaller accumulation of memory T cells, and a smaller decline in naïve T cells, then in young adults, one interpretation might be that exercise prevents, or at least delays immunosenescence, whereas in older adults, these associations could be interpreted as countering or limiting the development of an age-associated immune profile.

Experimental Evidence in Young People: Can Exercise Prevent or Delay Aging of the Adaptive Immune System?

The characteristics of the T cell pool have been examined in 16 young adults (50% male; 18 ± 2 years) classified as being very active (well-trained soccer players self-reporting 9–12 h of exercise per week) and compared to 16 young adults (50% male; 19 ± 2 years) classified as being untrained (individuals self-reporting 2–3 h of exercise per week). Untrained individuals showed the highest proportions of $CD4^+$ and $CD8^+$ memory T cells, and the lowest proportions of $CD8^+$ naïve T cells, defined on the basis of CD57 and CD28 expression (208). Although these results suggest that regular exercise might limit the age-associated accumulation of memory T cells and decline in naïve T cells, the effects were strongly influenced by sex: only untrained males exhibited high proportions of memory T cells and low proportions of naïve cells compared to trained males, and these effects were driven by changes in the $CD4^+$ T cell pool (208). Extending these findings by examining a female-only population of young adults, the same authors compared 13 well-trained soccer players (self-reporting around 12 h of exercise per week; 20 ± 2 years) to 13 untrained controls (self-reporting around 3 h of exercise per week; 21 ± 2 years) (209). Trained females exhibited a greater proportion of $CD8^+$ naïve T cells compared to untrained females, but these associations did not remain statistically significant after controlling for body fat percentage (trained 21.7 ± 4.0 versus untrained 25.1 ± 4.1%, $p < 0.05$) (209). Indeed, very few studies have considered whether relationships between an active lifestyle and markers of an aging immune system could be influenced by other factors, such as body composition. Recently, a very small study of 15 males aged 30 ± 4 years, categorized participants using a combination of gold standard methods for measuring physiological and lifestyle variables (210). Three groups were formed (sedentary, active, and very active) on the basis of objectively assessed habitual physical activity, directly measured cardiorespiratory fitness, and body composition assessed with dual energy X-ray absorptiometry (210). This work showed that sedentary individuals had higher proportions of memory $CD4^+$ T cells expressing CD45RO and PD-1, supporting the results of other published studies that have not taken body composition into consideration.

It should be emphasized, that among younger adults in particular, mixed results have been reported when investigating links between an active lifestyle and hallmarks of an aging immune system. Most investigations have been cross-sectional in design, or have made observations between groups over short periods. For example, one study has shown that national standard triathletes (age range 18–36 years, $n = 19$), appear to exhibit impaired thymic output, assessed by T cell receptor excision circle levels (211). Among $CD4^+$ and $CD8^+$ T cells, these athletes had lower absolute numbers of naïve cells and higher absolute numbers of memory cells compared to age-matched less active controls ($n = 16$), as shown by CD45RA, CD45RO, and CD27 expression (211). Similar observations have been made by examining individuals in the third decade of life, showing that endurance athletes, compared to less active controls, appear to exhibit slightly larger accumulations of memory T cells and slightly fewer naïve T cells, defined by CD45RA and CCR7 expression (212). Thus, it appears that among younger individuals (i.e., less than 40 years of age), if exercise is undertaken at very extreme volumes, such as more than 5–10 times the amount of physical activity recommended each week (i.e., 12–25 h per week), this might contribute toward a small decline in naïve T cells and a small increase in memory T cells. It is possible that these changes are due to reactivation of latent viruses, which could be independent of immune function, and driven by exercise-induced adrenergic activity, oxidative stress and inflammatory cytokines (48–50). However, these results might also be explained by fluctuations in cell numbers and cell sub-populations in peripheral blood over time. Such changes have been interpreted as being linked to exercise training load (213, 214), but it is also conceivable that these changes occur due to seasonal variation, as has been shown in non-exercise contexts (215, 216).

Experimental Evidence in Older People: Can Exercise Limit or Counter Aging of the Adaptive Immune System?

Although most studies have examined associations between biomarkers of an aging adaptive immune system in young adults, other studies have made measurements across a broader range of ages. For example, one study examined 102 men ranging in age from 18 to 61 years (mean 39 ± 6 years) (217). It was shown that the proportion of the $CD4^+$ and $CD8^+$ T cell pool comprising of memory cells (defined as $KLRG1^+CD57^+$ or $KLRG1^+CD28-$) was inversely correlated with cardiorespiratory fitness, which is largely indicative of an active lifestyle (217). The age-associated decrease of naïve T cells (defined as $KLRG1^-CD57^-$ or $KLRG1^-CD28^+$) and increase in memory T cells did not withstand statistical adjustment for cardiorespiratory fitness, but remained significant after adjusting statistically for body composition and *Cytomegalovirus* infection (217). Thus, it was concluded that fitter individuals exhibit a smaller age-associated decline of naïve T cells and a smaller accumulation of memory T cells.

As with work examining relationships between an active lifestyle and hallmarks of an aging adaptive immune system in young and middle-aged adults, similar associations have been shown in an older population of 61 men aged 65–85 years (218). In this study, participants self-reported to be "untrained" ($n = 15$), or to lead a "moderate" training lifestyle ($n = 16$;

taking part in team sports or running less than 6 km two to three times per week), or an "intense" training lifestyle ($n = 15$; running approximately 10 km at least 5 days per week). These categories were confirmed with a validated physical activity questionnaire and by measurement of cardiorespiratory fitness. Both training groups exhibited a lower proportion of $CD4^+$ and $CD8^+$ memory cells (defined as $CD45RA^+CCR7^-$), but these associations were largest and only statistically significant among men leading an "intense" training lifestyle, suggesting a dose–response effect of exercise. Although findings were less clear when examining other cell subpopulations based on CD45RA and CCR7 expression, and there were no effects of exercise when examining $CD28^-$ cells, men in the "trained" groups had T cells with the longest telomeres (218). Another recent study compared 125 adults (55–79 years of age) who maintained a high level of cycling throughout life to 75 age-matched inactive controls (219). Within the $CD4^+$ and $CD8^+$ T cell pool, the frequency of naive cells (defined as $CD45RA^+$) was greater, and the frequency of memory cells (defined as CD45RA-) was lower among cyclists. Extended phenotyping revealed that $CD4^+$ and $CD8^+$ CCR7-$CD45RA^+$ accumulation was less among cyclists, but no differences were found for CD28-$CD57^+$ cells. Cyclists also exhibited higher frequencies of recent thymic emigrants and regulatory B cells, lower Th17 polarization, and in plasma, higher IL-7 and lower IL-6 (219). Despite these findings, suggesting a beneficial effect of leading an active lifestyle on immunosenescence among older adults, there is some inconsistency in the literature. For example, comparing elderly athletes ($n = 12$, approximately 74 years of age) to less active age-matched controls ($n = 26$), there were no differences in thymic output, the proportions of naïve or memory $CD4^+$ and $CD8^+$ T cells (defined with CD45RA and CCR7 expression), or T cell activation in response to anti-CD3 stimulation (212). Most investigations of T cell immunosenescence and lifestyle among healthy elderly adults have had cross-sectional study designs. Longitudinal studies, or randomized and controlled trials of exercise training are lacking and might yield promising results. For example, one study has compared 6 months of exercise training in men and women ($n = 28$, aged 61–76 years) to a similar group who maintained their current lifestyle ($n = 20$, aged 62–79 years) (220). Using a simple immunophenotyping strategy, the results showed that the proportion of $CD4^+$ T cells expressing CD28 increased in the exercise group after 6 months, but not in those who maintained their lifestyle (220).

Summary of Experimental Evidence

In summary, evidence shows that the characteristics of the T cell pool appear to be influenced by leading an active lifestyle, determined by exercise training, physical activity level, or cardiorespiratory fitness. It seems that among both the young and elderly, an active lifestyle is generally linked to lower numbers and proportions of memory T cells and higher numbers and proportions of naïve T cells (10). This summary is partly supported by a recent systematic review, concluding that regular structured exercise increases the number of naïve T cells in peripheral blood at rest (221). Altogether, findings from recent studies examining relationships between an active lifestyle and the characteristics of the T cell pool—as robust and accepted biomarkers of immunosenescence—support observations from some cross-sectional and longitudinal studies, showing that other measures of immune competency, which typically decline with aging, can be improved with physical activity or regular structured exercise (163–165). However, further research is needed in this area that employs precise lifestyle measurements (e.g., using wearable technology to assess physical activity, and dual energy X-ray absorptiometry to measure body composition) and more robust measurements of immune competency (e.g., absolute cell counts rather than proportions, measurements of cell function, and *in vivo* antigen challenges) while controlling for factors that drive immunosenescence (e.g., inflammation and *Cytomegalovirus* infection).

Mechanisms

Links between a physically active lifestyle with lower numbers or proportions of memory T cells, and higher numbers or proportions of naïve T cells, have been hypothesized as being driven by the acute effects of exercise bouts. For example, it has been suggested that repeated bouts of exercise might prevent or delay immunological aging by limiting the accumulation of $CD4^+$ and $CD8^+$ antigen-experienced memory T cell clones, repopulating blood with antigen-inexperienced naïve T cells (11, 12). In this hypothesis, it is proposed that memory T cells are frequently mobilized into blood during regular bouts of exercise, followed by an extravasation to peripheral tissues, where these cells are exposed to pro-apoptotic stimuli, such as reactive oxygen species, glucocorticoids, and cytokines (11, 12). Subsequently, it is proposed that the number of naïve T cells increases as part of a negative feedback loop governing the number of naïve and memory cells in the immune system, which is bolstered by exercise-induced thymopoiesis and extra-thymic T cell development (11, 12). Supporting the mechanisms proposed in this hypothesis, many investigations have shown that memory T cells are mobilized into the circulation during exercise, followed by extravasation out of the bloodstream in the hours following (81, 123). In addition, studies in mice show that lymphocyte apoptosis occurs post-exercise in tissues thought to be the homing destination of mobilized cells (222). Although some T cells mobilized by exercise might be resistant to apoptosis, given that *Cytomegalovirus*-specific $CD8^+$ T cells express high levels of Bcl-2 (223), other work has shown that *Cytomegalovirus*-specific $CD8^+$ T cells, are equally as susceptible to Fas-induced apoptosis as the total pool of $CD8^+$ T cells (224). Further, irrespective of virus specificity, studies have shown that T cells expressing cell-surface proteins such as CD57 and KLRG1 are more susceptible to H_2O_2-induced apoptosis than total lymphocytes and naïve T cells (225, 226). Thus, the concept of exercise directly countering memory T cell accumulation is supported by evidence from human and animal studies.

It is unknown whether triggering apoptosis among expanded clones of memory T cells specific for viruses such as *Cytomegalovirus* is advantageous. For example, in a transplant

setting, *Cytomegalovirus* disease occurs when T cells fail to provide antiviral control (227) and a robust pro-inflammatory response to *Cytomegalovirus* has been associated with longer survival in the elderly (228). However, it remains to be determined what proportion of the T cell pool needs to be specific for *Cytomegalovirus* to limit viral reactivation. Infection with *Cytomegalovirus* results in approximately 10% of the $CD4^+$ and $CD8^+$ T cell pool becoming specific for this virus (229), although large inter-individual differences exist. For example, it has been reported that 23% of the $CD8^+$ T cell pool can become specific for a single *Cytomegalovirus* epitope (223). Traditionally, it has been considered disadvantageous for such a large proportion of the T cell pool to be specific for one virus. This view is linked to another age- or infection-associated change that occurs in parallel—a fall in the numbers and proportions of naïve cells—which has been interpreted as limiting capacity to engage novel antigens. These interpretations are based upon two assumptions. First, there is an upper limit to the size of the immune system, and second, thymic output is negligible after adolescence (230). Thus, it has been proposed that antigen-inexperienced naïve T cells could be "used up" due to ongoing differentiation into antigen-experienced memory T cells that "fill up" immunological "space" (230). It has also been proposed that this accumulation of antigen-experienced memory T cells leads to "squeezing out" of T cells targeting less dominant viruses leading to loss of viral control (231). This concept of a fixed amount of immunological space has since been debated (232, 233) and thymic output is now known to persist, albeit reduced, up until around 70 years of age (234). However, even if removal of some memory T cells is not essential for maintaining an effective T cell pool, assuming these cells contribute to systemic inflammation, their removal might limit "inflammageing" (230). Despite uncertainties over the susceptibility of memory T cells to undergo apoptosis, or whether it is advantageous to stimulate their removal, it seems that exercise-induced immune cell death in the tissues has relevance to other processes. For example, apoptotic cells and cell debris stimulates hematopoietic stem cell mobilization into blood (95) perhaps promoting trafficking to the thymus or extrathymic sites facilitating output of naïve T cells (235). Additional support for exercise stimulating production of naïve T cells is provided by work showing that contracting skeletal muscle produces IL-7 (236) which might increase thymic mass and function (237).

A physically active lifestyle might also counter T cell immunosenescence indirectly, perhaps by limiting adipose tissue accumulation and dysfunction that occurs with aging and obesity (174, 238, 239). Indeed, obesity has been linked with impaired lymphocyte proliferation (240), shorter leukocyte telomere length (241), and a skewing of the T cell pool toward a regulatory and Th2-phenotype (242). In addition, large expansions of differentiated αβ T cells and γδ T cells have been shown among people with obesity, with γδ T cells exhibiting impaired antiviral function (243–245). It is generally accepted that repeated stimulation with antigens from *Cytomegalovirus* drives immunosenescence (185, 186, 193, 194). With obesity, adipose tissue is the primary source of pro-inflammatory cytokines and reactive oxygen species (174, 246) which can reactivate *Cytomegalovirus* directly (48, 49). Thus, exercise might limit T cell immunosenescence by decreasing visceral and subcutaneous adipose tissue (238), providing a potent anti-inflammatory and anti-oxidative stimulus (247, 248). In turn, lower systemic inflammation and better redox balance might limit viral reactivation, reducing stimulation with antigens from viruses such as *Cytomegalovirus*. In addition, T cell dysfunction might also be prevented, in part, by limiting reactive oxygen species production (249).

In summary, leading a physically active lifestyle appears to limit the age-associated changes to the cellular composition of the adaptive immune system, but the mechanisms are yet to be determined. Exercise might counter the expansion of memory T cells directly, which is desirable assuming these cells contribute to systemic inflammation and not all are required to control latent viruses. Limiting the expansion of memory T cells also assumes the "size" of the immune system is fixed, the capacity to produce antigen-naïve T cells is limited, and these constraints contribute to immune decline in the elderly. However, exercise might affect memory T cell accumulation indirectly, by reducing viral reactivation, or preventing T cell senescence, by controlling adipose tissue deposition and dysfunction that drives inflammation and oxidative stress with aging and obesity.

CONCLUDING REMARKS

Contemporary evidence from epidemiological studies shows that leading a physically active lifestyle reduces the incidence of communicable (e.g., bacterial and viral infections) and non-communicable diseases (e.g., cancer), implying that immune competency is enhanced by regular exercise bouts. However, to this day, research practice, academic teaching, and even physical activity promotion and prescription continues to consider a prevailing myth that exercise can temporarily suppress immune function. We have critically reviewed related evidence, and conclude that regular physical activity and frequent exercise are beneficial, or at the very least, are not detrimental to immunological health. We summarize that (i) limited reliable evidence exists to support the claim that exercise suppresses cellular or soluble immune competency, (ii) exercise *per se* does not heighten the risk of opportunistic infections, and (iii) exercise can enhance *in vivo* immune responses to bacterial, viral, and other antigens. In addition, we present evidence showing that regular physical activity and frequent exercise might limit or delay immunological aging. We conclude that leading an active lifestyle is likely to be beneficial, rather than detrimental, to immune function, which may have implications for health and disease in older age.

AUTHOR CONTRIBUTIONS

JC and JT contributed equally toward literature searching and retrieval, the ideas and interpretation of the studies described, drafting and revision of the manuscript, and approval of the final version to be published. JT and JC both agreed to be accountable for the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Impact of Aging on the Frequency, Phenotype, and Function of CD161-Expressing T Cells

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Immune-aging is associated with perturbed immune responses in the elderly. CD161-expressing T cells, i.e., the previously described subsets of CD161$^+$ CD4$^+$ T cells, CD161^{high} CD8$^+$ T cells, and CD161^{int} CD8$^+$ T cells, are highly functional, pro-inflammatory T cells. These CD161-expressing T cells are critical in immunity against microbes, while possibly contributing to autoimmune diseases. So far, little is known about the impact of aging on the frequency, phenotype, and function of these CD161-expressing T cells. In the current study, we investigated the impact of aging on CD161$^+$ CD4$^+$ T cells, CD161^{high} CD8$^+$ T cells, and CD161^{int} CD8$^+$ T cells in peripheral blood samples of 96 healthy subjects (age 20–84). Frequencies of CD161$^+$ CD4$^+$ T cells and CD161^{int} CD8$^+$ T cells were stable with aging, whereas frequencies of CD161^{high} CD8$^+$ T cells declined. Although CD161^{high} CD8$^+$ T cells were mostly T cell receptor-Vα7.2$^+$ mucosal-associated invariant T cells, CD161 expressing CD4$^+$ and CD8$^+$ T cells showed a limited expression of markers for gamma–delta T cells or invariant natural killer (NK) T cells, in both young and old subjects. In essence, CD161-expressing T cells showed a similar memory phenotype in young and old subjects. The expression of the inhibitory NK receptor KLRG1 was decreased on CD161$^+$ CD4$^+$ T cells of old subjects, whereas the expression of other NK receptors by CD161-expressing T cells was unaltered with age. The expression of cytotoxic effector molecules was similar in CD161^{high} and CD161^{int} CD8$^+$ T cells of young and old subjects. The ability to produce pro-inflammatory cytokines was preserved in CD161^{high} and CD161^{int} CD8$^+$ T cells of old subjects. However, the percentages of IFN-γ^+ and interleukin-17$^+$ cells were significantly lower in CD161$^+$ CD4$^+$ T cells of old individuals than those of young individuals. In addition, aging was associated with a decrease of nonclassic T helper 1 cells, as indicated by decreased percentages of CD161-expressing cells within the IFN-γ^+ CD4$^+$ T cell compartment of old subjects. Taken together, aging is associated with a numerical decline of circulating CD161^{high} CD8$^+$ T cells, as well as a decreased production of pro-inflammatory cytokines by CD161$^+$ CD4$^+$ T cells. These aging-associated changes could contribute to perturbed immunity in the elderly.

Keywords: aging, T lymphocytes subsets, CD161, cytokines, T helper 1 cells, T helper 17 cells, mucosal-associated invariant T cell

Abbreviations: CM, central memory; CMV, cytomegalovirus; DN, double negative; DNAM-1, DNAX accessory molecule-1; EM, effector memory; IL, interleukin; LLT1, lectin-like transcript 1; iNKT cell, invariant natural killer T cell; MAIT cell, mucosal-associated invariant T cell; NK, natural killer; TCR, T cell receptor; TD, terminally differentiated; Th1, T helper 1; Th17, T helper 17; TNF-α, tumor necrosis factor-α.

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INTRODUCTION

Aging-associated perturbations of the immune system have been linked to increased risks for infections and autoimmune diseases in the elderly (1, 2). Insight into these immunological changes may eventually help to develop strategies to correct immunological disturbances in aged subjects.

The T cell compartment is especially affected by aging. The production of naive T cells shows a strong aging-associated decline, which starts already early in life (3). By contrast, a shift toward the memory T cell compartment develops upon antigenic stimulation by environmental stimuli (4). Consequently, the maintenance of the already existing naive and memory T cells is important to preserve immunity during adult life (5). Another aging-associated change in the T cell compartment of humans includes the acquisition of natural killer (NK) receptors and cytotoxic effector molecules by aged T cells (6, 7). In addition, the balance between pro-inflammatory and anti-inflammatory T cells is disturbed in the elderly (8). Thus, aging affects the numerical, phenotypic, and functional aspects of both the naive and memory T cell compartment in humans.

Cytomegalovirus (CMV) infection also has a broad impact on the T cell compartment (9). Latent infection with CMV is associated with skewing of the T cell receptor (TCR) repertoire (4). CMV drives the expansion of memory T cells, both in the circulation and in the peripheral tissues (10, 11). CMV-specific memory T cells may express NK receptors and are potent producers of pro-inflammatory cytokines, such as IFN-γ and tumor necrosis factor-α (TNF-α) (12–14). Furthermore, CMV has been linked to poor vaccination responses and a slightly increased mortality rate in the elderly (9, 15–17). Overall, CMV contributes to immune senescence in humans (9).

Ample evidence indicates that the expression of the C-type lectin receptor CD161 identifies subsets of $CD4^+$ and $CD8^+$ T cells with a strong pro-inflammatory phenotype (18–20). These CD161-expressing T cells are considered important for antimicrobial immunity in humans (20–24). However, CD161-expressing T cells may also contribute to the development of various autoimmune diseases (25–28). CD161 is expressed by $CD4^+$ T helper 17 (Th17) lineage cells and nonclassic T helper 1 (Th1) cells (18, 29–31). In addition, two subsets of $CD8^+$ T cells with different expression levels of CD161 have been identified (19, 20). Firstly, high expression levels of CD161 ($CD161^{high}$) are found on a subset of innate-like $CD8^+$ T cells termed mucosal-associated invariant T (MAIT) cells (32, 33). Another population of $CD8^+$ T cells is characterized by intermediate expression levels of CD161 ($CD161^{int}$) and contributes to antiviral immunity (20). Although several studies have shown that frequencies of MAIT cells decrease with age (34–36), little is known about the effect of aging on the other CD161-expressing T cell subsets.

In the current study, we assessed the impact of aging on the frequencies, phenotype, and function of CD161-expressing $CD4^+$ and $CD8^+$ T cells in the peripheral blood of humans. While taking into account CMV serostatus, we investigated the effect of age on the numbers of circulating CD161-expressing T cells in a large group of healthy subjects. Furthermore, we studied the expression of T cell differentiation markers, NK receptors, cytotoxic effector molecules, and pro-inflammatory cytokines by CD161-expressing T cells of young and old subjects. Taken together, our study provides a unique and comprehensive insight into the effect of aging on highly functional, pro-inflammatory T cell populations in humans.

MATERIALS AND METHODS

Study Subjects and Samples

Blood samples were obtained from 96 healthy individuals (age range 20–84, of which 26 were males) Subjects underwent a thorough examination of their health status, as described previously (8). Exclusion criteria included infection, malignancy, autoimmune disease, chronic liver or kidney disease, alcohol or drug abuse, diabetes mellitus, current pregnancy, or immunosuppressive treatment. Written informed consent was obtained from all study participants. The study was approved by the Medical Ethical Committee of the UMCG. All procedures were in accordance with the Declaration of Helsinki.

Flow Cytometry

Blood samples (EDTA) were stained with the following monoclonal antibodies: CD3-eFluor605, CD4-eFluor450, TCRγδ-PE (eBioscience), CD45RO-FITC, CCR7-PE-Cy7, CD4-PerCP, CD8-PerCP, CD8-APC-H7, DNAX accessory molecule-1 (DNAM-1)-FITC (BD), CD161-PE, CD161-APC (Miltenyi), 2B4-PE, NKG2D-PE-Cy7, TCR-Vα7.2-FITC, TCR-Vα24-Jα18-FITC (Biolegend), TCR-Vβ11-PE (Beckman Coulter), and KLRG1-FITC (generous gift from H. Pirchner). An overview of antibody panels is shown in Table S1 in Supplementary Material. Samples were subsequently treated with BD Lysing Solution (BD Biosciences) according to instructions of the manufacturer. Samples were measured on a LSR-II flow cytometer (BD) and analyzed with Kaluza Analysis Software (Beckman Coulter). In addition, the absolute numbers of circulating lymphocyte subsets were determined according to the MultiTest TruCount method (BD), as described by the manufacturer. Data were acquired on a FACSCanto-II flow cytometer (BD) and analyzed with FACSCanto Clinical Software (BD). The number of events for a particular T cell population needed to be more than 100 to allow for subsequent analysis of cellular markers, cytokines, and cytotoxic molecules.

Intracellular Cytokine Staining

Blood samples (heparin) were diluted 1:1 with RPMI and stimulated with 40 nM PMA and 2 nM Ca^{2+} ionophore A23187 in the presence of 3 μM brefeldin A (all Sigma) for 4 h. Subsequently, red blood cells were lysed with ammonium chloride. The remaining cells were treated with Fix/Perm reagents A and B (Invitrogen) and stained with the following antibodies: CD3-eFluor 605, CD4-eFluor450, interleukin (IL)-17-AF488, Perforin-FITC (eBioscience), CD161-APC (Miltenyi), CD8-APC-H7, Granzyme-B-PE (BD), IFN-γ-PerCP-Cy5.5, IL-4-PE, and TNF-α-PerCP-Cy5.5 (Biolegend). Samples were measured on an LSR-II flow cytometer (BD) and analyzed with Kaluza Analysis Software (Beckman Coulter).

Measurement of CMV-Specific IgG

As previously described (4), 96-well ELISA plates (Greiner) were coated overnight with lysates of CMV-infected fibroblasts. Lysates of non-infected fibroblasts were used as negative controls. Following the coating, diluted serum samples were incubated for 1 h. Goat anti-human IgG was added and incubated for 1 h. Samples were incubated with phosphatase for 15 min, and the reaction was stopped with NaOH. The plates were scanned on a Versamax reader (Molecular Devices). A pool of sera from three CMV-seropositive individuals with known concentrations of CMV-specific IgG was used to quantify CMV-specific IgG in the tested samples. Detailed information on CMV serostatus of the healthy donors is shown for experiments reporting the phenotype and function of CD161-expressing T cells in Table S2 in Supplementary Material.

Statistics

The Mann–Whitney *U*-test was used to compare continuous variables between different age groups. Correlations were determined with Spearman's rank correlation coefficient. Univariate and multivariate linear regression analyses were used to further determine the impact of age and CMV serostatus on CD161-expressing T cell counts in healthy subjects. Firstly, each variable was tested in a univariate linear regression analysis. Subsequently, variables with $p < 0.3$ in the univariate analysis were used in the multivariate analysis. Reported *B*-coefficients indicate how much cell counts (10^9/L) change with every unit increase of the tested variable. The categorical variable CMV serostatus was assigned a value of 0 or 1. CMV: 0 = seronegative, 1 = seropositive. Non-normally distributed outcome variables were transformed (i.e., log or square root). Analysis was performed with SPSS 23.0 Software and GraphPad Prism 5.0. *P*-values less than 0.05 were considered to be significant.

RESULTS

Characterization of Healthy Subjects

To investigate the impact of aging on CD161-expressing T cells, we recruited a group of healthy subjects with a wide age range. Detailed characteristics of the healthy subjects are shown in **Table 1**. Young (age 18–39), intermediate (age 40–59), and old (age ≥60) subjects showed a similar distribution of gender and CMV serostatus. Furthermore, general laboratory parameters were comparable among the different age groups.

TABLE 1 | Subject characteristics.

	Young (age 18–39)	Intermediate (age 40–59)	Old (age ≥60)
No.	22	24	50
Age, years, median (range)	27 (20–38)	57 (40–59)	69 (60–84)
No. male (%)	6 (27)	6 (25)	14 (28)
No. CMV positive (%)	12 (55)	11 (46)	27 (54)
Hemoglobin, mmol/L, median (range)[a]	8.3 (7.3–9.5)	8.5 (7.5–10.0)	8.6 (7.1–10.2)
Leukocyte count, 10^9/L, median (range)[a]	4.7 (2.8–7.5)	5.3 (3.5–8.2)	5.8 (2.9–9.1)
Thrombocyte count, 10^9/L, median (range)[a]	275 (173–422)	240 (161–381)	240 (121–350)
ESR, mm/h, median (range)[a]	6 (1–16)	8 (1–15)	8 (2–26)
Creatinine, μmol/L, median (range)[a]	62 (53–90)	71 (58–89)	69 (49–97)

No., number; ESR, erythrocyte sedimentation rate.
[a]Performed in 11/22 young individuals, 19/24 intermediate age individuals, and 50/50 aged individuals.

Aging Is Associated With a Decrease of Circulating CD161^{high} CD8$^+$ T Cells

Firstly, we determined the impact of age on total CD3$^+$, CD4$^+$, and CD8$^+$ T cell numbers in our study population. The numbers of CD3$^+$ T cells, CD4$^+$ T cells, and CD8$^+$ T cells were stable with age (**Figures 1A,B**).

Next, we studied the effect of age on the numbers of CD161-expressing T cells. In accordance with prior studies (18–20), we identified CD161$^+$ cells within the CD4$^+$ T cell compartment, as well as CD8$^+$ T cells with high and intermediate expression levels of CD161, i.e., CD161^{high} and CD161^{int} cells (**Figure 1C**). The absolute numbers of CD161$^+$ CD4$^+$ T cells showed a slight decrease with age ($p = 0.04$), whereas the absolute numbers of CD161$^-$ CD4$^+$ T cells remained stable (**Figure 1D**). The percentages of CD161$^+$ CD4$^+$ T cells showed a statistically significant inverse correlation with age (Figure S1A in Supplementary Material). The absolute numbers of CD161^{high} and CD161$^-$ CD8$^+$ T cells showed a negative correlation with age, while the absolute numbers of CD161^{int} CD8$^+$ T cells remained stable (**Figure 1E**). Percentages of CD161^{high} CD8$^+$ T cells were also negatively associated with age (Figure S1B in Supplementary Material). However, due to the absolute decrease of CD161^{high} and CD161$^-$ CD8$^+$ T cells, the percentage of CD161^{int} CD8$^+$ T cells increased with age. These findings suggest that aging might impact the numbers of CD161-expressing T cells.

Subsequently, we performed a multivariate analysis in order to evaluate the impact of both aging and CMV serostatus on the absolute numbers of CD161-expressing T cells. In this analysis, neither aging nor CMV showed an effect on the numbers of CD161$^+$ and CD161$^-$ CD4$^+$ T cells (**Table 2**). Aging was associated with declining numbers of CD161^{high} CD8$^+$ T cells, whereas CMV seropositivity was linked to a higher number of CD161^{int} CD8$^+$ T cells. Independent effects of aging and CMV serostatus were also observed on the absolute numbers of CD161$^-$ CD8$^+$ T cells. Taken together, aging is associated with a decrease of CD161^{high} CD8$^+$ T cells, whereas the absolute numbers of CD161^{int} CD8$^+$ T cells and CD161$^+$ CD4$^+$ T cells remain stable.

Aging Does Not Affect the Expression of Innate-Like T Cell Markers by CD161-Expressing T Cells

Previous studies have reported that gamma–delta T cells, invariant natural killer T (iNKT) cells, and MAIT cells may express CD161 (33, 37, 38). Therefore, we investigated if CD161-expressing T cells of young and old subjects express markers defining these innate-like T cells. Gamma–delta T cells were identified by the expression of the TCRγδ receptor (**Figure 2A**) in young and old subjects with comparable CMV-seropositivity rates (Figure S2 in Supplementary Material). The TCRγδ receptor was present on few CD161$^+$ CD4$^+$ T cells, both in young and in old subjects

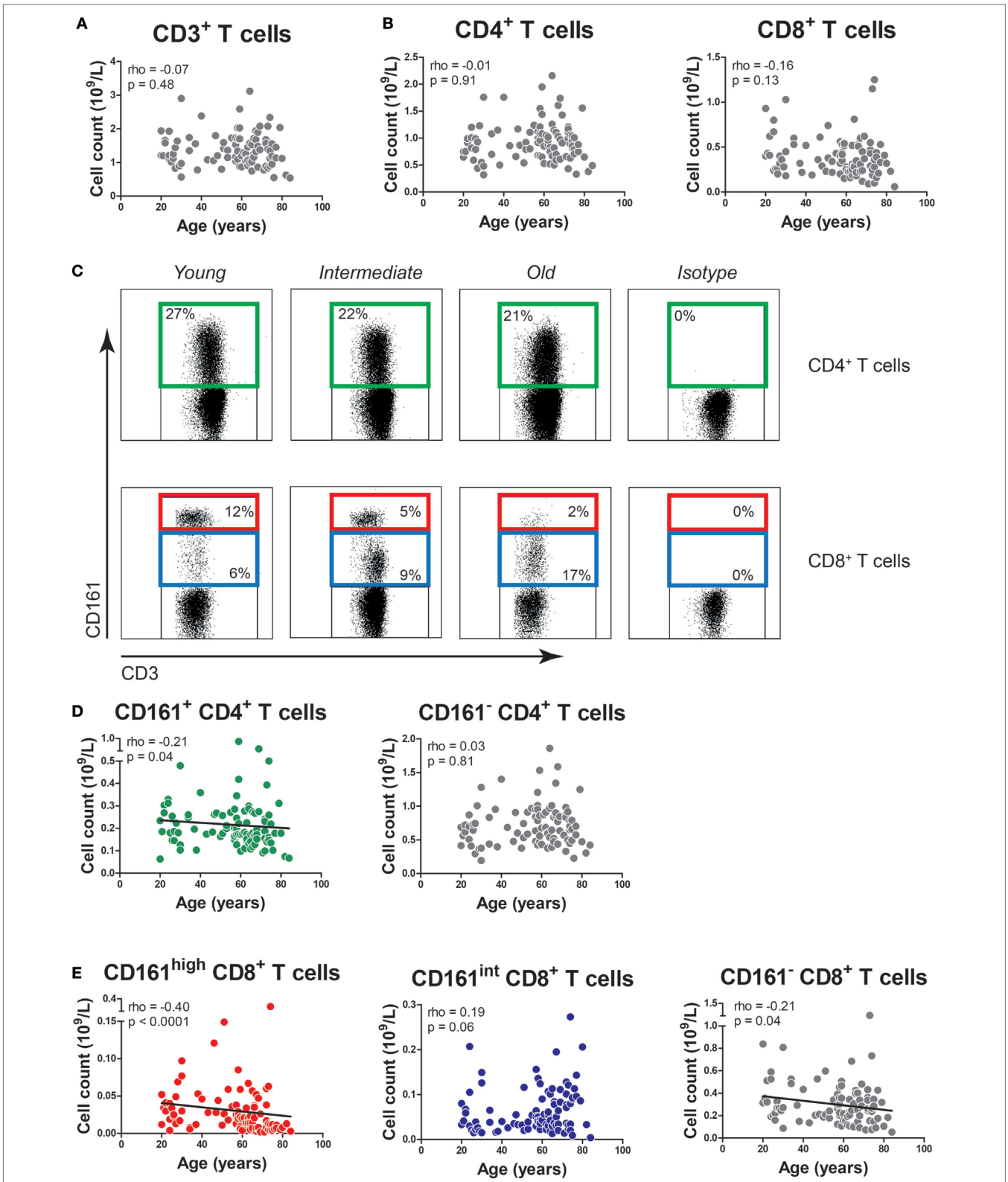

FIGURE 1 | The effect of age on the absolute numbers of CD161-expressing T cells. **(A)** The absolute numbers of CD3+ T cells, as well as **(B)** CD4+ T cells and CD8+ T cells in the peripheral blood of 96 healthy subjects (age range 20–84). **(C)** Flow cytometric gating of CD161+ CD4+ T cells, CD161^{high} CD8+ T cells, and CD161^{int} CD8+ T cells in the peripheral blood of healthy young, intermediate, and old subjects. **(D)** The absolute numbers of CD161+ CD4+ T cells, CD161− CD4+ T cells, and **(E)** CD161^{high} CD8+ T cells, CD161^{int} CD8+ T cells, and CD161− CD8+ T cells in the peripheral blood of same donors as mentioned at **(A,B)**. Statistical significance by Spearman's rank test is shown.

TABLE 2 | Univariate and multivariate linear regression analysis for the absolute numbers of CD161-expressing T cell subsets.

Dependent variable	Predicting variables	Univariate analysis *B* (95% CI)	*p*-value	Multivariate analysis *B* (95% CI)	*p*-value
CD161+ CD4+ (10^9/L)	Age	−0.001 (−0.002 to 0.000)	0.176	−0.001 (−0.002 to 0.000)[a]	0.158
	CMV	0.027 (−0.008 to 0.068)	0.133	0.034 (−0.008 to 0.084)[a]	0.120
CD161− CD4+ (10^9/L)	Age	0.001 (−0.002 to 0.004)	0.426	†	
	CMV	0.030 (−0.083 to 0.152)	0.614	†	
CD161^{high} CD8+ (10^9/L)	Age	−0.001 (−0.002 to 0.000)	0.001	−0.001 (−0.002 to 0.000)[b]	0.001
	CMV	−0.004 (−0.010 to 0.004)	0.294	−0.010 (−0.026 to 0.012)[b]	0.318
CD161^{int} CD8+ (10^9/L)	Age	0.000 (0.000 to 0.001)	0.156	0.000 (0.000 to 0.000)[c]	0.159
	CMV	0.030 (0.014 to 0.051)	0.000	0.021 (0.010 to 0.037)[c]	0.000
CD161− CD8+ (10^9/L)	Age	−0.003 (−0.006 to −0.001)	0.016	−0.003 (−0.005 to −0.001)[d]	0.006
	CMV	0.122 (0.059 to 0.200)	0.000	0.199 (0.101 to 0.320)[d]	0.000

Data are obtained for 96 healthy individuals (age range 20–84). Firstly, each variable was tested in a univariate linear regression analysis. Subsequently, variables with $p < 0.3$ in the univariate analysis were used in the multivariate analysis. Reported B-coefficients indicate how much cell counts (10^9/L) change with every unit increase of the predicting variable. The categorical variable CMV was assigned a value of 0 or 1, where 0 = CMV seronegative and 1 = CMV seropositive. Age: in years. CI = confidence interval.

[a]*Model $R^2 = 0.045$.*

[b]*Model $R^2 = 0.120$.*

[c]*Model $R^2 = 0.184$.*

[d]*Model $R^2 = 0.233$.*

†*Not tested in multivariate analysis due to $p > 0.3$ in univariate regression analysis.*

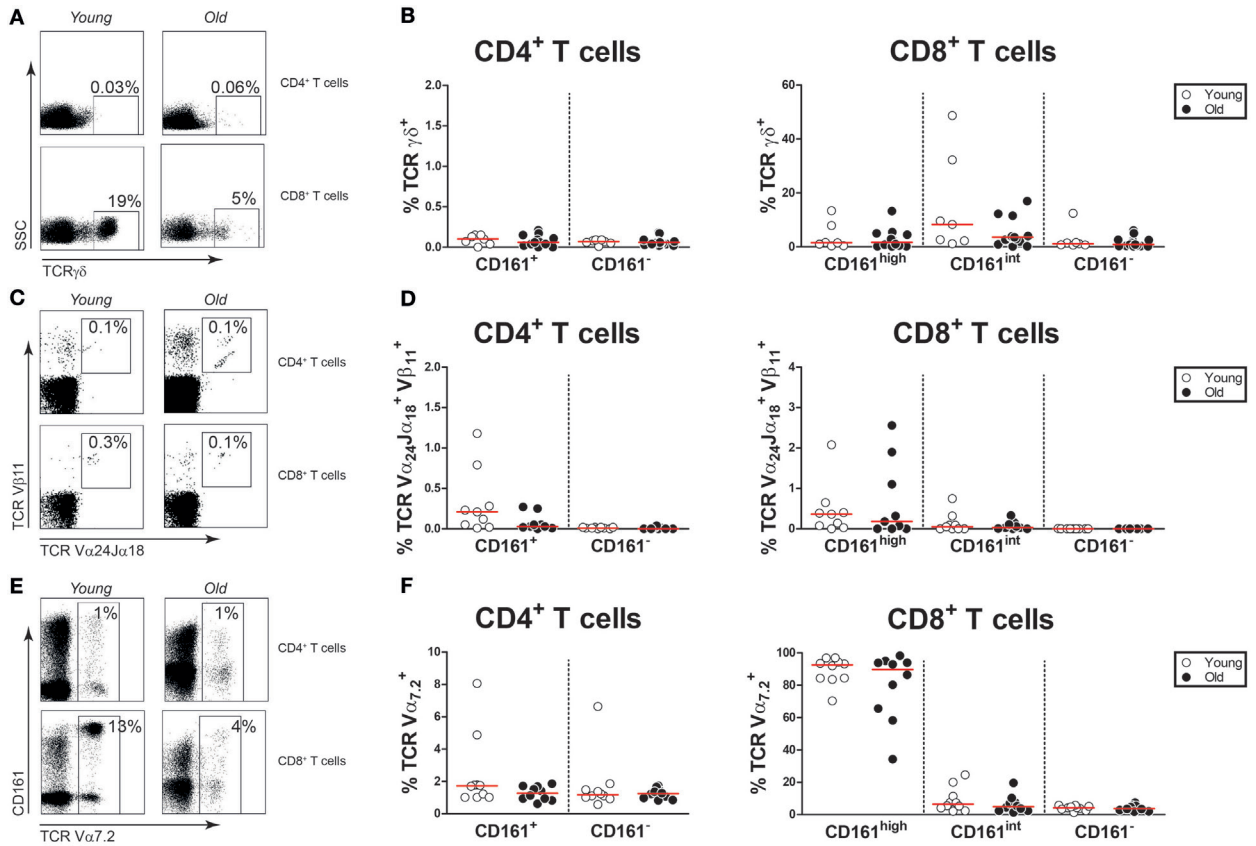

FIGURE 2 | Innate-like T cell markers on CD161-expressing T cells. **(A)** Flow cytometric gating of TCRγδ+ cells in the CD4+ and CD8+ T cell compartment of a young and old subject. **(B)** Percentages of TCRγδ+ cells within the CD161-defined CD4+ and CD8+ T cell subsets of 7 young [of which 4 were cytomegalovirus (CMV) seropositive] and 16 old (of which 8 were CMV seropositive) subjects. **(C)** Flow cytometric gating of TCR-Vα24-Jα18+TCR-Vβ11+ cells in the CD4+ and CD8+ T cell compartment of a young and old subject. **(D)** Percentages of TCR-Vα24-Jα18+ TCR-Vβ11+ cells within the CD161-defined CD4+ and CD8+ T cell subsets of nine young (of which four were CMV seropositive) and nine old (of which four were CMV seropositive) subjects. **(E)** Flow cytometric gating of TCR-Vα7.2+ cells in the CD4+ and CD8+ T cell compartment of a young and old subject. **(F)** Percentages of TCR-Vα7.2+ cells within the CD161-defined CD4+ and CD8+ T cell subsets of 10 young (of which 5 were CMV seropositive) and 10 old (of which 5 were CMV seropositive) subjects. Graphs in which CMV-seropositive young and old subjects are highlighted in the figures are shown in Figure S2 in Supplementary Material.

(**Figure 2B**). Percentages of TCRγδ+ cells were limited within the CD161high and CD161int CD8+ T cell subsets, and no differences were observed between young and old subjects. iNKT cells were identified as TCR-Vα24-Jα18 and TCR-Vβ11 co-expressing cells (**Figure 2C**). Overall, percentages of iNKT cells were low and similar among the CD161-expressing T cell subsets of young and old subjects (**Figure 2D**). To delineate MAIT cells, additional staining was performed for the TCR-Vα7.2 receptor (**Figure 2E**). Although limited expression of TCR-Vα7.2 was observed among all CD161-defined T cell populations, the expression of this receptor was largely restricted to CD161high CD8+ T cells, with similar percentages observed in young and old subjects (**Figure 2F**). Overall, the expression of innate-like T cell markers by CD161-expressing CD4+ and CD8+ T cells was not affected by age.

We subsequently investigated the effect of aging on proportions of the CD161-expressing innate-like T cell subsets within the total CD3+ T cell compartment. Percentages of TCRγδ+ cells, i.e., gamma–delta T cells, were similar in young and old subjects (**Figure 3A**). The same was true for percentages of CD161+ TCRγδ+ CD8+ T cells (**Figure 3B**). CD161+ TCRγδ+ CD4+ T cells were nearly absent, both in young and in old subjects (data not shown). However, a small portion of TCRγδ+ cells showed a CD4/CD8 double-negative (DN) phenotype. No difference was observed for percentages of CD161+ TCRγδ+ DN T cells within the CD3+ T cell pool of young and old subjects (**Figure 3C**). Proportions of TCR-Vα24-Jα18 and TCR-Vβ11 co-expressing cells, i.e., iNKT cells, were comparable in young and old subjects (**Figure 3D**). The low frequencies of iNKT cells precluded any further sub-analyses of these cells. Percentages of CD161high

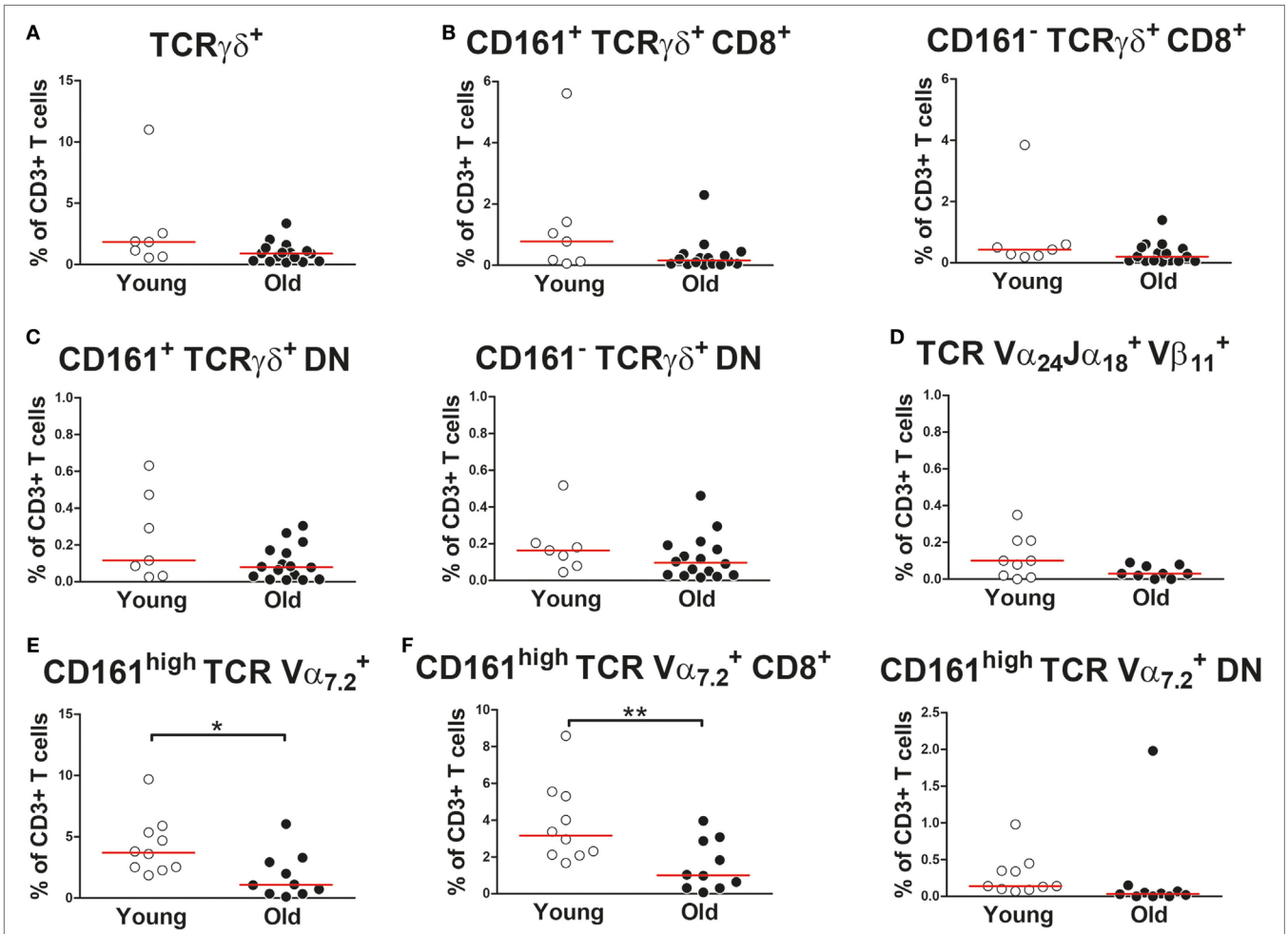

FIGURE 3 | Proportions of CD161-expressing innate-like T cells in the CD3+ T cell compartment **(A)** Percentages of total TCRγδ+ cells (i.e., gamma–delta T cells) [**(B)** left panel], CD161+ CD8+ gamma–delta T cells [**(B)** right panel], CD161− CD8+ gamma–delta T cells [**(C)**, left panel], CD161+ double-negative (DN) gamma–delta T cells, and [**(C)**, right panel] CD161− DN gamma–delta T cells within the CD3+ T cell compartment of 7 young [of which 4 were cytomegalovirus (CMV) seropositive] and 16 old (of which 8 were CMV seropositive) subjects. **(D)** Percentages of TCR-Vα24-Jα18+TCR-Vβ11+ cells (i.e., invariant natural killer T cells) within the CD3+ T cell compartment of nine young (of which four were CMV seropositive) and nine old (of which four were CMV seropositive) subjects. **(E)** Percentages of total CD161high TCR-Vα7.2+ mucosal-associated invariant T (MAIT) cells, [**(F)**, left panel] CD8+ MAIT cells, and [**(F)**, right panel] DN MAIT cells within the CD3+ T cell compartment of 10 young (of which 5 were CMV seropositive) and 10 old (of which 5 were CMV seropositive) subjects. Statistical significance by Mann–Whitney *U*-test is shown as $^{*}p < 0.05$ or $^{**}p < 0.01$. Graphs in which CMV-seropositive young and old subjects are highlighted in the figures are shown in Figure S3 in Supplementary Material.

TCR-Vα7.2+ MAIT cells were lower in the CD3+ T cell compartment of old subjects than that of young subjects (**Figure 3E**). This decrease resulted from a decrease of CD8+ MAIT cells rather than DN MAIT cells (**Figure 3F**). As previously reported by others (32, 35), CD4+ MAIT cells were nearly absent (data not shown). Thus, aging is associated with a decrease of CD8+ MAIT cells.

Aging Is Not Associated With Major Changes in the Memory Phenotype of CD161-Expressing T Cells

Next, we questioned if aging would impact the differentiation status of CD161-expressing CD4+ and CD8+ T cells. Based on the expression of CD45RO and CCR7, we identified naive (N; CD45RO−CCR7+), central memory (CM) (CD45RO+CCR7+), effector memory (EM) (CD45RO+CCR7−), and terminally differentiated (TD) (CD45RO−CCR7−) cells in the peripheral blood of the healthy subjects (**Figure 4A**). CD161+ CD4+ T cells were predominantly CM and EM cells, irrespective of age (**Figure 4B**). Nearly all CD161high CD8+ T cells showed an EM cell phenotype (**Figure 4C**). Similar percentages of EM cells were observed among CD161high CD8+ T cells of young and old subjects, whereas a small increase of CM cells was observed in the old. CD161int CD8+ T cells were mostly EM and TD cells, with similar percentages in young and old subjects. In contrast to the relatively stable differentiation phenotype of CD161-expressing T cells, a clear

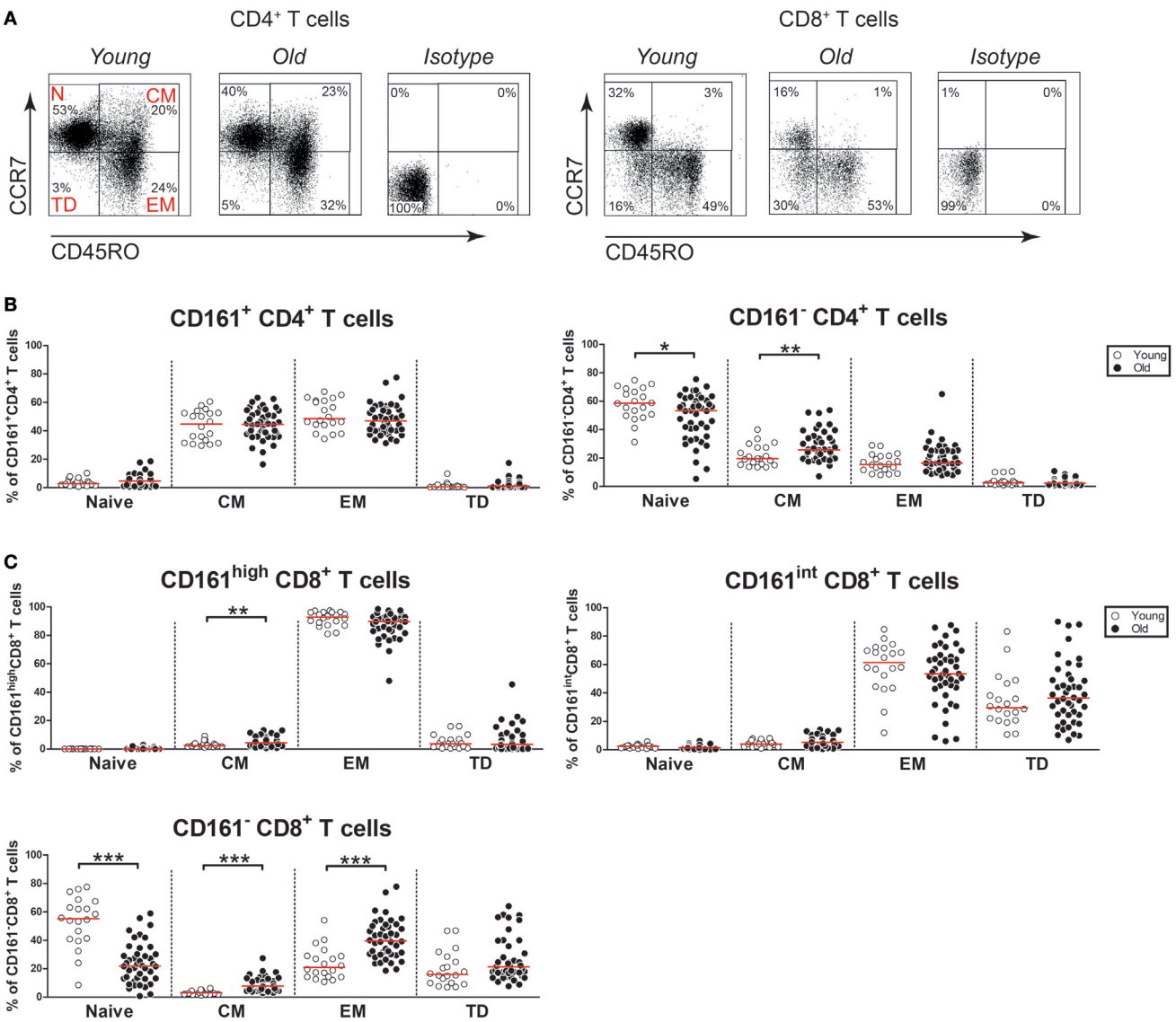

FIGURE 4 | Differentiation markers on CD161-expressing T cells. **(A)** Flow cytometric gating of naive cells (N; CD45RO−CCR7+), central memory cells (CM) (CD45RO+CCR7+), effector memory cells (EM) (CD45RO+CCR7−), and terminally differentiated cells (TD) (CD45RO−CCR7−) within the CD4+ and CD8+ T cell compartment of a young and old subject. **(B)** Percentages of differentiation subsets within the CD161-defined CD4+ T cell subsets of 20 young [of which 10 were cytomegalovirus (CMV) seropositive] and 44 old (of which 22 were CMV seropositive) subjects. **(C)** Percentages of differentiation subsets within the CD161-defined CD8+ T cell subsets of the same donors as mentioned at **(B)**. Statistical significance by Mann–Whitney *U*-test is shown as $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$. Graphs in which CMV-seropositive young and old subjects are highlighted in the figures are shown in Figure S4 in Supplementary Material.

shift from naive toward memory cells was observed among the CD161⁻ CD4⁺ and CD161⁻ CD8⁺ T cell fractions (**Figures 4B,C**). Thus, aging showed no substantial effect on the differentiation status of CD161-expressing T cells.

Aging Has Limited Effect on NK Receptor Expression by CD161-Expressing T Cells

As aging is associated with the expression of NK receptors by T cells (6, 7), we next investigated the expression of NK receptors on CD161-expressing CD4⁺ and CD8⁺ T cells of young and old subjects. Firstly, we evaluated the activating NK receptor 2B4 on the CD4⁺ and CD8⁺ T cell subsets (**Figure 5A**). Few CD161⁺ CD4⁺ T cells expressed 2B4, without any difference between young and old subjects (**Figure 5B**). Nearly all CD161^{high} and CD161^{int} CD8⁺ T cells showed the expression of 2B4, irrespective of age. Next, we studied the activating NK receptor DNAM-1 (**Figure 5C**). The majority of CD161⁺ CD4⁺ T cells, CD161^{high} CD8⁺ T cells, and CD161^{int} CD8⁺ T cells expressed DNAM-1 (**Figure 5D**). Among these T cell subsets, the percentages of DNAM-1⁺ cells were similar in young and old

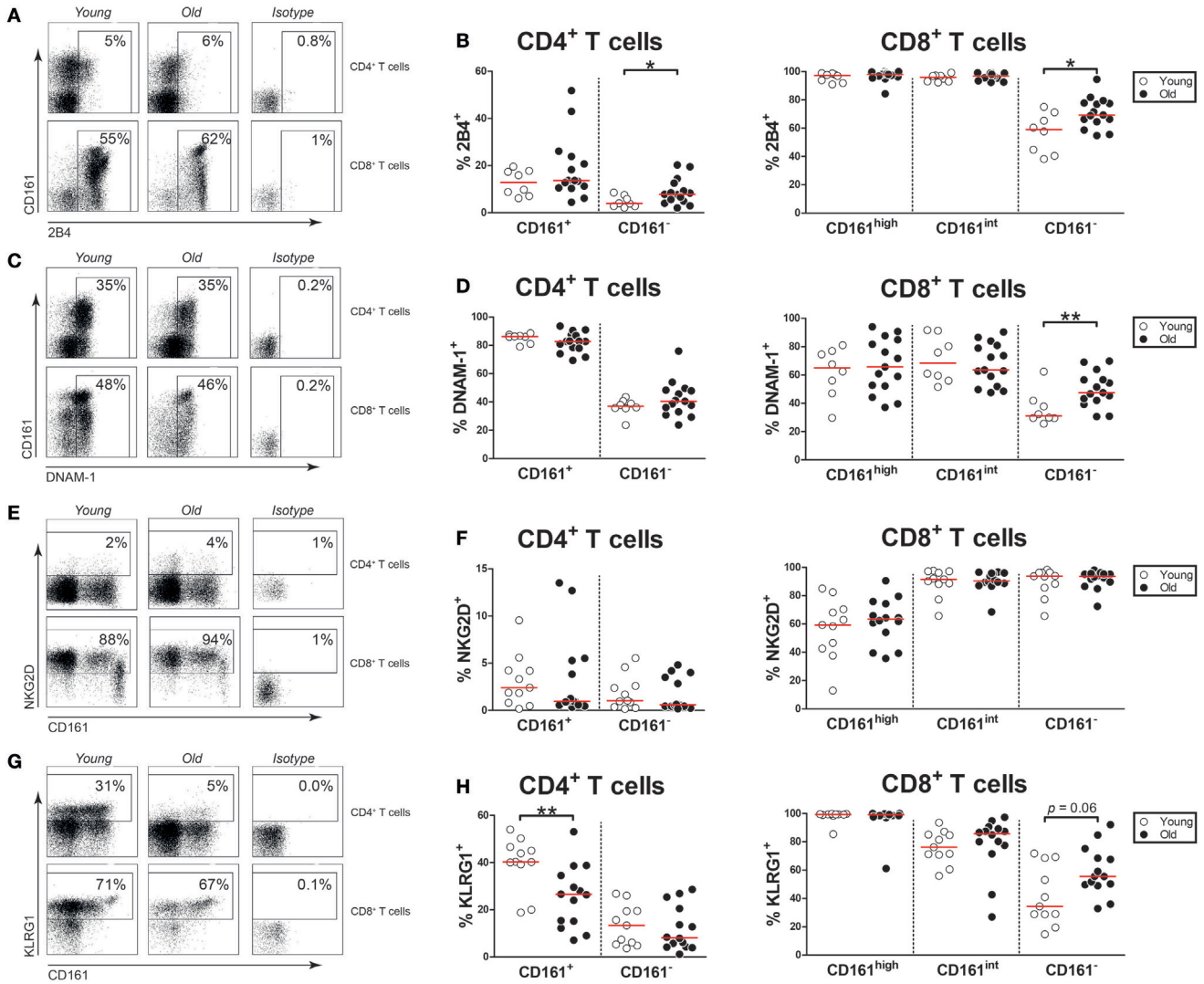

FIGURE 5 | Natural killer markers on CD161-expressing T cells. **(A)** Flow cytometric gating of 2B4⁺ cells within the CD4⁺ and CD8⁺ T cell compartment of a young and old subject. **(B)** Percentages of 2B4⁺ cells within the CD161-defined CD4⁺ and CD8⁺ T cell subsets of 8 young [of which 4 were cytomegalovirus (CMV) seropositive] and 15 old (of which 7 were CMV seropositive) subjects. **(C)** Flow cytometric gating of DNAX accessory molecule-1 (DNAM-1)⁺ cells within the CD4⁺ and CD8⁺ T cell compartment of a young and old subject. **(D)** Percentages of DNAM-1⁺ cells within the CD161-defined CD4⁺ and CD8⁺ T cell subsets of the same subjects as shown in **(B)**. **(E)** Flow cytometric gating of NKG2D⁺ cells within the CD4⁺ and CD8⁺ T cell compartment of a young and old subject. **(F)** Percentages of NKG2D⁺ cells within the CD161-defined CD4⁺ and CD8⁺ T cell subsets of 11 young (of which 6 were CMV seropositive) and 14 old (of which 8 were CMV seropositive) subjects. **(G)** Flow cytometric gating of KLRG1⁺ cells within the CD4⁺ and CD8⁺ T cell compartment of a young and old subject. **(H)** Percentages of KLRG1⁺ cells within the CD161-defined CD4⁺ and CD8⁺ T cell subsets of the same subjects as shown in **(F)**. Statistical significance by Mann–Whitney *U*-test is shown as $^{*}p < 0.05$ or $^{**}p < 0.01$. Graphs in which CMV-seropositive young and old subjects are highlighted in the figures are shown in Figure S5 in Supplementary Material.

subjects. Subsequently, the expression of the activating receptor NKG2D was evaluated (**Figure 5E**). Equally low percentages of NKG2D+ cells were observed among CD161+ CD4+ T cells of young and old individuals (**Figure 5F**). A significant proportion of CD161^{high} CD8+ T cells and nearly all CD161^{int} CD8+ T cells expressed NKG2D, with similar percentages in young and old subjects. Next, we evaluated the expression of the inhibitory receptor KLRG1 (**Figure 5G**). A significant portion of CD161+ CD4+ T cells expressed KLRG1 in young subjects, but the percentage of KLRG1+ cells was significantly lower in CD161+ CD4+ T cells of old subjects (**Figure 5H**). Most CD161^{high} and CD161^{int} CD8+ T cells expressed KLRG1, with similar percentages in young and old subjects. Taken together, aging was associated with a decreased expression of KLRG1 on CD161+ CD4+ T cells, while the expression of the other NK receptors was stable.

Aging Has Limited Effect on Cytotoxic Effector Molecule Expression by CD161-Expressing T Cells

Next, we questioned if aging would affect the expression of cytotoxic effector molecules, i.e., perforin and granzyme B, by CD161-expressing CD4+ and CD8+ T cells. Few CD161+ CD4+ T cells and approximately half of CD161^{high} and CD161^{int} CD8+ T cells expressed perforin (**Figures 6A,B**). In this respect, no difference was observed between young and old subjects. The expression of granzyme B was limited in CD161+ CD4+ T cells and CD161^{high} CD8+ T cells, whereas a significant portion of CD161^{int} CD8+ T cells expressed this cytotoxic effector molecule (**Figures 6C,D**). The expression of granzyme B was similar among the CD161-expressing subsets of young and old individuals. Overall, the expression of perforin and granzyme B among CD161-expressing cells was rather stable with age.

Aging Is Associated With Decreased IFN-γ and IL-17 Expression by CD161+ CD4+ T Cells

As CD161-expressing T cells are potent producers of pro-inflammatory cytokines, we investigated if aging impacts this function of CD161-expressing T cells. In contrast to CD161− CD4+ and CD8+ T cells, a substantial portion of CD161+ CD4+ T cells and the vast majority of CD161^{high} and CD161^{int} CD8+ T cells were capable of producing IFN-γ upon PMA/Ca^{2+}-ionophore stimulation (**Figures 7A–C**). Interestingly, percentages of IFN-γ producing cells were lower among CD161+ CD4+ T cells of old subjects than those of young subjects. By contrast, slightly more CD161^{int} CD8+ T cells were capable of producing IFN-γ in old subjects than young subjects. IL-17 was mostly produced by CD161+ CD4+ T cells and CD161^{high} CD8+ T cells (**Figures 7D–F**). Whereas the percentage of IL-17-producing cells was similar in CD161^{high} CD8+ T cells of young and old subjects, the percentage of IL-17+ cells was decreased in CD161+ CD4+ T cells of old individuals. A small fraction of CD161+ CD4+ T cells was capable of producing IL-4, with no differences between young and old subjects (**Figures 7G,H**). The expression of IL-4 was nearly absent in CD161^{high} and CD161^{int} CD8+ cells, irrespective

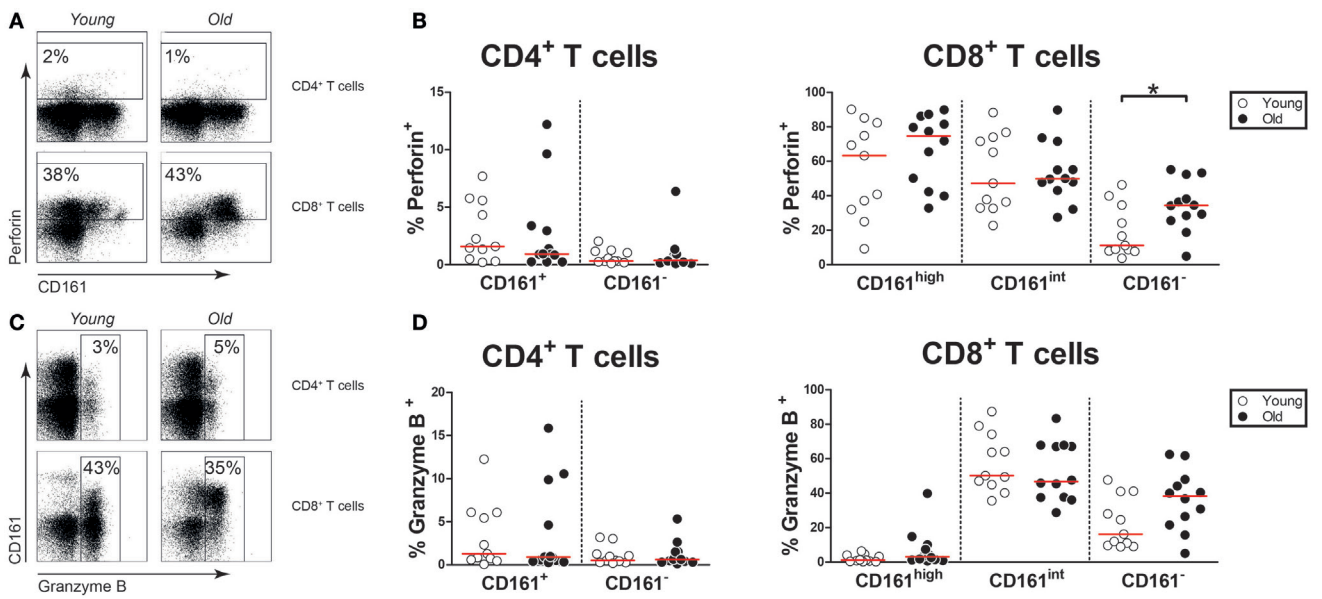

FIGURE 6 | Cytotoxic effector molecules in CD161-expressing T cells. Intracellular staining for perforin and granzyme B was performed on non-stimulated blood samples. **(A)** Flow cytometric gating of perforin+ cells within the CD4+ and CD8+ T cell compartment of a young and old subject. **(B)** Percentages of perforin+ cells within the CD161-defined CD4+ and CD8+ T cell subsets of 11 young [of which 7 were cytomegalovirus (CMV) seropositive] and 12 old (of which 8 were CMV seropositive) subjects. **(C)** Flow cytometric gating of granzyme B+ cells within the CD4+ and CD8+ T cell compartment of a young and old subject. **(D)** Percentages of granzyme B+ cells within the CD161-defined CD4+ and CD8+ T cell subsets of the same subjects as shown in **(B)**. Statistical significance by Mann–Whitney *U*-test is shown as $*p < 0.05$. Graphs in which CMV-seropositive young and old subjects are highlighted in the figures are shown in Figure S6 in Supplementary Material.

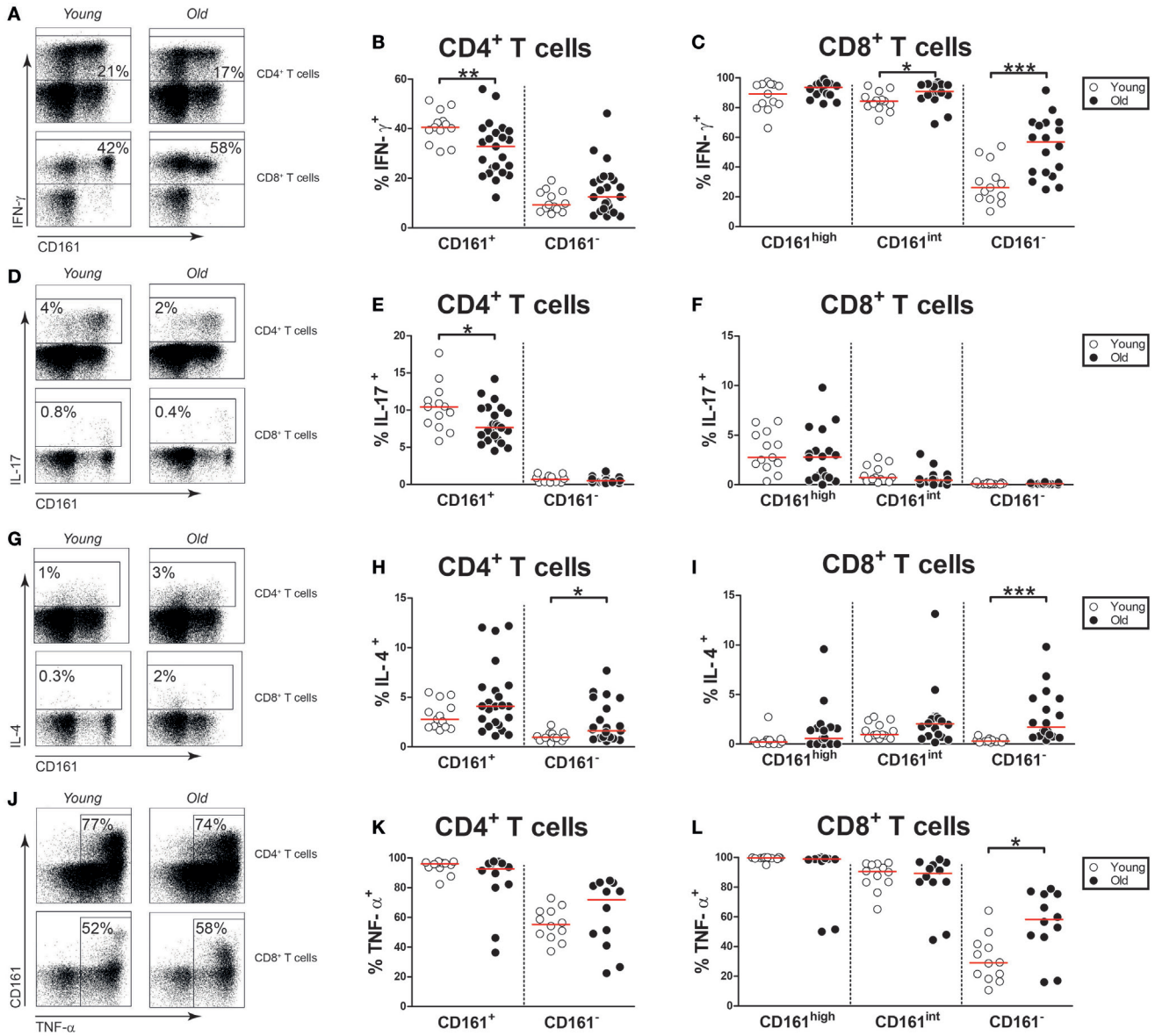

FIGURE 7 | Cytokine expression by CD161-expressing T cells. Intracellular staining for pro-inflammatory cytokines was determined on blood samples that were stimulated with PMA and calcium ionophore in the presence of brefeldin A. **(A)** Flow cytometric gating of IFN-γ+ cells within the CD4+ and CD8+ T cell compartment of a young and old subject. **(B)** Percentages of IFN-γ+ cells within the CD161-defined CD4+ T cell subsets of 13 young [of which 8 were cytomegalovirus (CMV) seropositive] and 23 old (of which 15 were CMV seropositive) subjects. **(C)** Percentages of IFN-γ+ cells within the CD161-defined CD8+ T cell subsets of 13 young (of which 8 were CMV seropositive) and 18 old (of which 12 were CMV seropositive) subjects. **(D)** Flow cytometric gating of interleukin (IL)-17+ cells within the CD4+ and CD8+ T cell compartment of a young and old subject. **(E)** Percentages of IL-17+ cells within the CD161-defined CD4+ T cell subsets of the same subjects as shown in **(B)**. **(F)** Percentages of IL-17+ cells within the CD161-defined CD8+ T cell subsets of the same subjects as shown in **(C)**. **(G)** Flow cytometric gating of IL-4+ cells within the CD4+ and CD8+ T cell compartment of a young and old subject. **(H)** Percentages of IL-4+ cells within the CD161-defined CD4+ T cell subsets of the same subjects as shown in **(B)**. **(I)** Percentages of IL-4+ cells within the CD161-defined CD8+ T cell subsets of the same subjects as shown in **(C)**. **(J)** Flow cytometric gating of TNF-α+ cells within the CD4+ and CD8+ T cell compartment of a young and old subject. **(K)** Percentages of TNF-α+ cells within the CD161-defined CD4+ T cell subsets and **(L)** CD161-defined CD8+ T cell subsets of 12 young (of which 7 were CMV seropositive) and 12 old (of which 7 were CMV seropositive) subjects. Statistical significance by Mann–Whitney *U*-test is shown as $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$. Graphs in which CMV-seropositive young and old subjects are highlighted in the figures are shown in Figure S7 in Supplementary Material.

of age (**Figures 7G,I**). Finally, we investigated CD161-expressing T cells for their ability to produce TNF-α (**Figure 7J**). Nearly all CD161+ CD4+ T cells, CD161high CD8+ T cells, and CD161int CD8+ T cells were capable of producing TNF-α (**Figures 7K,L**). Similar percentages of TNF-α+ cells were observed in these CD161-expressing T cell subsets of young and old subjects. Taken together, our findings confirm that CD161-expressing cells are potent producers of pro-inflammatory cytokines. However,

CD161+ CD4+ T cells of old subjects show a diminished capacity to produce IFN-γ and IL-17 upon stimulation.

Aging Is Associated With a Numerical Decline of Nonclassic Th1 Cells

CD4+ T cells may co-express pro-inflammatory cytokines such as IFN-γ, IL-17, and IL-4 (39). Therefore, we next investigated CD161+ and CD161− CD4+ T cells for the co-expression of the Th1, Th17, and Th2 lineage cytokines, i.e., IFN-γ, IL-17, and IL-4, respectively. The CD161+ CD4+ T cell pool contained cells solely producing IFN-γ, IL-17, or IL-4, but also IFN-γ+ IL-17+, and IFN-γ+ IL-4+ cells (**Figure 8A**). Interestingly, the percentages of both IFN-γ+ and IFN-γ+ IL-17+ cells were decreased among CD161+ CD4+ T cells of old subjects when compared to those of young subjects. In essence, the CD161− CD4+ T cell pool showed limited potential to produce pro-inflammatory cytokines, with only few cells producing solely IFN-γ or IL-4. The percentages of IL-4-producing cells were slightly higher in CD161− CD4+ T cells of old subjects than those of young subjects. Thus, the percentages of Th1 and Th1/Th17 cells are decreased in the CD161+ CD4+ T cell compartment of aged subjects.

Prior reports indicate that Th17 cells show plasticity toward Th1/Th17 cells and ultimately Th1 cells. The latter cells have been termed nonclassic Th1 cells and are characterized by the expression of CD161 (39). To assess the impact of aging on frequencies of nonclassic Th1 cells, we next investigated CD161 expression on Th1, Th1/Th17, and Th17 cells (**Figure 8B**, left panel). Percentages of CD161-expressing cells were equally high among Th17 and Th1/Th17 cells of young and old subjects (**Figure 8B**, right panel). Percentages of CD161-expressing cells were clearly lower among Th1 cells than among Th17 and Th1/Th17 cells. Moreover, fewer Th1 cells expressed CD161 in old subjects than in young subjects. This finding suggests that aging is associated with decreased frequencies of nonclassic Th1 cells.

DISCUSSION

We here provide the first comprehensive analysis of the impact of aging on CD161-expressing T cells in humans. We show that the numbers of CD161high CD8+ T cells decline with age, whereas the numbers of CD161int CD8+ T cells and CD161+ CD4+ T cells remain stable. In respect to the expression of innate-like T cell markers, differentiation markers, and NK receptors, the phenotype of CD161-expressing T cell subsets appeared rather stable with age. Overall, the expression of pro-inflammatory cytokines and cytotoxic effector molecules was comparable in CD161-expressing T cells of young and old subjects. However, the ability to produce IFN-γ and IL-17 upon stimulation was

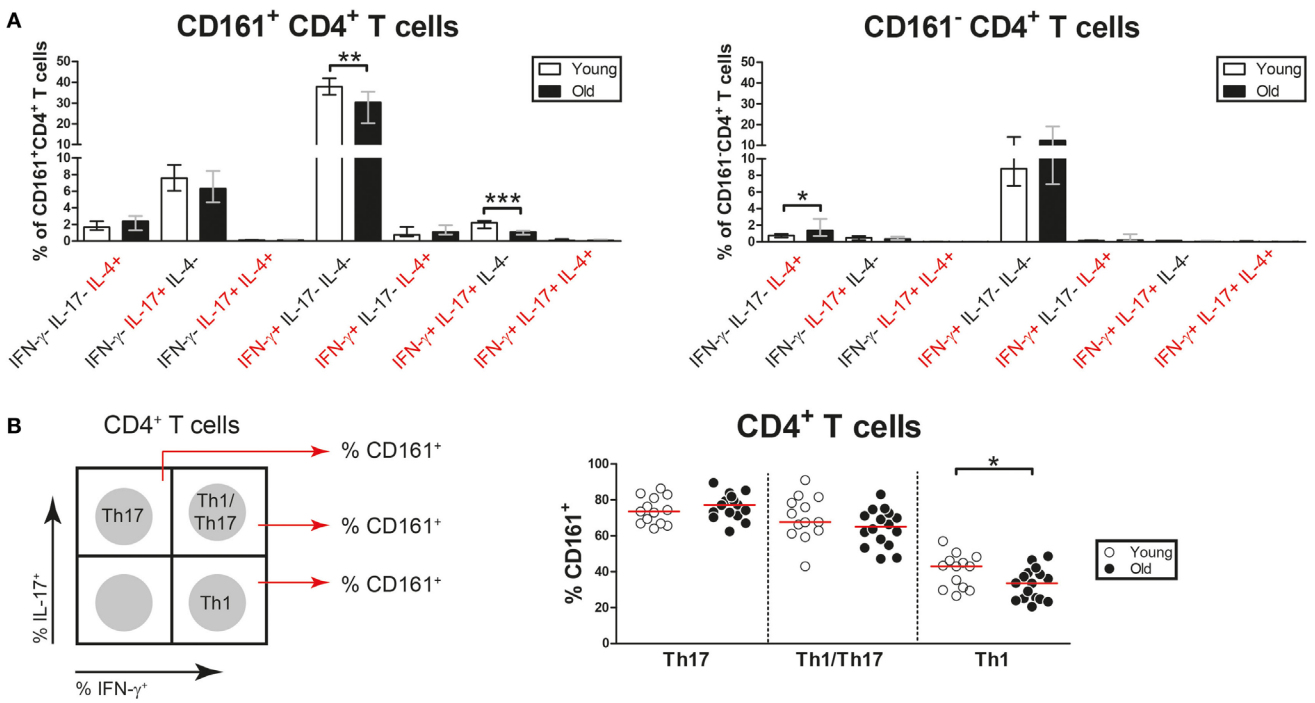

FIGURE 8 | Co-expression of pro-inflammatory cytokines by CD4+ T cells. Intracellular staining for pro-inflammatory cytokines was determined on blood samples that were stimulated with PMA and calcium ionophore in the presence of brefeldin A. **(A)** Analysis of co-expression of IFN-γ, interleukin (IL)-17, and IL-4 by CD161+ CD4+ T cells and CD161− CD4+ T cells of 13 young [of which 8 were cytomegalovirus (CMV) seropositive] and 23 old (of which 15 were CMV seropositive) subjects. Percentages of cells co-expressing different combinations of these cytokines within the CD161+ and CD161− CD4+ T cell pool are shown. **(B)** Analysis of CD161 expression by Th17 cells (IL-17+ IFN-γ−), Th1/Th17 cells (IL-17+ IFN-γ+), and Th1 cells (IL-17− IFN-γ+) in 13 young (of which 8 were CMV seropositive) and 16 old (of which 11 were CMV seropositive) subjects. A schematic illustration of the gating strategy is shown in the left panel. Percentages of CD161+ cells among Th17, Th1/Th17, and Th1 cells are shown in the right panel. Bars and whiskers indicate median and interquartile range. Statistical significance by Mann–Whitney *U*-test is shown as $^{*}p < 0.05$ and $^{**}p < 0.01$.

diminished among CD161$^+$ CD4$^+$ T cells of old individuals. Thus, aging is associated with both numerical and functional changes of CD161-expressing T cells, whereas we observed no substantial phenotypic alterations of these cells.

Aging was associated with stable frequencies of circulating CD161$^+$ CD4$^+$ T cells and a diminished production of pro-inflammatory cytokines by these cells. So far, the impact of age on the numbers of CD161$^+$ CD4$^+$ T cells remained unclear. The absolute numbers of CD161$^+$ CD4$^+$ T cells were not affected by aging in our multivariate linear regression analysis. We confirmed that CD161$^+$ CD4$^+$ T cells primarily show a CM or an EM phenotype, both in young and in old subjects. In accordance with their pro-inflammatory function, CD161$^+$ CD4$^+$ T cells produced more IFN-γ, IL-17, and TNF-α than CD161$^-$ CD4$^+$ T cells. Previously, we have shown that frequencies of Th17 cells and Th1 cells are decreased within the memory CD4$^+$ T cell compartment of elderly subjects (8). In the current study, we add that the proportions of IL-17$^+$ and IFN-γ$^+$ cells are decreased among CD161$^+$ CD4$^+$ T cells of old subjects. Thus, the pro-inflammatory function of CD161$^+$ CD4$^+$ T cells declines with age.

Frequencies of nonclassic Th1 cells were found to be decreased in old subjects. Previous studies have shown that most Th17 cells are contained within the CD161$^+$ CD4$^+$ T cell compartment (18). It has been suggested that these Th17 cells show plasticity toward Th1/Th17 cells and eventually Th1 cells under pro-inflammatory conditions (30, 31). The latter Th1 cells have been termed nonclassic Th1 cells, while maintaining their expression of CD161 (39). In the current study, we show that the proportions of Th1/Th17 and Th1 cells are decreased among CD161$^+$ CD4$^+$ T cells of old subjects when compared to young subjects. Moreover, we show that fewer Th1 cells expressed CD161 in old subjects. Together, these findings suggest that aging affects the plasticity of Th17 cells toward nonclassic Th1 cells. It would be interesting to further study the mechanisms explaining this decreased plasticity in the elderly.

The numbers of circulating CD161^{high} CD8$^+$ T cells decreased with age, whereas their ability to produce pro-inflammatory cytokines remained intact. As previously shown by others (32, 33), we observed that most CD161^{high} CD8$^+$ T cells are MAIT cells, as evidenced by the expression of the TCR-Vα7.2 receptor. Proportions of MAIT cells were uniformly high among CD161^{high} CD8$^+$ T cells of young and old subjects. In accordance with prior reports (34–36), we observed that the numbers of CD161^{high} TCR-Vα7.2$^+$ CD8$^+$ T cells decline with age. We confirmed that CD161^{high} CD8$^+$ T cells are mostly contained within the EM compartment (19). Prior reports indicate that CD161^{high} CD8$^+$ T cells are strong producers of pro-inflammatory cytokines (19). In the current study, we show that this function of CD161^{high} CD8$^+$ T cells is not affected by aging. CD161^{high} CD8$^+$ T cells of young and old subjects showed similar ability to produce IFN-γ, IL-17, and TNF-α. This finding seems in agreement with a prior study showing that the percentages of IFN-γ$^+$ and IL-17$^+$ cells are comparable in the CD8$^+$ MAIT cell compartment of young and old individuals (34). Thus, only the frequency, but not the pro-inflammatory function, of CD161^{high} CD8$^+$ T cells declines with age.

Neither the numbers of CD161^{int} CD8$^+$ T cells nor their cytokine-producing potential was affected by age. The absolute numbers of CD161^{int} CD8$^+$ T cells were comparable in young and old subjects. Nevertheless, the percentages of CD161^{int} CD8$^+$ T cells showed an increase with age due to loss of CD161^{high} and CD161$^-$ CD8$^+$ T cells from the CD8$^+$ T cell compartment. CD161^{int} CD8$^+$ T cells primarily resided within the EM and TD compartments, as previously shown by others (20). In this respect, we observed no difference between CD161^{int} CD8$^+$ T cells of young and old subjects. We confirmed that most CD161^{int} CD8$^+$ T cells are able to produce IFN-γ and TNF-α (19, 20). In addition, CD161^{int} CD8$^+$ T cells of old subjects more frequently produced IFN-γ than those of young subjects, whereas no difference was observed for TNF-α. Thus, both the frequencies and cytokine-producing potential of CD161^{int} CD8$^+$ T cells are preserved on to high age.

The expression of NK receptors was comparable in CD161-expressing T cells of young and old subjects. In addition to signaling *via* the TCR and conventional co-stimulation molecules, T cell activation may be influenced by NK receptors. In particular, late-stage T cells of aged subjects may express activating and inhibitory NK receptors (6, 7). We here examined CD161-expressing T cells for the presence of three well-defined activating NK receptors (i.e., 2B4, DNAM-1, and NKG2D), as well as one inhibitory NK receptor (i.e., KLRG1). CD161^{high} and CD161^{int} CD8$^+$ T cells showed prominent expression of all four NK receptors, without any difference between young and old subjects. By contrast, CD161$^+$ CD4$^+$ T cells primarily expressed DNAM-1 and KLRG1. DNAM-1 expression was similar in CD161$^+$ CD4$^+$ T cells of young and old subjects, but the percentage of KLRG1$^+$ cells was decreased among CD161$^+$ CD4$^+$ T cells of old subjects. Although our analysis was restricted to only four NK receptors, a decreased expression of the latter inhibitory NK receptor could indicate that CD161$^+$ CD4$^+$ T cells of old subjects might be more prone to activation.

The expression of cytotoxic effector molecules by CD161-expressing T cells was not affected by age. CD161$^+$ CD4$^+$ T cells showed little expression of perforin and granzyme B, irrespective of age. Approximately half of the CD161^{int} CD8$^+$ T cells expressed perforin and granzyme B in young and old subjects. This finding underscores the prominent cytotoxic potential of these cells. Similar percentages of perforin expressing CD161^{high} CD8$^+$ T cells were observed in young and old individuals. In accordance with prior studies, few CD161^{high} CD8$^+$ T cells expressed granzyme B (19, 40), both in young and in old subjects. It has been demonstrated that CD161^{high} CD8$^+$ T cells primarily express granzymes A and K (40). Although the latter cytotoxic effector molecules were not analyzed in the current study, the stable expression of perforin by CD161^{high} CD8$^+$ T cells suggests that the cytotoxic potential of these cells remains intact with age.

Limited data suggest that CD161-mediated signaling promotes the secretion of pro-inflammatory cytokines by T cells. Lectin-like transcript 1 (LLT1) has been identified as the ligand for CD161 (41, 42). LLT1 is expressed by antigen-presenting cells, including B cells and macrophages (43, 44). It has been shown that the engagement of CD161 by LLT1 enhances the production of

pro-inflammatory cytokines by T cells. For instance, the ligation of CD161 when given in addition to TCR stimulation increased the production of IFN-γ and TNF-α by MAIT cells (37). Similar experiments with CD161-expressing T cell clones also indicate that CD161 ligation in the presence of TCR stimulation promotes IFN-γ production by T cells (41). Thus, current evidence suggests that CD161 may act as a co-stimulatory receptor on T cells. An early study has also suggested that CD161 may be directly involved in transendothelial migration of T cells (45). However, this observation has not yet been confirmed by others. It would be interesting to study the effect of aging on the function of CD161 itself.

Given the broad antimicrobial functions of CD161-expressing T cells (20–24), it is likely that the aging-associated changes of $CD161^+$ $CD4^+$ T cells and $CD161^{high}$ $CD8^+$ T cells may compromise immunity in the elderly. The $CD161^+$ $CD4^+$ T cell compartment contains antiviral Th1-like cells (22), as well as Th17 cells promoting immunity against bacteria and yeasts (46). In patients undergoing treatment for hematological malignancies, $CD161^+$ $CD4^+$ T cells have recently been linked to preserved immunity against CMV and lower risks for neutropenic infections (22, 47). $CD161^{high}$ $CD8^+$ T cells, which primarily consist of MAIT cells, are critical for immunity against common bacteria and viruses, such as *Escherichia coli* and influenza, respectively (23, 48). Numerical decreases of MAIT cells might put elderly subjects at risk for infections with these microbes. Indeed, sepsis due to Gram-negative bacteria and pneumonia occurs more frequently and with a higher severity in the elderly (49). The $CD161^{int}$ $CD8^+$ T cell compartment has been identified as a polyclonal $CD8^+$ T cell population that contributes to antiviral immunity (20). The stable frequencies of $CD161^{int}$ $CD8^+$ T cells, and the ability to produce cytotoxic effector molecules and pro-inflammatory cytokines, likely contribute to immunity throughout adult life. Thus, not all CD161-expressing T cell subsets are compromised with age. It remains to be elucidated if the aging-related effects on $CD161^+$ $CD4^+$ T cells and $CD161^{high}$ $CD8^+$ T cells might be advantageous in the context of autoimmune diseases (25, 26, 50).

We precluded that CMV confounded our findings regarding the impact of aging on CD161-expressing T cells. Indeed, CMV markedly influences the T cell compartment and might potentially compromise immunity to other pathogens in the elderly (9–11, 17). Therefore, we delineated the effects of aging and CMV on the absolute numbers of CD161-expressing cells by performing multivariate linear regression analyses. We confirmed that the aging-associated decline of $CD161^{high}$ $CD8^+$ T cell numbers occurred independently from CMV serostatus. These analyses also demonstrated that CMV seropositivity by itself is associated with higher numbers of $CD161^{int}$ $CD8^+$ T cells. Interestingly, a recent report indicates that $CD161^{int}$ $CD8^+$ T cells are highly functional memory cells that contribute to antiviral immunity (20). Ample evidence also suggests that CMV-specific memory cells upregulate NK receptors and have a strong potential to produce cytotoxic effector molecules and pro-inflammatory cytokines (12–14). Importantly, CMV-seropositivity rates were comparable between young and old subjects in our extensive phenotypical and functional analyses of CD161-expressing T cells. Thus, CMV unlikely influenced the findings in the current study. Indeed, it would be interesting to further perform a comprehensive analysis of CMV and CD161-expressing T cells.

In conclusion, we here show that aging affects the frequencies and function of CD161-expressing T cells. Aging-associated changes of CD161-expressing T cells might potentially contribute to a decreased antimicrobial immunity in the elderly. Future studies should evaluate the mechanisms and implications of aging-associated changes of CD161-expressing T cells in humans.

ETHICS STATEMENT

Written informed consent was obtained from all study participants. The study was approved by the Medical Ethical Committee of the UMCG. All procedures were in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

KG, B-JK, WA, EB, and AB conceived the study and designed the experiments. KG and EB recruited the study participants. KG and GH performed the experiments and acquired data. All authors were involved in data analysis and interpretation. KG and AB wrote the manuscript, and all authors revised it critically for important intellectual content. All authors approved the final version of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Parameters of the Immune System and Vitamin D Levels in Old Individuals

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Aim: The increased number of individuals older than 80 years, centenarians, and supercentenarians is not a synonym for healthy aging, since severe infections, hospitalization, and disability are frequently observed. In this context, a possible strategy is to preserve the main characteristics/functions of the immune system with the aim to cause less damage to the organism during the aging process. Vitamin D acts on bone marrow, brain, breast, malignant cells, and immune system and has been recommended as a supplement. We aimed to evaluate whether immune parameters and vitamin D serum levels are correlated.

Methods: We evaluated some features of the immune system using the peripheral blood of individuals older than 80 years (n = 12) compared to young subjects (n = 10). In addition, we correlated these findings with vitamin D serum levels.

Results: Old individuals presented metabolic parameters of healthy aging and maintained preserved some features of immunity such as CD4/CD8 ratio, and low production of pro-inflammatory cytokines after stimulus. On the other hand, we observed increase in the frequency of myeloid-derived suppressor cells, reduction in circulating leukocytes, in the percentage of total CD8+, and in CD8+ Naïve T cells, in addition to increase in the percentage of CD8+ effector memory re-expressing CD45RA (EMRA) T cells. We found seropositivity for CMV in 97.7%, which was correlated with the decrease of CD8+ Naïve T cells and increase in CD8+ EMRA T cells. Vitamin D levels were insufficient in 50% of old individuals and correlated positively with total CD8+ T cells and negatively with CD8+ EMRA T cells.

Conclusion: In the studied population, longevity was correlated to maintenance of some immune parameters. Considering the limitations of the study as size of the sample and lack of functional assays, it was found that vitamin D in old individuals was correlated to some features of the immune system, mainly in the CD8 compartment.

Keywords: longevity, immunity, vitamin D, myeloid-derived suppressor cells, T cells

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INTRODUCTION

In several developed and developing countries, human longevity has been achieved (1–3) but insufficient function of the immune system in the old population leads to severe infections, frequent hospitalizations, and immunization reduced after vaccination (4–6).

Therefore, to reach the longevity with good quality of life, a possible strategy is to preserve the main characteristics/functions of the immune system with the aim to cause less damage to the organism

during the aging process. Aging has been associated with lower generation of progenitors from pluripotent hematopoietic stem cells (HSC) located in the bone marrow. In addition, there occurs a myeloid-biased differentiation of HSC due to selection pressures from cell-intrinsic and extrinsic mechanisms (7–10). These factors lead to a decrease in the number of circulating leukocytes and increased frequency in the myeloid lineage. The increase in the frequency of myeloid cells may be the reason for the recently described increase in myeloid-derived suppressor cells (MDSC) in aging individuals. MDSC membrane markers are $CD11b^+$ $CD33^+HLA^-DR^{-/low}$, which can be subtyped in monocytic MDSC ($CD14^+$) or granulocytic ($CD15^+$) MDSC.

The suppressive actions of MDSC are mainly based on the production of arginase-1, reactive oxygen species, nitric oxide (NO), IL-10, and TGF-β1. These cells exert several effects on lymphocytes such as impairment in the antigen presentation and recognition by T cells, deficient B and T cells activation, and accumulation of regulatory T cells (11, 12).

Changes in hematopoiesis have also been related to the decreased percentage of $CD4^+$ and $CD8^+$ T cells in the circulating blood. In addition, the inverted CD4/CD8 ratio was reported by Swedish OCTO and NONA immune longitudinal studies as a blood marker predictive for a high rate of mortality in 2, 4, and 6 years (immune risk profile) (13).

Another aspect of aging is the thymic involution which has been linked to less diversity in the T-cell receptor and decreased frequency of Naive T cells (14, 15), while the peripheral homeostatic proliferation compensates for the T-cell loss (16, 17).

The phenotype of T cells has been used to characterize the immune system status and Hamann et al. (18, 19) proposed that in humans, the $CD8^+$ T cell compartment presents four different phenotypes based on membrane markers and cellular function. The phenotype $CD45RA^+CD27^+$ represents undifferentiated Naive cells and $CD45RA^-CD27^+$ lymphocytes are antigen-experienced T cells (central memory) with increased frequency of lymphotoxicity precursors (CTLp). $CD45RA^+CD27^-$ cells present features of antigen-stimulated cells with cytolytic potential, production of IFN-γ and tumor necrosis factor-alpha (TNF-α), high amounts of perforin and granzyme B, Fas ligand mRNA expressed in abundancy, and cells exerting potent cytotoxic activity without previous *in vitro* stimulation [effector memory re-expressing CD45RA (EMRA)]. $CD45RA^-CD27^-$ T cells are observed in low frequency and express perforin and granzyme B (effector memory). The correlation between aging and increased frequency of $CD8^+CD45RA^+CD27^-$ has been reported. The same phenotypes were identified in different stages of $CD4^+$ T cells mainly during stimulation by viral infections (CMV, EBV, HSV, and VZV) (20, 21).

In order to preserve the functional characteristics of the immune system that could in turn prevent and/or delay age-related diseases, health professionals have proposed physical activity, control of diet, supplements, and probiotics (22–24).

The main actions of vitamin D in bone tissue and the latest reports of its effects on bone marrow, brain, colon, breast, malignant cells, and immune system (25) raised the interest of researchers to investigate the role played by vitamin D in the immunity of old individuals.

Considering that low levels of vitamin D are common in older individuals, some health professionals have recommended vitamin D supplementation to the aging population in general and especially for aged-care residents and critically ill patients (26–28). However, the benefits arising on the immunity with vitamin D supplementation are not consistent in the literature. Upper respiratory infections (URI) in non-hospitalized middle-aged and older individuals with vitamin D supplementation have been associated with a lower incidence of infection (29), discrete decrease of infections events (30), or no alteration in severity and duration of infection (31, 32). In addition, residents of sheltered accommodation supplemented with vitamin D showed increase in URI and duration of symptoms and no changes to the risk or duration of lower respiratory infections (33). However, in patients with antibody deficiency or increased susceptibility to respiratory tract infections (RTI), supplementation with vitamin D was beneficial and associated with fewer episodes of RTI and increased time for first infection compared to placebo group (34).

As the extension of life expectancy is a reality, it is a challenge to understand how the aging population deals with the remodeling of the immune system and if interventions as vitamin D could provide extra years of life with good health. In this study, our goal was to investigate changes that occur in some parameters of the immune system in individuals reaching longevity (80–100 years) and the possible correlation with vitamin D levels.

MATERIALS AND METHODS

The present study is part of a larger epidemiologic survey called the health, well-being, and aging study (SABE), which was coordinated by the Pan-American Health Organization, Washington, and in Brazil by the School of Public Health of the University of São Paulo. From 2000 to 2001, SABE evaluated a sample of 2,143 non-institutionalized individuals, representing 836,204 aging people (60 years and older) living in the municipality of São Paulo, who were selected through multi-stage sampling. In 2006, the School of Public Health continued the survey in São Paulo and transformed it into a multi-cohort study with 1,115 individuals from the previous study who agreed to participate in the follow-up. Since then, the survey has been repeated every 5 years. In this study, the inclusion/exclusion criteria were applied as cited above, except that we used blood only from individuals older than 80 years (male, female) and they were enrolled as their biological samples were received. Young individuals (20–30 years, male and female) were master and Ph.D. students from UNIFESP.

The blood samples were collected with 12 h of fasting (6:00 a.m.–9:00 a.m.) during the summer months (December, January, and February) in Brazil.

The Ethics Committee of the Federal University of São Paulo—UNIFESP approved all procedures (Protocol number 10904).

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll–Hypaque density gradient (Amersham Biosciences, Uppsala, Sweden) and centrifugation. Viable cells were counted, adjusted for 2×10^6/100 μL in 80% fetal bovine serum and 20% dimethylsulfoxide (Sigma, St. Louis, MO, USA), and frozen stored (−80°C) until the phenotyping and cell culture.

Cell Culture

Cells were diluted in RPMI 10, counted, and adjusted for 1×10^6/100 mL. The assessment of cell proliferation was based on a substance (CFSE) that once in the cell cytoplasm halves its content to each cell division. Cells were incubated with 5(6)-carboxyfluorescein acetate (CFSE, CFDA Vybrant IF Cell Tracer Kit Invitrogen) for 10 min. The cells were washed, counted, and adjusted for plating (2×10^5/100 μL of RPMI10 per well). Culture conditions were phytohemagglutinin (PHA^+, 5 μg/mL, Sigma) or absence of stimulus (PHA) for 72 h in 5% CO_2, humidity controlled and 37°C. After 3 days of culture, the cell suspension was collected, centrifuged, and the proliferation (CFSE) was measured in flow cytometer (35). The cell culture supernatant was frozen (−80°C) for the measurement of cytokines (ELISA).

Cell Phenotype

The cells were stained with monoclonal antibodies to T-cell phenotype CD3 APC, CD4 PerCP Cy 5.5, CD8 APC Cy7, CD27 FITC, CD45RA PE (eBioscience, CA, USA). The cells were also stained with monoclonal antibodies to MDSC phenotype CD3 APC, CD19 APC, CD56 APC, HLA-DR APC e-fluor 780, CD33 PerCP Cy5.5, CD11b PE, CD14 PE Cy7, CD15 FITC (eBioscience, CA, USA). After 30 min of incubation with monoclonal antibodies, in the dark and at 4°C, the cells were washed with PBS and centrifuged. Living cells (based on forward and side scatter) were acquired in the FACS Canto II using the DIVA software (Becton Dickinson, USA). Further analyses of FACS data were performed using the 9.3 FLOWJO software (Tree Star, USA).

T lymphocytes were characterized as described previously (36).

Naïve: $CD3^+CD4^+CD45RA^+CD27^+$ or $CD3^+CD8^+CD45RA^+CD27^+$ (Naïve).
Central memory: $CD3^+CD4^+CD45RA^-CD27^+$ or $CD3^+CD8^+CD45RA^-CD27^+$ (CM).
Effector memory: $CD3^+CD4^+CD45RA^-CD27^-$ or $CD3^+CD8^+CD45RA^-CD27^-$ (EM).
Effector memory re-expressing CD45RA: $CD3^+CD4^+CD45RA^+CD27^-$ or $CD3^+CD8^+CD45RA^+CD27^-$ (EMRA).

Myeloid-derived suppressor cells were characterized as:

$CD3^-CD19^-CD56^-HLA^-DR^{-/low}CD33^+CD11b^+CD15^+$ granulocytic or
$CD3^-CD19^-CD56^-HLA^-DR^{-/low}CD33^+CD11b^+CD14^+$ monocytic.

ELISA

The frozen culture supernatants were thawed and cytokines [IL-1, IL-2 α, interleukin-6 (IL-6), IFN-γ, and TNF-α] were evaluated by ELISA assay according to the manufacturer's instructions (DuoSet ELISA Development Systems R&D). ELISA reading PerkinElmer—EnSpire, quantified the samples.

Metabolic Data

Obtained from the databank of SABE study.

Cytomegalovirus IgM and IgG

Serum was previously isolated by centrifugation and frozen stored (−80°C). IgG and IgM levels were measured in serum by electrochemiluminescence immunoassay according to the manufacturer's instructions (cobas® http://e-labdoc.roche.com REF 04784618 190).

Measurement of Vitamin D

Serum of studied individuals was previously isolated by centrifugation and frozen stored (−80°C) until the measurement of vitamin D. 25-Hydroxyvitamin D value was obtained in accordance with the manufacturer's instructions (cobas® http://e-labdoc.roche.com 05894913 190 V7).

Statistics

After testing the variables for normality (Shapiro–Wilk) it was used the unpaired Student's *t*-test (Vitamin D) or Mann–Whitney

TABLE 1 | Metabolic parameters in old individuals (80–100 years) and reference values of 80-year-old individuals (37).

Metabolic parameters

Age	Gender	Cholesterol total mg/dL	HDL mg/dL	Triglycerides mg/dL	Glucose mg/dL	Urea mg/dL	Creatinine mg/dL	Albumin mg/dL
100	F	179	38	183	162	35	0.91	3.1
90	F	224	53	178	80	33	1.09	3.4
90	F	188	73	125	86	42	0.67	3.9
93	M	181	43	47	80	57	0.98	3.9
93	F	186	51	121	93	28	0.93	3.6
88	M	275	68	114	104	36	1.01	4
94	M	177	39	86	71	47	1.26	3.4
90	M	163	43	72	82	30	1.1	3.7
90	M	181	38	135	95	43	1.22	3.9
94	M	132	33	80	90	61	1.97	3.9
91	F	207	63	106	81	41	0.73	4.3
90	F	236	58	109	90	29	0.94	4
Mean ± SD		194.1±37.1	50 ± 13.1	113 ± 40.2	92.8 ± 2	40.2 ± 10.7	1.1 ± 0.3	3.8 ± 0.3
Range		132–275	33–73	47–183	71–162	28–61	0.67–1.97	3.1–4.3
Reference values (37)		108.3–313.2	27.1–104.4	43.8–287.8	126	19.8–89.5	0.52–1.94	3.2–4.7

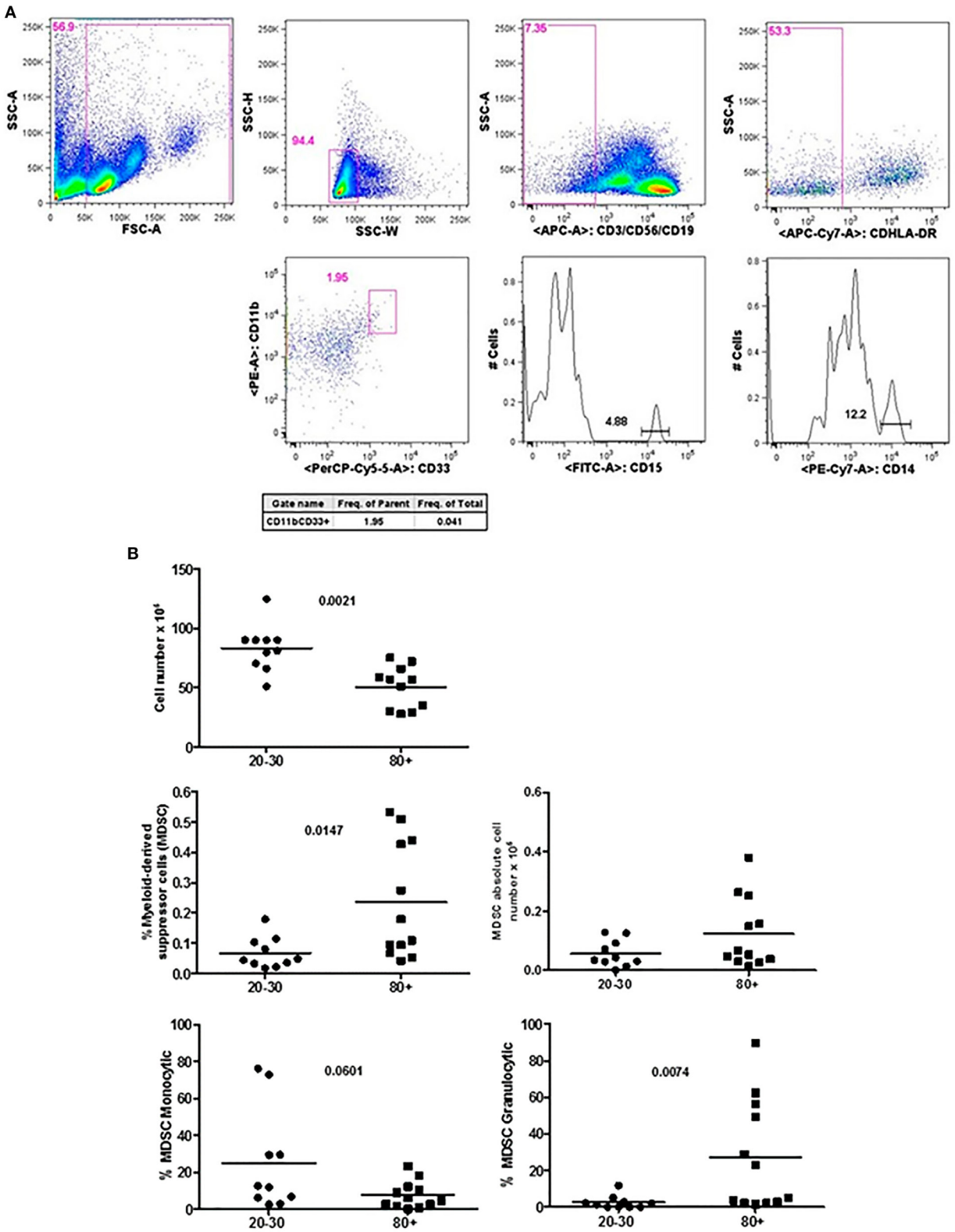


FIGURE 1 | Representative flow cytometry plots showing gate strategy for myeloid-derived suppressor cells (MDSC) frequency. Gate in live cells (FSC-A × SSC-A), doublets exclusion (SSC-H × SSC-W), gate in lineage negative cells ($CD3^-CD56^-CD19^-$), gate in HLA-$DR^{low/neg}$ cells, gate in $CD33^+CD11b^+$ cells. Gate in granulocytic ($CD15^+$) and monocytic ($CD14^+$) cells **(A)**. Number of circulating leukocytes, MDSC (% MDSC) frequency, absolute number of MDSC, and frequency of monocytic and granulocytic MDSC in individuals of 20–30 years and 80+ years old individuals **(B)**.

(parameters of the immune system) for comparisons between young and old groups. The correlation between parameters of the immune system and serum levels of IgG (CMV) or vitamin D was performed by Spearman test. Values of $p < 0.05$ were considered statistically significant. All statistical analyses were performed with the aid of the Graph Pad PRISM software (Graph pad, La Jolla, CA, USA).

RESULTS

Young ($n = 10$, 5 male and 5 female, 20–30 years old) and old ($n = 12$, 6 male and 6 female, 80–100 years old) individuals were evaluated. **Table 1** shows some metabolic parameters that highlight the health status of the old population evaluated. In agreement with Helmersson-Karlqvist et al. (37), our old population may be considered healthy for most of the parameters evaluated in spite of great variability observed. Only one female (100 years) presented glucose (162 mg/dL) higher than the reference values established by Helmersson-Karlqvist et al. (37).

Figure 1 shows that old individuals (80–100 years) presented a significantly high percentage of MDSC (%MDSC). However, as this group had a significantly lower number of leukocytes, the absolute cell number of MDSC (leukocytes cell number × percentage of MDSC) was not different compared to young individuals (20–30 years). There was significant predominance of granulocytic MDSC in individuals older than 80 years, while the subtype monocytic was statistically higher in young individuals. The percentage of T CD4$^+$ lymphocytes was similar in both groups while there was a trend toward lower percentage of CD8$^+$ T cells in old individuals ($p = 0.0572$). The CD4/CD8 ratio was significantly higher in old individuals except in a woman (96 years) with the CD4/CD8 ratio inverted (0.542) (**Figure 2**).

The evaluation of T cells phenotypes showed a significantly lower percentage of CD4$^+$ central memory in elderly individuals (**Figure 3**). In the CD8 compartment, Naive cells were statistically less expressed in old individuals and CD8$^+$ EMRA T cells presented significantly higher expression in old individuals

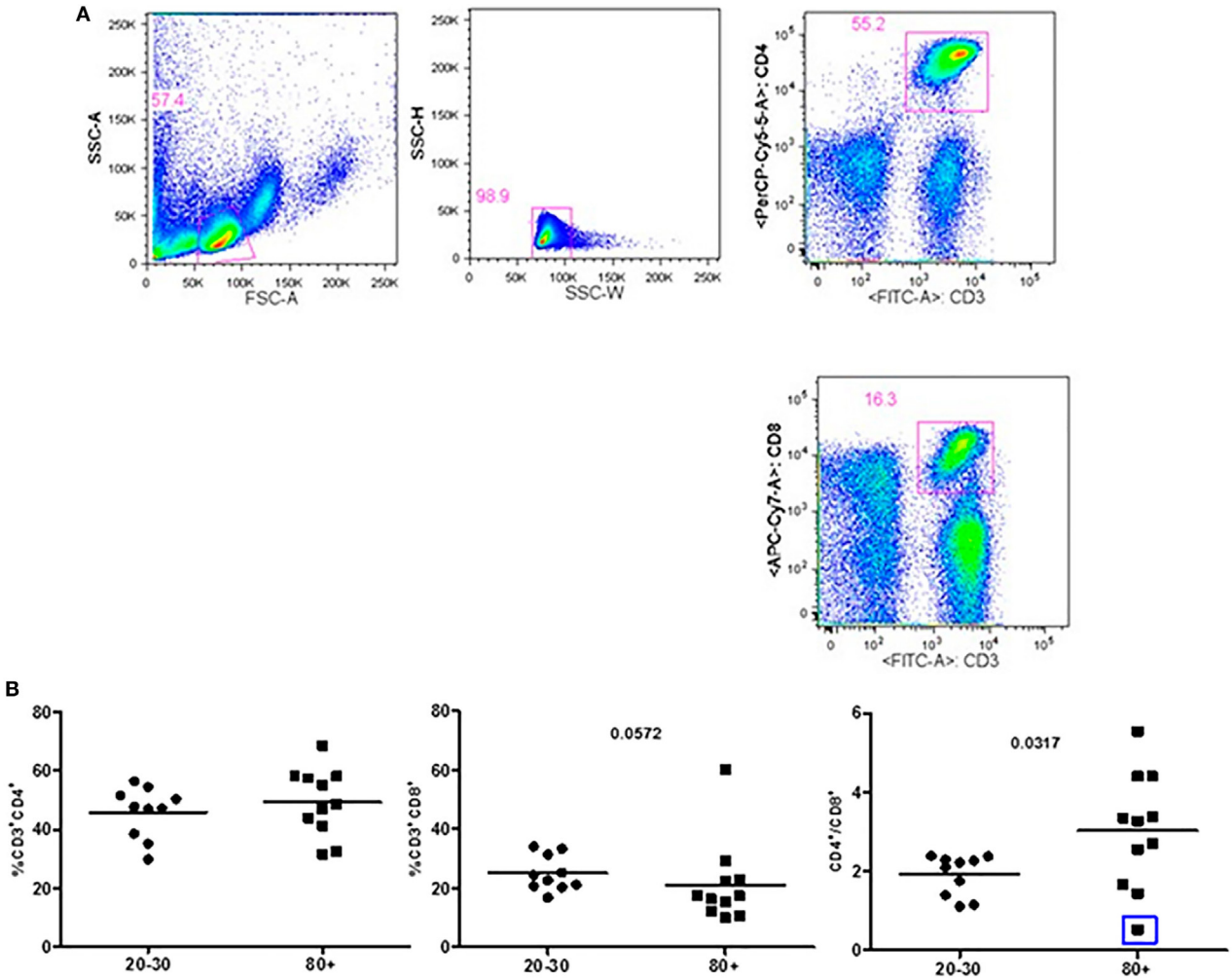


FIGURE 2 | Representative flow cytometry plots showing gate strategy for the frequency of CD4$^+$ and CD8$^+$ T cells. Gate in live lymphocytes (FSC-A × SSC-A), doublets exclusion (SSC-H × SSC-W), gate in CD3$^+$CD4$^+$ T cells, gate in CD3$^+$CD8$^+$ T cells **(A)**. Frequency of T lymphocytes CD3$^+$CD4$^+$, CD3$^+$CD8$^+$, and CD4/CD8 ratio in 20–30 and 80$^+$ years old individuals. The CD4/CD8 ratio lower than 1 (0.542; blue square) **(B)**.

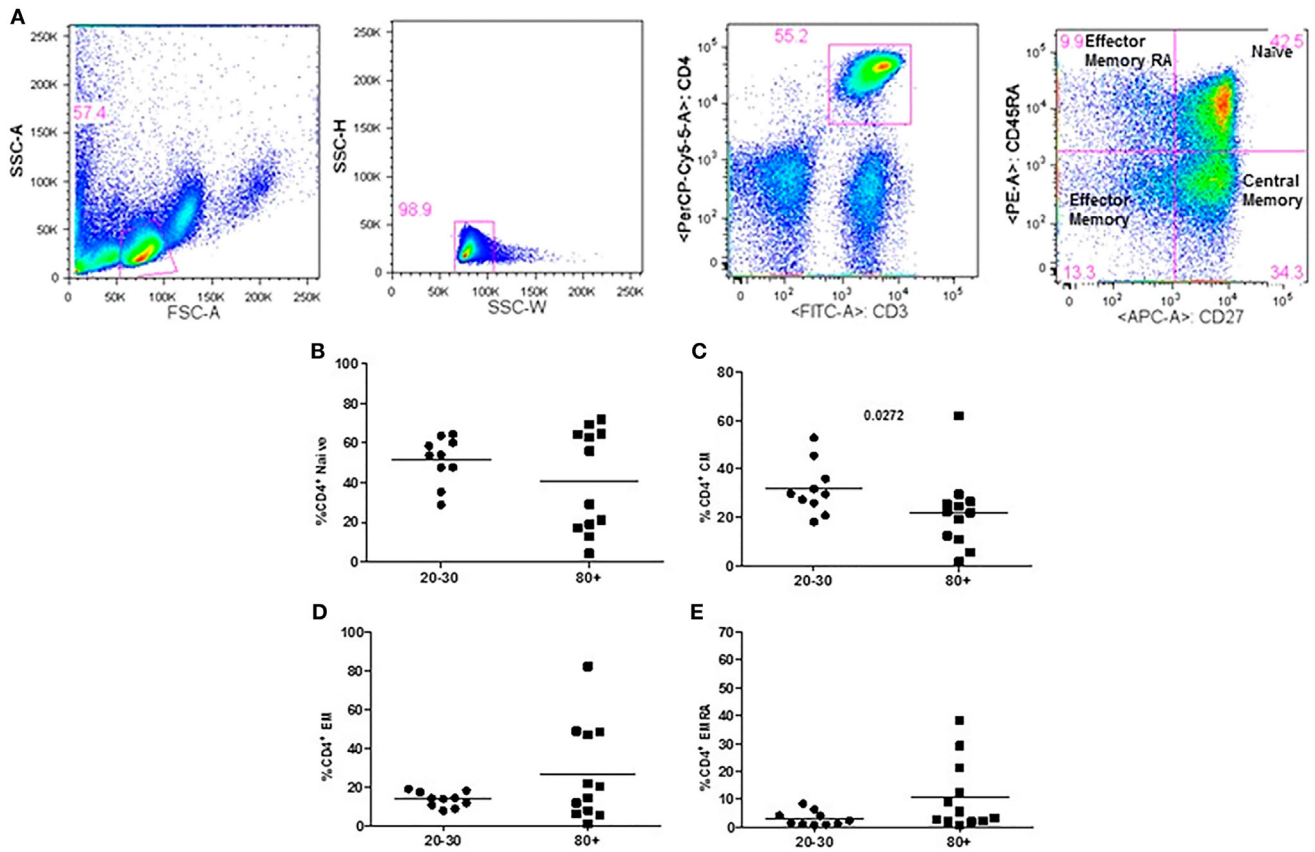


FIGURE 3 | Representative flow cytometry plots showing gate strategy for the frequency of CD4+ T cells phenotype. Gate in live lymphocytes (FSC-A × SSC-A), doublets exclusion (SSC-H × SSC-W), gate in CD3+CD4+ T cells, gate in CD45RA+CD27+ (Naïve) T cells, gate in CD45RA−CD27+ (central memory) T cells, gate in CD45RA−CD27− (effector memory), gate in CD45RA+CD27− (effector memory re-expressing CD45RA) T cells **(A)**. Percentage (%) of CD4+ T cells phenotypes: Naïve **(B)**, central memory **(C)**, effector memory **(D)**, effector memory RA re-expressing CD45RA **(E)** in 20–30 and 80+ years old individuals.

(**Figure 4**). Considering the functions of T cells after stimulation in culture with PHA, the percentage of proliferation was lower in old individuals both in the compartment CD4+ and CD8+ (**Figure 5**). The production of cytokines was reduced in old individuals with statistical difference for IL-6 and TNF-α (**Figure 6**). As cytomegalovirus infection/latency has been related to immunosenescence, we evaluated IgM and IgG against CMV in serum of old (80–100 years) individuals (**Table 2**) and the possible correlation with immune parameters (**Table 3**).

Table 2 shows that in 12-old individuals evaluated, only one male (88 years old) can be considered negative for cytomegalovirus as the IgM level was <0.7 (0.158) and IgG level was <0.5 (<0.25). In addition, only one male (90 years old) could be considered as recently infected by CMV as the IgM level was >1.0 (1.240) and the IgG level was relatively low (55.85) in comparison to other old individuals studied. Levels of IgG >500 U/mL were observed in four individuals (3 females and 1 male).

The levels of IgG against CMV were negatively correlated ($p = 0.027$) with the percentage of Naïve CD8+T cells. There was a trend of negative correlation ($p = 0.06$) between the percentage of CD4+ central memory T cells and IgG levels (CMV). A trend of positive correlation ($p = 0.055$) between the percentage of CD8+ EMRA T cells and IgG levels (CMV) was observed (**Table 3**).

The next question was whether total vitamin D levels were different in the studied groups (**Figure 7**). Vitamin D levels were lower in old individuals ($p = 0.050$). In 50% ($n = 6$) of aged individuals vitamin D levels were <20 ng/mL (deficiency) and in 90% ($n = 9$) of young individuals vitamin D levels were greater than 20 ng/mL. Insufficiency (21–29 ng/mL) was present in 25% ($n = 3$) of old individuals and 50% ($n = 5$) of young individuals. Sufficient levels (30 ng/mL or more) were observed in 25% ($n = 3$) of aged and 40% ($n = 4$) of young individuals.

It was analyzed whether total vitamin D was correlated with the immunological parameters evaluated previously. We observed correlation of vitamin D levels only for the CD8 compartment (**Table 4**). The percentage of total CD8 T cells was positively correlated ($p = 0.006$) with vitamin D levels in old individuals, whereas there was a trend toward positive correlation ($p = 0.074$) in young individuals. CD8+ effector memory T cells were positively correlated with vitamin D levels in young individuals and CD8+ EMRA T cells were negatively correlated ($p = 0.05$) with vitamin D levels in old individuals.

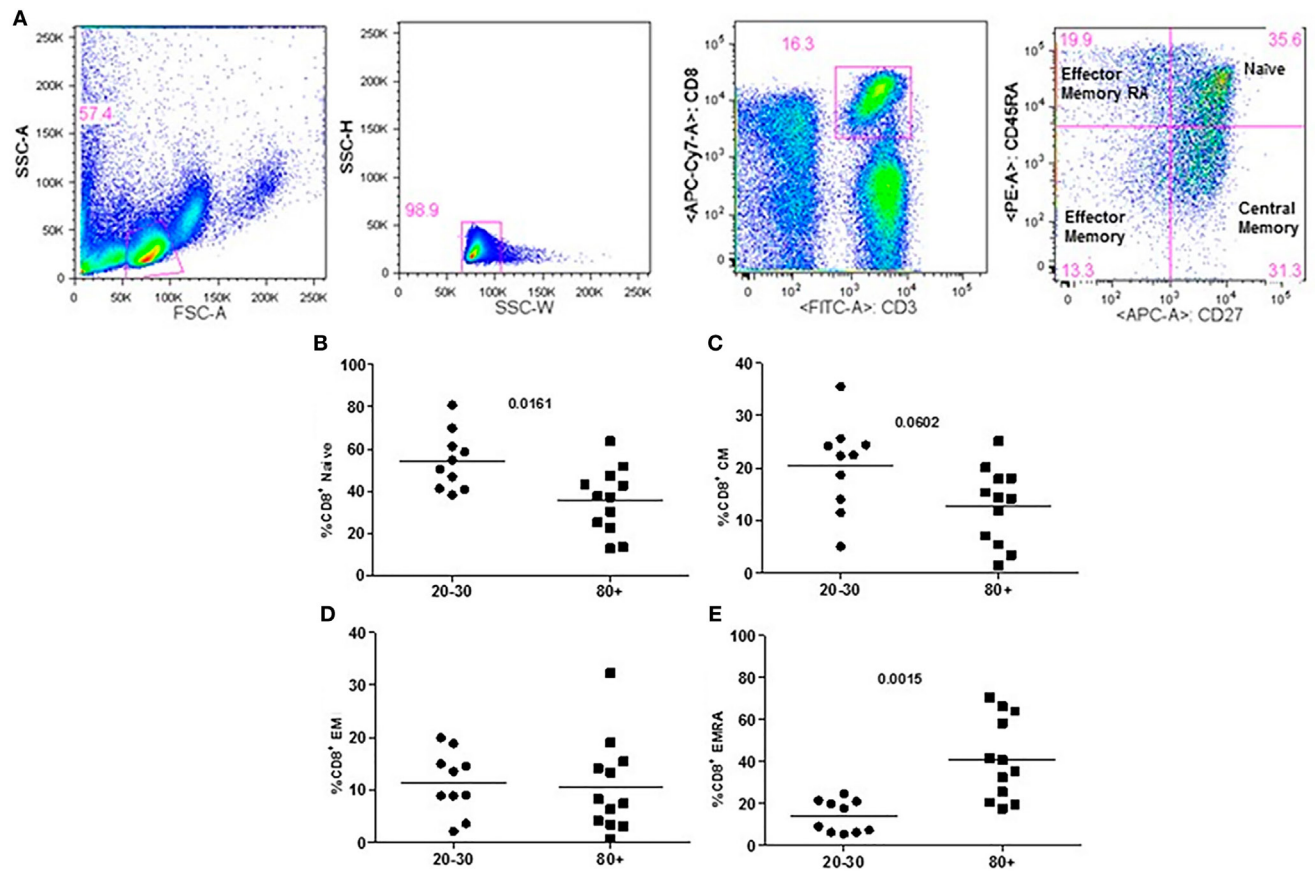


FIGURE 4 | Representative flow cytometry plots showing gate strategy for the frequency of CD8+ T cells phenotype. Gate in live lymphocytes (FSC-A × SSC-A), doublets exclusion (SSC-H × SSC-W), gate in CD3+CD8+ T cells, gate in CD45RA+CD27+ (Naïve) T cells, gate in CD45RA−CD27+ (central memory) T cells, gate in CD45RA−CD27− (effector memory), gate in CD45RA+CD27− (effector memory re-expressing CD45RA) T cells **(A)**. Percentage (%) of CD8+ T cells phenotypes: Naïve **(B)**, central memory **(C)**, effector memory **(D)**, effector memory RA re-expressing CD45RA **(E)** in 20–30 and 80+ years old individuals.

DISCUSSION

Morbidities associated with aging contribute to organ failure that leads to a pathway of poor quality of life and/or death. In addition, the impairment in the protective functions of the immune system promoting infections and tumors, and the increase of inflammatory factors causing tissue damage and contributing for age-related diseases have also been reported in old individuals.

In our study, it was observed that old individuals presented metabolic parameters consistent with healthy aging except for a female (100 years) with blood glucose higher than 126 mg/dL. Our data are in agreement with the report of Helmersson-Karlqvist et al. (37) who have developed reference intervals of metabolic parameters adjusted for very old (80 years) individuals.

Despite our studied population could be considered healthy, we observed characteristics reported as immune senescence.

Myeloid-derived suppressor cells were only recently described as potential markers of the aging process, since these cells were initially associated with tumor development (12). We observed in old individuals that MDSC were present in high percentage with predominance of the granulocytic subtype. It has been reported that the accumulation of MDSC with aging may contribute to some of the immune disorders and pathologies observed in older adults (12). In aging individuals, Verschoor et al. observed a significant increase in the frequency of MDSC compared to young adults in addition to the higher number of MDSC in older individuals with frailty and previous history of cancer (11). In addition to the increased percentage of MDSC, our old population presented decreased number of circulating leukocytes, reduced percentage of total CD8+ and CD8+ Naïve T cells, and increase in the percentage of terminally differentiated CD8+ EMRA T cells. Significant changes in the phenotype and function occurred mainly in CD8+ T cells in these individuals and are in agreement with the literature (38–43). In addition, it has been shown that the homeostatic proliferation is less effective for CD8 than for CD4 Naïve T cells (44).

In old individuals, it has been shown that many aspects of immunosenescence are related to the seropositivity for cytomegalovirus (CMV). However, it seems that the effects of CMV in the immune system of healthy old individuals are dependent on the increased latency of the virus (45, 46). In our study, 11 out of 12 old individuals were seropositive for CMV and four individuals presented

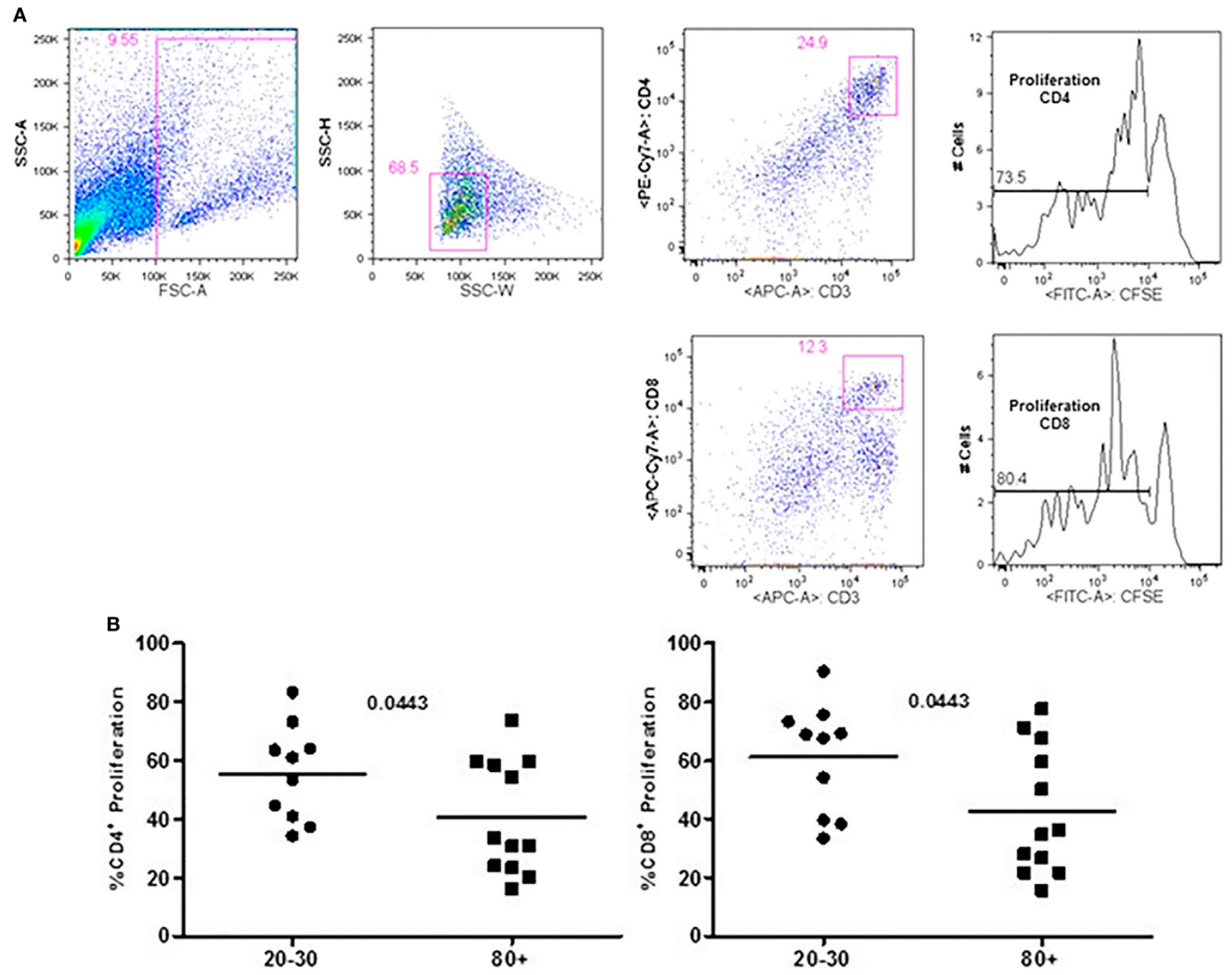


FIGURE 5 | Representative flow cytometry plots showing gate strategy for the frequency of proliferation in CD4+ and CD8+ T cells after stimulus with phytohemagglutinin (PHA) in culture. Gate in live cells (FSC-A × SSC-A), doublets exclusion (SSC-H × SSC-W), gate in CD3+CD4+ T cells, gate in the decrease of CFSE, gate in CD3+CD8+ T cells, gate in the decrease of CFSE **(A)**. Percentage of CD3+CD4+ and CD3+CD8+ T cells proliferation after stimulus with PHA according to age: 20–30 and 80+ years old **(B)**.

IgG levels >500 U/mL. The seropositivity to CMV was correlated with the decrease of Naïve CD8+ T cells and with a trend toward decrease in CD4+ central memory T cells and increase in CD8+ EMRA T cells. The impact of CMV infection/latency in immunity can exacerbate the features of immunosenescence. However, some of the features reported in literature were not in agreement with our studied old individuals such as the decrease in Naïve CD4+ T cells and CD4/CD8 ratio in addition to the increase in CD4+ and CD8+ central memory, CD8+ effector memory T cells, and pro-inflammatory cytokines (39, 47, 48). These differences may be due to the small number of individuals and great variability observed in the frequency of cell subtypes and cytokine production in our study population. Nonetheless, it cannot be ruled out by the possibility that the features preserved in immune system and observed in the old individuals studied are the key to achieve the longevity (13, 49, 50). Arai et al. (49) found in two different Japanese cohorts ($n = 1{,}554$) evaluating very old individuals (85–99 years), centenarians, and individuals ≥105 years that the lowest levels of inflammation correlated with the main indicators markers of successful aging, such as survival, capability, and cognition.

In old individuals, we observed lower rates of proliferation in CD4 and CD8 compartments in addition to the reduced levels of cytokines after stimulation with PHA. In association, we found lower percentage of CD4+ central memory T cells, which have been described as highly proliferative, and producers of IL-2 (13, 49–53). Corroborating with our data, Whisler et al. (51) showed that in *in vitro* there were diminished proliferative capacity and decreased production of IL-2 after stimulation with

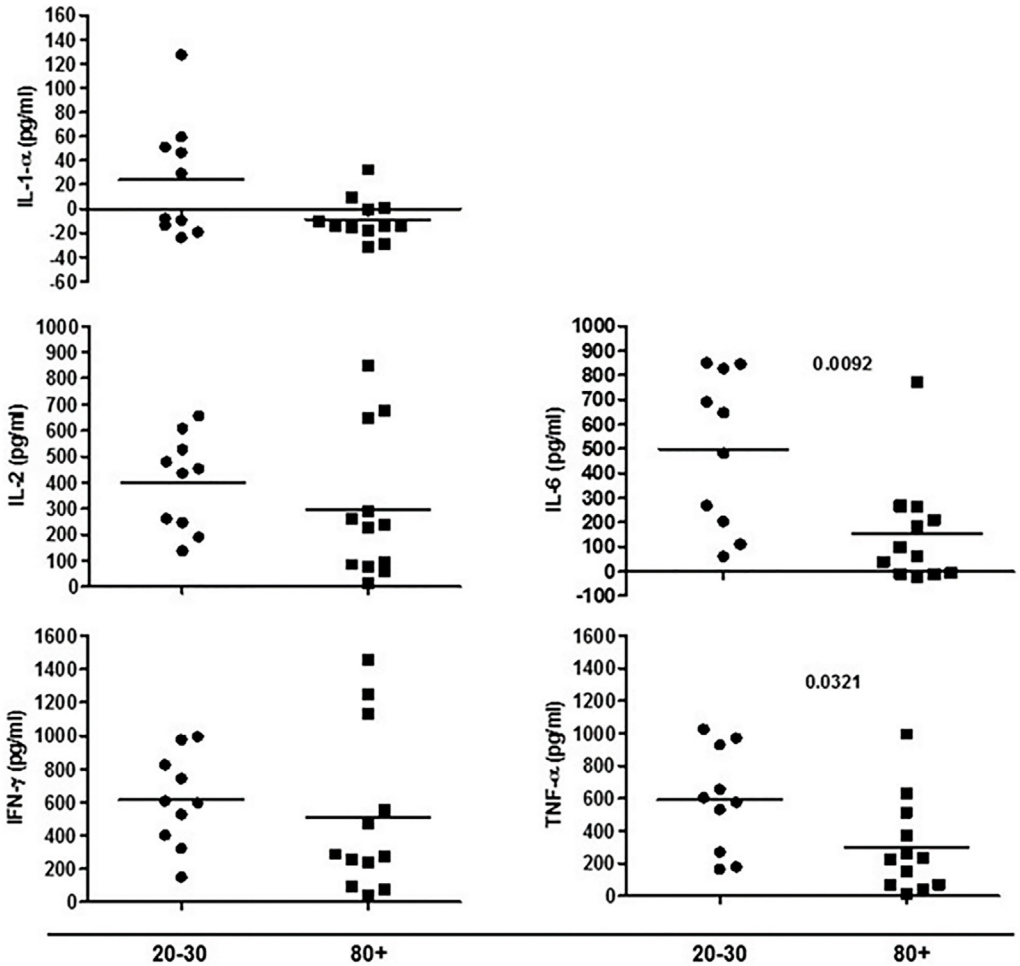


FIGURE 6 | Cytokines produced by cells in culture under phytohemagglutinin stimulus according to age: 20–30 and 80+ years old.

TABLE 2 | Serum levels of IgM and IgG to cytomegalovirus in old (80–100 years) individuals.

Age	Gender	IgM	IgG
100	F	0.159	>500
90	F	0.296	240.60
90	F	0.164	>500
93	M	0.237	316.30
93	F	0.160	31.36
88	M	0.158	<0.25
94	M	0.436	153.90
90	M	1.240	55.85
90	M	0.189	>500
94	M	0.157	180.60
91	F	0.488	>500
90	F	0.428	87.39

IgM cutoff index negative: <0.7; indeterminate: 0.7–0.99; positive: ≥1.0.
IgG negative <0.5 U/mL; indeterminate: 0.5–0.99 U/mL; positive: ≥1.0 U/mL.

PHA in 7 out of 12 old individuals evaluated (mean age 78 years) in association with deficient activation of transcriptional factor AP-1 and nuclear factor of activated T cells.

Others have reported that age interferes negatively with the expansion of T cells due to telomere erosion (52, 53). On the other hand, after vaccination with live attenuated virus varicella zoster Qi et al. (54) observed that the majority of activated T cells were $CD4^+$ and age (50–70 years) did not interfere with the expansion of antigen-specific T cells. However, the long-lived memory T cells (production of IFN-γ *in vitro*) decreased from day 14 to 28 post-vaccination.

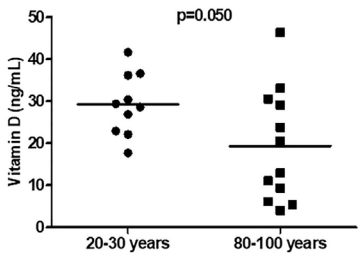
The study of Shahid et al. (55) showed reduced expression of IFN-γ and granzyme B in $CD8^+$ T cells of older adults vaccinated against influenza.

Our findings show that despite the common features of immune senescence presented by old individuals, they managed to achieve longevity.

Health professionals have proposed alternatives to circumvent age-associated diseases, such as physical activity, diet control, supplements, and probiotics (22–24). Vitamin D has been recommended due to its action on the immunity, but data from literature are inconclusive regarding the benefits of supplementation with vitamin D.

TABLE 3 | IgG levels against cytomegalovirus and correlation with parameters of the immune system in old (80–100 years) individuals.

Parameters	*r* Spearman	*p*
Cell number × 10^5	−0.164	0.6054
% Myeloid-derived suppressor cells (MDSC) ($CD33^+CD11b^+$)	0.071	0.8267
MDSC absolute cell number × 10^5	0.046	0.883
% CD15 MDSC	−0.267	0.397
% CD14 MDSC	−0.377	0.225
% $CD3^+CD4^+$	−0.209	0.532
% $CD3^+CD8^+$	0.107	0.753
CD4/CD8	−0.191	0.566
% Proliferation $CD4^+$	0.259	0.410
% Proliferation $CD8^+$	0.085	0.792
% $CD4^+$ Naïve	−0.135	0.673
% $CD4^+$ central memory	−0.559	0.06
% $CD4^+$ effector memory	0.295	0.347
% $CD4^+$ effector memory RA	0.499	0.100
% $CD8^+$ Naïve	−0.648	0.027
% $CD8^+$ central memory	−0.420	0.174
% $CD4^+$ effector memory	0.085	0.792
% $CD8^+$ effector memory RA	0.573	0.055
IL-1α	−0.346	0.276
IL-2	0.025	0.941
Interleukin-6	0.143	0.652
IFN-γ	0.110	0.731
Tumor necrosis factor-alpha	0.075	0.818
Vitamin D	0.302	0.336



Parameter	Median (variation)	p
Vitamin D (20-30 years old)	29.0 (17.7 - 41.7) ng/mL	0.050
Vitamin D (80-100 years old)	16.7 (4.01 - 46.4) ng/mL	

FIGURE 7 | Vitamin D [25(OH)D3] levels in young (*n* = 10; 20–30 years) and old (*n* = 12; 80–100 years) individuals.

The US Endocrine Society defined vitamin D levels of 20 ng/mL or less as deficiency, 21–29 ng/mL as insufficiency, and 30 ng/mL or more as sufficiency (56). However, suboptimal levels of vitamin D have been reported worldwide and depending on the lifestyle and environmental conditions, hypovitaminosis D could be observed in all age groups (57). In old adults, the diminished sun exposure, skin atrophy with decreased amounts of the precursor 7-dehydrocholesterol, and the reduced content of vitamin D in the diet leads to lower serum levels of vitamin D (58, 59). In accordance, blood samples were collected in summer and yet we observed that 50% of individuals older than 80 years showed vitamin D deficiency, while the insufficiency was observed in 50% of young individuals.

In innate immunity, macrophages and dendritic cells can convert vitamin D3 on biological active 1,25OH (60). In addition, immune cells also express the vitamin D receptor (VDR) and thus 1,25OH can act on immune microenvironment in paracrine and autocrine pathways (61).

Human monocytes activated through TLR upregulate the expression of genes associated with the conversion of 25OH to 1,25OH (Cyp27B1) and VDR. The addition of vitamin D to monocytes in culture leads to upregulation of VDR downstream genes, such as the antimicrobial cathelicidin (62–64). In opposition, it was observed that in adaptive immunity, vitamin D added in culture abolished the production of IFN-γ and IL-17 by $CD4^+$ memory T cells co-cultured with activated dendritic cells (pneumococci products) (65). Accordingly, Rode et al. (66) found that human $CD4^+$ T cells stimulated with CD3/CD28 beads in the presence of 25OH or 1,25OH present reduced production of IFN-γ.

In this study, there was a trend toward negative correlation between the absolute number of MDSC and vitamin D levels ($r = -0563$ $p = 0.089$) in young individuals. This is an important finding, since there was a higher percentage of MDSC in old individuals with predominance of the granulocytic phenotype ($CD33^+CD11b^+CD15^+$) that has been associated with some types of cancer in humans. In addition, these cells have recently been associated with frailty and Alzheimer's disease (11, 67).

Interventions to circumvent the MDSC increase have been proposed such as the use of vitamin D to induce the differentiation of non-mature suppressive myeloid cells into mature effector non-suppressive cells (68). In patients (49–71 years old) with head and neck cancer, vitamin D3 (20, 40, 60 μg/day) decreased the number of progenitor cells with suppressive phenotype, promoted the proliferation of T cells after *in vitro* stimulation, and increased the levels of effector cytokines (IL-12 and IFN-γ) (69).

Regarding to the $CD4^+$ T cell compartment there was no correlation with the levels of vitamin D which is in agreement with literature data showing that for young adults, vitamin D levels (70), or supplement (71, 72) previously to vaccination did not cause enhanced humoral immunity that is dependent of $CD4^+$ T cells help. However, Khoo et al. (73) showed that during winter the level of vitamin D decreases and is associated with lower percentage of Naive $CD4^+$ T cells suggesting a role played by vitamin D in this cell compartment.

Our data points for correlation of vitamin D levels with some parameters of $CD8^+$ T cells. In old individuals, vitamin D had a positive correlation with total $CD8^+$ T cells. Considering that we observed a trend toward lower percentage of $CD8^+$ T cells in old individuals (**Figure 2**), vitamin D could be beneficial in preventing the decrease of this cell subtype. In young individuals, vitamin D levels correlated positively with the frequency of $CD8^+$ effector memory T cells.

Another important result was the negative correlation between vitamin D and CD8 EMRA T cells in old individuals suggesting that higher levels of vitamin D would be linked to less

TABLE 4 | Vitamin D [25(OH)D3] levels and correlation with parameters of the immune system in young (20–30 years) and old (80–100 years) individuals.

Parameters	*r* Spearman 20–30 years	*p*	*r* Spearman 80–100 years	*p*
Cell number × 10^5	0.012	0.973	−0.182	0.571
% Myeloid-derived suppressor cells (MDSC) ($CD33^+CD11b^+$)	−0.527	0.117	0.370	0.235
MDSC absolute cell number × 10^5	−0.563	0.089	0.181	0.571
% CD15 MDSC	0.281	0.431	0.167	0.602
% CD14 MDSC	−0.176	0.626	0.168	0.602
% $CD3^+CD4^+$	0.442	0.200	0.190	0.574
% $CD3^+CD8^+$	0.588	0.074	0.764	0.006
CD4/CD8	−0.090	0.803	−0.547	0.082
% Proliferation $CD4^+$	0.345	0.328	−0.238	0.457
% Proliferation $CD8^+$	0.236	0.511	−0.126	0.697
% $CD4^+$ Naïve $CD3^+CD4^+CD45RA^+CD27^+$	−0.109	0.764	−0.027	0.931
% $CD4^+$ central memory $CD3^+CD4^+CD45RA^-CD27^+$	−0.079	0.829	0.287	0.366
% $CD4^+$ effector memory $CD3^+CD4^+CD45RA^-CD27^-$	−0.042	0.907	0.028	0.931
% $CD4^+$ effector memory RA $CD3^+CD4^+CD45RA^+CD27^-$	0.150	0.701	−0.192	0.548
% $CD8^+$ Naïve $CD3^+CD8^+CD45RA^+CD27^+$	−0.297	0.405	0.378	0.269
% $CD8^+$ central memory $CD3^+CD8^+CD45RA^-CD27^+$	0.236	0.511	0.462	0.131
% $CD8^+$ effector memory $CD3^+CD8^+CD45RA^-CD27^-$	0.697	0.025	0.260	0.467
% $CD8^+$ effector memory RA $CD3^+CD8^+CD45RA^+CD27^-$	0.188	0.603	−0.552	0.050
IL-1α (pg/mL)	−0.472	0.169	0.505	0.094
IL-2 (pg/mL)	0.030	0.934	−0.266	0.404
Interleukin-6 (pg/mL)	−0.151	0.676	−0.246	0.442
IFN-γ (pg/mL)	−0.491	0.150	−0.014	0.966
Tumor necrosis factor-alpha (pg/mL)	0.212	0.556	−0.133	0.681

accumulation of cells that have been described as a marker of senescence (74, 75).

There was no correlation of vitamin D levels and proliferation of T cells ($CD4^+$ and $CD8^+$) or production of cytokines after stimulation with PHA. In agreement, the addition of vitamin D to culture of T cells stimulated with CD3 and CHO-CD80 cell line did not increase the proliferative capacity (individuals from 32 to 57 years old) (76). In addition, PBMCs stimulated *in vitro* in the presence of vitamin D showed diminished IFN-γ and increased IL-4 production in culture supernatant (77).

Aging has been related to chronic low-grade inflammation (inflammaging) with increased levels of circulating C-reactive protein (CPR), IL-6, and TNF-α. The InCHIANTI study found that individuals ($n = 867$, mean age 75.1 years) with vitamin D levels lower than 31.4 nmol/L presented high circulating IL-6, but not TNF-α, IL-1α, and IL-18 (78).

The English Longitudinal Study of Aging assessed community-dwelling individuals ($n = 5{,}870$, 50–80 years) and reported that low levels of 25OH (≤30 nmol/L) were negatively associated with CPR (79). A follow-up of old individuals ($n = 23$, 55–86 years) for 12 months showed that vitamin D levels were significantly lower in the winter with an increase in the number of individuals presenting deficiency. In the same season, there was a significant increase of circulating IL-6, IL-8, IL-β-1, MCP-1, and TNF-α (80). The low-grade chronic inflammation has been associated with aging-related diseases, and suboptimal levels of vitamin D have been related to chronic diseases/overall mortality (81–84), suggesting that adequate levels of vitamin D could benefit the aging population.

Despite the size of the sample be a limitation of the study and not allow more detailed statistical analyses, after testing the variables for normality and applying the adequate statistics, we obtained some important results. We found that the old population evaluated could be considered healthy based on the metabolic parameters. In this sample, 11 out of 12 were CMV^+ and still maintained preserved some features of immunity such as CD4/CD8 ratio, and low production of inflammatory cytokines after stimulus. On the other hand, we observed increased frequency of MDSC, reduced number of circulating leukocytes, reduced percentage of total $CD8^+$ and Naïve $CD8^+$ T cells, and increased percentage of terminally differentiated $CD8^+$EMRA T cells. CMV^+ was correlated with the decrease of $CD8^+$ Naïve T cells and increase in $CD8^+$ EMRA T cells. Vitamin D levels were insufficient in 50% of old individuals and correlated positively with total $CD8^+$ T cells and negatively with CD8 EMRA T cells. Our next step is to develop an *ex vivo* model to study the action of vitamin D in $CD4^+$ and $CD8^+$ T cells, associated phenotypes, proliferation, and cytokines production.

CONCLUSION

In the studied population, longevity was correlated to maintenance of some immune parameters. Considering the limitations of the study as size of the sample and lack of functional assays showing the direct effect of vitamin D in immunity, it was found that vitamin D in old individuals was correlated to some features of the immune system, mainly in the CD8 compartment.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "UNIFESP CEP Committee of Ethics in Research" 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 "CEP Committee of Ethics in Research."

AUTHOR CONTRIBUTIONS

All authors contributed significantly to this study and have read and approved the submitted manuscript. AA—laboratorial experiments, data analysis, figures. MI—laboratorial experiments. YD—SABE coordinators of study teams responsible for recruitment and sample collection. VB—laboratorial experiments and data analysis, figures, manuscript writing, and primary responsibility for final content.

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CD8+HLADR+ Regulatory T Cells Change With Aging: They Increase in Number, but Lose Checkpoint Inhibitory Molecules and Suppressive Function

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CD4+ regulatory T cells have been intensively studied during aging, but little is still known about age-related changes of other regulatory T cell subsets. It was, therefore, the goal of the present study to analyze CD8+human leukocyte antigen–antigen D related (HLADR)+ T cells in old age, a cell population reported to have suppressive activity and to be connected to specific genetic variants. We demonstrate a strong increase in the number of CD8+HLADR+ T cells with age in a cohort of female Sardinians as well as in elderly male and female persons from Austria. We also show that CD8+HLADR+ T cells lack classical activation molecules, such as CD69 and CD25, but contain increased numbers of checkpoint inhibitory molecules, such as cytotoxic T lymphocyte-associated antigen 4, T cell immunoglobulin and mucin protein-3, LAG-3, and PD-1, when compared with their HLADR− counterparts. They also have the capacity to inhibit the proliferation of autologous peripheral blood mononuclear cells. This suppressive activity is, however, decreased when CD8+HLADR+ T cells from elderly persons are analyzed. In accordance with this finding, CD8+HLADR+ T cells from persons of old age contain lower percentages of checkpoint inhibitory molecules than young controls. We conclude that in spite of high abundance of a CD8+ regulatory T cell subset in old age its expression of checkpoint inhibitory molecules and its suppressive function on a per cell basis are reduced. Reduction of suppressive capacity may support uncontrolled subclinical inflammatory processes referred to as "inflamm-aging."

Keywords: CD8+ T cells, CD8+human leukocyte antigen–antigen D related+, aging, checkpoint inhibitory molecules, regulatory T cells

Abbreviations: BM, bone marrow; BMMCs, bone marrow mononuclear cells; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; CXCR3, C-X-C motif chemokine receptor 3; FMO, fluorescence minus one; FOXP3, forkhead box protein P3; HLADR, human leukocyte antigen–antigen D related; LAG-3, lymphocyte activation gene-3; PB, peripheral blood; PBMCs, peripheral blood mononuclear cells; PD-1, programmed death 1; TIM-3, T cell immunoglobulin and mucin protein-3; Tregs, regulatory T cells.

INTRODUCTION

We have recently reported genetic contributions to quantitative levels of 95 immune cell types encompassing 272 immune traits in a cohort of 1,629 individuals from four clustered Sardinian villages (1). One of these 95 cell types are human leukocyte antigen–antigen D related (HLADR)$^+$ T cells. In the past, the expression of HLADR on human T cells has mainly been regarded as a marker of activated T cells (2–4). However, HLADR expression on CD4$^+$ regulatory T cells (Tregs) has also been interpreted as a marker of a functionally distinct population of mature Tregs (5). Recently, HLADR expression on CD8$^+$ T cells has been suggested to represent a marker of a natural human CD8$^+$ regulatory T cell subset (6). This subset was shown to suppress the proliferation of autologous peripheral blood mononuclear cells (PBMCs) presumably by cell-to-cell contact with cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) signaling playing an essential role in this process. In the past, CD8$^+$ T cells were the first to be described to have immunosuppressive properties (7, 8). However, difficulties in characterizing these "suppressor" CD8$^+$ T cells and lack of specific markers delimited this area noticeably. Only when CD4$^+$CD25$^+$FOXP3$^+$ T cells were shown to be strongly immunosuppressive (9–13), a global interest in the downregulation of immune activity by T cells re-emerged. Apart from the description of many interesting specific properties, CD4$^+$ Tregs were shown to increase in number in some important conditions, such as in patients with tumors (14) or during aging [reviewed in Ref. (15)]. In the latter context it was of interest that some Treg populations, specifically naturally occurring Tregs, seemed to accumulate in old age, whereas inducible Tregs decrease in elderly persons. Little information is, however, still available on the functional competence of CD4$^+$ Tregs in old age. The worldwide strong interest in CD4 Tregs also stimulated new research efforts in the analysis of potential CD8$^+$ suppressor cells. Different subsets were defined, such as CD8$^+$CD28 low cells (16–20), CD8$^+$CD122$^+$ cells in mice (21–23), CD8$^+$C-X-C motif chemokine receptor 3$^+$ cells in humans (24) and CD8$^+$CD39$^+$FOXP3$^+$ cells (25). CD8$^+$HLADR$^+$ T cells were therefore just one of several CD8$^+$ T cell subsets believed to have regulatory properties (6). In view of the clear genetic regulation of this population exerted by a genetic variant in the *CIITA* gene region (1) and the fact that the composition of the CD8$^+$ population characteristically changes with age (26), we became interested in elucidating potential age-related changes in the number and function of CD8$^+$HLADR$^+$ T cells. We now demonstrate that CD8$^+$HLADR$^+$ T cells increase in number with aging, but lose suppressive activity on a per cell basis. This may challenge the homeostatic balance between immune cell sub-populations in old age and support the development of inflammation.

MATERIALS AND METHODS

Study Subjects

Samples from three different cohorts were used for this study. Details regarding the probands' characteristics are summarized in **Table 1**.

Cohort A

Peripheral blood (PB) samples were obtained from 91 healthy females recruited in Lanusei, Sardinia, Italy. The exclusion criteria were the following:

Persons who experienced significant variation of body temperature or received vaccination in the 2 weeks before the blood draw, as well as persons under antimicrobial treatment were excluded from the study. Persons with pathological conditions were also excluded.

Cohort B

Peripheral blood samples were obtained from healthy Austrian 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 impairment.

Cohort C

Bone marrow (BM) and PB-paired samples were obtained from patients who underwent hip replacement surgery in Wels, Austria. The patients did not receive immunomodulatory drugs or suffer from diseases known to influence the immune system, such as autoimmune diseases and cancer.

Mononuclear Cell Isolation

Peripheral blood mononuclear cells from heparinized blood were purified by Ficoll-Hypaque density gradient centrifugation (GE Healthcare Life Sciences). Cells were washed with complete RPMI medium (RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin; Invitrogen). PBMCs from cohort A were frozen according to a standard protocol using 10% DMSO as cryoprotective agent. PBMCs from Cohort B and C were freshly used.

Bone marrow samples (cohort C) were obtained from the femur shaft of patients of varying age (**Table 1**) during hip replacement surgery. A biopsy of *Substantia spongiosa ossium*, 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). BMMCs were freshly used.

Cell Sorting

T cells were isolated from fresh PBMCs by magnetic cell sorting using the MACS human Pan T cell isolation kit (Miltenyi Biotech) following manufacturer's protocol. Purified T cells were then stained with anti-CD8 PerCP (RPA-T8), anti-CD28 BV421 (L293), anti-HLADR PeCy7 (G46-6) Abs, all from BD Biosciences. Labeled cells were sorted with a FACSAria II flow cytometer (BD Biosciences). Cells were either sorted into CD8$^+$HLADR$^+$, CD8$^+$HLADR$^-$ or into CD8$^+$CD28$^+$HLADR$^+$, CD8$^+$CD28$^+$HLADR$^-$, CD8$^+$CD28$^-$HLADR$^+$ or CD8$^+$CD28$^-$

TABLE 1 | Demographic data on the cohorts used.

Cohort	Material	*n*	*n* Female	Age (median)	Age (range)	Site of recruitment
A	Peripheral blood (PB)	91	91 (100%)	49 years	18–89 years	Lanusei, Sardinia, Italy
B	PB	26	16 (61%)	26 years	21–29 years	Innsbruck, Austria
B	PB	22	13 (59%)	77 years	71–88 years	Innsbruck, Austria
C	Bone marrow	29	17 (58%)	68 years	39–87 years	Wels, Austria
C	PB	29	17 (58%)	68 years	39–87 years	Wels, Austria

HLADR$^-$ T cells. Fluorescence minus one (FMO) was used as control. The cells were collected into RPMI 1640 medium containing 15% FCS and washed once prior to further studies. The purity of each subset was >95% as determined by flow cytometry.

Flow Cytometric Analyses

Peripheral blood mononuclear cells and BMMCs were stained with anti-CD4 VioGreen (REA623), anti-CD3 Vio770 (REA613) from Miltenyi Biotech, anti-CD45RA PerCp (HI100), anti-CD28 APC (28.2), anti-CTLA-4 PE (BNI3), anti-T cell immunoglobulin and mucin protein-3 (TIM-3) PerCP (F38-2E2), anti-programmed death 1 (PD-1) FITC (EH12.2H7), anti-lymphocyte activation gene-3 (LAG-3) APC (7H2C65) from Biolegend, anti-HLADR PeCy7 (G46-6), anti-CD28 BV421 (28.2), anti-CD25 APC (2A3), and anti-CD69 PE (FN 50) from BD Biosciences. Intracellular detection of FOXP3 with anti-FOXP3 FITC Abs was performed using fixed and permeabilized cells following the manufacturer's instructions. FMO was used as control. Dead cells were excluded by forward and side scatter characteristics and by using either 7-AAD or fixable viability dye Zombie violet (Biolegend).

For each sample at least 2×10^6 total events were acquired using a FACScanto II and FACSSymphony (BD Biosciences). The data were analyzed using FlowJo software.

Suppression Assay

The capacity of sorted CD8$^+$HLADR$^+$, CD8$^+$HLADR$^-$, CD8$^+$CD28$^+$HLADR$^+$, CD8$^+$CD28$^+$HLADR$^-$, CD8$^+$CD28$^-$HLADR$^+$, and of CD8$^+$CD28$^-$HLADR$^-$ T cells to suppress the proliferation of responder autologous PBMCs was analyzed by CFSE dilution.

CFSE dilution method: responder autologous PBMCs (1×10^5) labeled with 1 μM CFSE (Invitrogen) were cultured with highly purified unlabeled CD8$^+$HLADR$^+$, CD8$^+$HLADR$^-$, CD8$^+$CD28$^+$HLADR$^+$, CD8$^+$CD28$^+$HLADR$^-$, CD8$^+$CD28$^-$HLADR$^+$, and CD8$^+$CD28$^-$HLADR$^-$ T cells at different responder: suppressor cell ratios (2:1, 4:1, and 8:1). Cells were stimulated with 1 μg/ml anti-CD3 (BD Pharmingen) and 0.5 μg/ml anti-CD28 (BD Pharmingen) Abs in 96-well round-bottom plate and cultured in complete medium. After 4 days of culture cells were stained with anti-CD4 BV 510 (BD Pharmingen), anti-CD8 PeCy7 (BD Pharmingen) and proliferation of CFSE-labeled cells was assessed by flow cytometry. Percentage of suppression was calculated as 100 minus the percentage of responder PBMCs, which underwent one or more cell divisions (proliferated PBMCs) in the presence of suppressor cells divided by the percentage of proliferated PBMCs when cultured alone, and multiplied by 100 as shown in the following formula:

$$\%\ \text{suppression} = 100 - \frac{\left(\begin{array}{c}\text{proliferated PBMCs cultured}\\ \text{with suppressor cells}\end{array}\right)}{\text{proliferated PBMCs cultured alone}} \times 100$$

The following controls for the suppression assay were used:

- CFSE-unlabeled and unstimulated (in the absence of stimulatory antibodies) cells,
- CFSE-labeled and unstimulated cells,
- CFSE-labeled and stimulated cells in the absence of suppressor cells.

Neutralization Assay

To test the involvement of the checkpoint inhibitory molecules in the suppression mediated by CD8$^+$HLADR$^+$CD28$^+$ T cells, neutralizing Abs against CTLA-4 (BD Biosciences), TIM-3 (Invitrogen), LAG-3 (Adipogen Life Sciences), and PD-1 (Invitrogen) were added to the co-cultures. All neutralization assays were performed under the culture conditions described above. The corresponding isotype-matched mAb IgG1 (Biozym) was used as control. Optimal neutralizing Ab concentrations were determined in pilot experiments.

Statistical Analysis

Statistical significance was assessed by Spearman correlation analysis, Mann–Whitney *U*-test and Wilcoxon matched pairs test. *p*-Values below 0.05 were considered as significant.

Study Approval

Cohort A

The study was approved by the Research Ethics and Bioethics Committee of the Consiglio Nazionale delle Ricerche (Italy). Written informed consent was received from participants prior to their inclusion in the study.

Cohort B

The study was approved by the Ethics Committees of the Medical University of Innsbruck (Austria). Written informed consent was received from participants prior to their inclusion in the study.

Cohort C

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+HLADR+ T Cells Increase With Age

The percentage of HLADR+ cells within the CD8 T cell fraction was analyzed by FACS (**Figure 1**), first in a cohort of 91 female Sardinians (**Figure 1A**) who had previously been recruited and genotyped (1) and second in a young (<30 years) and an elderly (≥70 years) cohort of Austrian males and females (**Figure 1B**). When HLADR+ T cells were correlated with age, there was a highly significant positive correlation in the Sardinian cohort. In the Austrian cohort, CD8+HLADR+ T cells were compared in young and elderly persons and there was a significant difference between the groups, the elderly persons having a higher percentage of CD8+HLADR+ T cells. In order to define whether the phenotype of HLADR+ T cells corresponded to previous reports (6), FACS analysis of HLADR+ cells from young persons was performed (**Figure 2**). The gating strategy for HLADR+ cells and the assessment of CD28 and CD45RA on HLADR+ T cells are shown in **Figure 2A**. The percentages of CD28+CD45RA+, CD28+CD45RA−, CD28−CD45RA−, and CD28−CD45RA+ cells indicating different differentiation stages from naïve to T_{EMRA}-like cells are shown in CD8+HLADR+ and in CD8+HLADR− cells in **Figure 2B**. **Figures 2C,D** demonstrate the gating strategy as well as the expression of CD28, FOXP3, CD25, and CD69 in the CD8+HLADR+ as well as in the CD8+HLADR− cell populations. The figures show that CD8+HLADR+ and CD8+HLADR− cells contained cells of every CD8 T cell subset when defined according to the expression of CD28 and CD45RA, although there were fewer cells in the CD28+CD45RA+ (naïve) subset and more cells in the CD28+CD45RA− and the CD28−CD45RA− (memory) subsets (**Figures 2A,B**). In accordance with previous reports (6) neither CD8+HLADR+ nor CD8+HLADR− T cells expressed the classical activation markers CD69 and CD25 and they were negative for FOXP3. The percentage of CD28+ cells was identical in the two T cell subsets (**Figures 2C,D**).

Expression of Checkpoint Inhibitory Molecules in CD8+HLADR+ and CD8+HLADR− T Cells

The checkpoint inhibitory molecules CTLA-4, TIM-3, LAG-3, and PD-1 were analyzed in CD8+HLADR+ and CD8+HLADR− T cells in the resting state (**Figures 3A,C**). The HLADR+ subset contained between 7% (LAG-3) and 28% (PD-1) cells which carried the inhibitory molecules. In contrast, CD8+HLADR− cells did not contain checkpoint inhibitory molecules in the resting state. Following stimulation with anti-CD3 and anti-CD28, the percentage of total HLADR+ cells expectedly increased (**Figure 3B**; $p < 0.0001$). The percentage of inhibitory molecule-expressing cells also increased in the stimulated CD8+HLADR+ as well as in the CD8+HLADR− subsets, but there was always a difference between the two populations. CD8+HLADR+ cells contained more cells expressing inhibitory molecules than CD8+HLADR− cells (**Figures 3B,D**). When one assessed the percentage of cells positive for checkpoint inhibitory molecules in HLADR+CD28+ and HLADR+CD28- T cells, the CD28+ subset always contained more cells expressing inhibitory molecules than the CD28− subset (**Figure 3E**). The same pattern was observed following stimulation of the cells with anti-CD3 and anti-CD28 antibodies (**Figure 3F**). When we assessed the percentage of cells positive for checkpoint inhibitory molecules in the four populations defined by divergent expression of CD45RA and CD28 (**Figure 2**) in CD8+HLADR+ T cells, the CD28+CD45RA+ population contained more cells expressing CTLA-4, TIM-3, and LAG-3 than the CD28+CD45RA−, CD28−CD45RA−, and CD28−CD45RA+ populations (Figure S1 in Supplementary Material). In contrast, PD1+HLADR+ cells were more frequent in CD45RA− than in CD45RA+ cells. A similar marker profile was observed following stimulation (Figure S1B in Supplementary Material). In CD8+ HLADR− cells the expression of checkpoint inhibitory molecules was generally very low even after stimulation (Figures S1C,D in Supplementary Material).

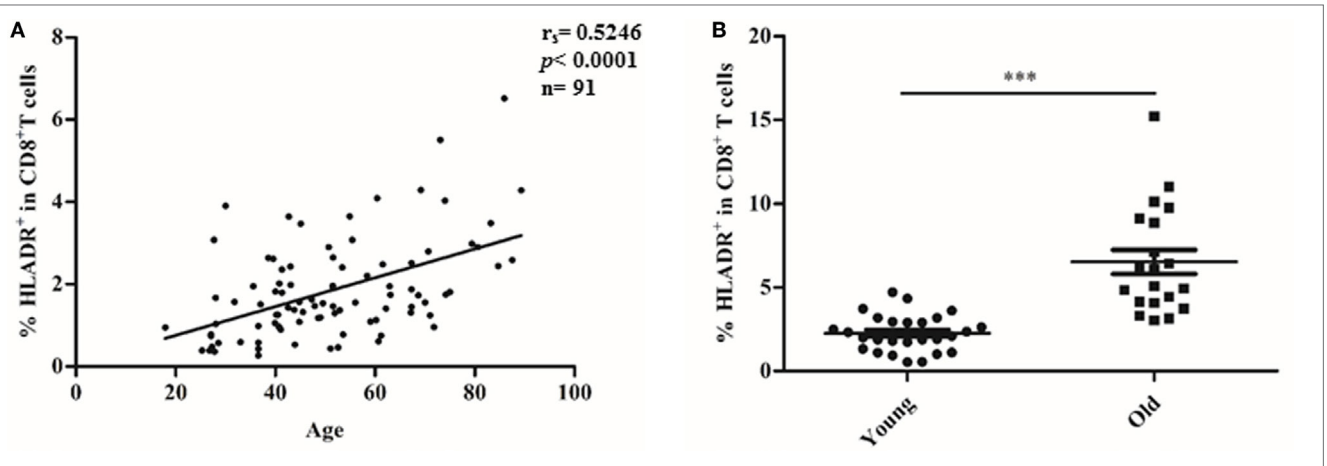

FIGURE 1 | CD8+human leukocyte antigen–antigen D related (HLADR)+ T cells increase with age. HLADR+ cells (%) within CD3+CD8+ (100%) were measured by FACS analysis. **(A)** Correlation of CD8+HLADR+ cells in 91 female donors from Sardinia with age. Relationship between CD8+HLADR+ T cells (%) and age was assessed by Spearman correlation analysis; (r_s), p value, and sample size (n) are indicated in **(B)** CD8+HLADR+ T cells in young vs elderly donors from Austria. Mean ± SEM are indicated for each group; young (<30 years), $n = 26$, old (≥70 years), $n = 20$. Statistical significance was assessed by Mann–Whitney test, ***$p < 0.0001$.

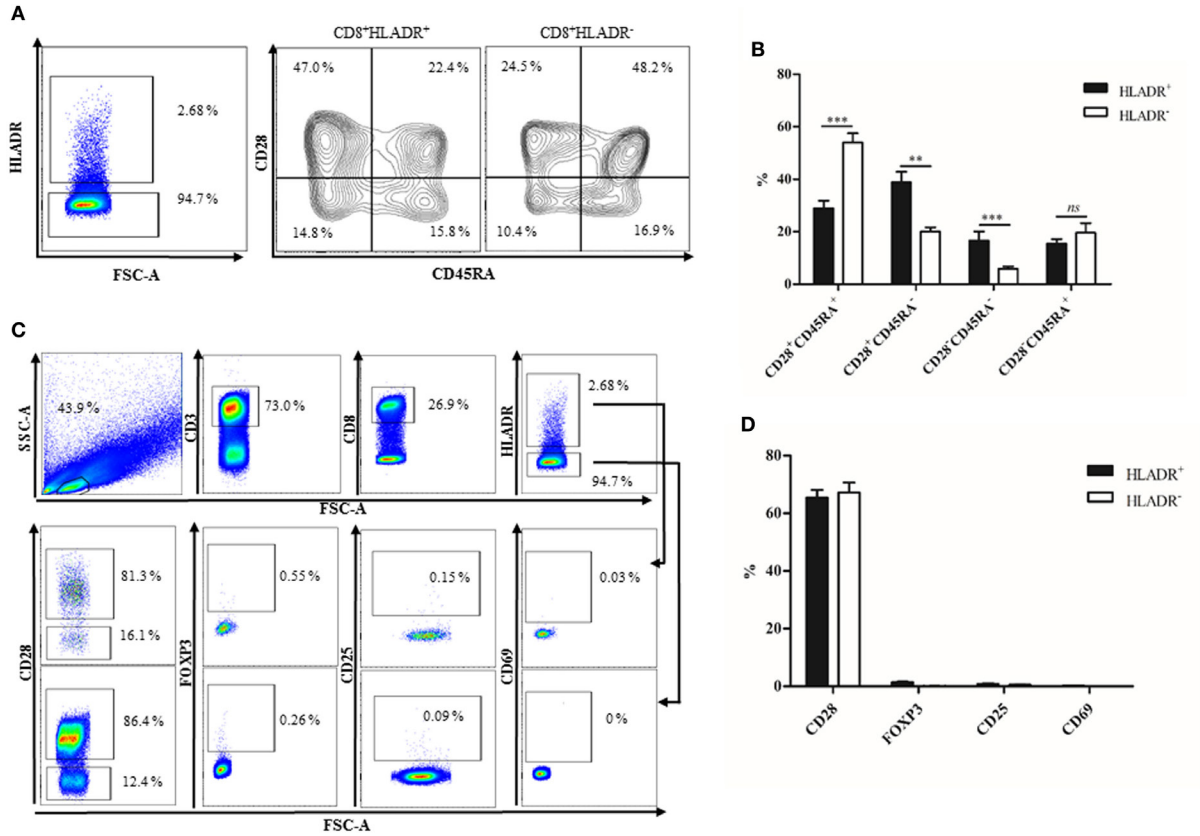

FIGURE 2 | Phenotypical characterization of CD8+human leukocyte antigen–antigen D related (HLADR)+ and CD8+HLADR− T cells. **(A)** Representative FACS plots demonstrating the expression of CD45RA and CD28 in HLADR+ and HLADR− cells from a young donor. **(B)** Percentages of HLADR+ and HLADR− cells (CD28+CD45RA+, CD28+CD45RA−, CD28−CD45RA−, and CD28−CD45RA+ cells), young donors $n = 24$ (<30 years), mean ± SEM, $^{**}p < 0.001$, $^{***}p < 0.0001$, ns, not significant. **(C)** Representative FACS plots of activation markers and other molecules in CD8+HLADR+ and CD8+HLADR− cells. The upper panel represents the gating strategy. **(D)** Percentages of CD28, FOXP3, CD25, and CD69 in CD8+HLADR+ vs CD8+HLADR− cells; mean ± SEM, young donors $n = 22$ (<30 years).

CD8+HLADR+ T Cells Have Suppressive Activity Toward the Proliferation of Autologous Stimulated PBMCs

CD8+HLADR+ T cells have been referred to as "suppressor cells" (6). It was, therefore, our next goal to confirm this assumption in cells from young persons. CD3+ cells were purified with magnetic beads and CD8+HLADR+ T cells were sorted according to a strategy depicted in Figure S2A in Supplementary Material. CD8+HLADR+ T cells were additionally sub-divided into CD28+ and CD28− T cells. The purity of the different fractions was always >95%. The purified subsets were then co-cultured with CFSE- labeled autologous PBMCs and the proliferation of the labeled cells was measured 4 days after stimulation with anti-CD3 and anti-CD28 (Figures S2B–E in Supplementary Material). In the first set of experiments, HLADR+ cells were compared with HLADR− cells without considering the expression of CD28 (Figures S2A–D in Supplementary Material). CD8+HLADR+ T cells suppressed the proliferation of autologous PBMCs in a dose-dependent manner. At a ratio of 4:1 they were still suppressive (Figures S2C,D in Supplementary Material). In Figure S2D in Supplementary Material mean suppressive activities of CD8+HLADR+ and CD8+HLADR− T cells on PBMC proliferation are shown. In spite of a minor not dose-dependent suppressive effect of the CD8+HLADR− population, suppression of the CD8+HLADR+ subset was always higher. In view of the difference in the expression of inhibitory molecules on HLADR+CD28+ and HLADR+CD28− T cells we were interested whether these two subsets had the same suppressive activity. For this reason, the purified HLADR+CD28+ and HLADR+CD28− T cells were added to CFSE-labeled autologous PBMCs. At least at a ratio of 1:2, HLADR+CD28+ cells had a more pronounced suppressive effect than their CD28− counterparts. At lower cell concentrations there was still a tendency toward increased suppression, but this did not reach statistical significance.

CD8+HLADR+ T Cells Occur Also in the BM Where They Have a Similar Phenotype as in the PB

In order to define whether CD8+HLADR+ T cells occur only in the PB or in other lymphatic organs, we investigated BM samples

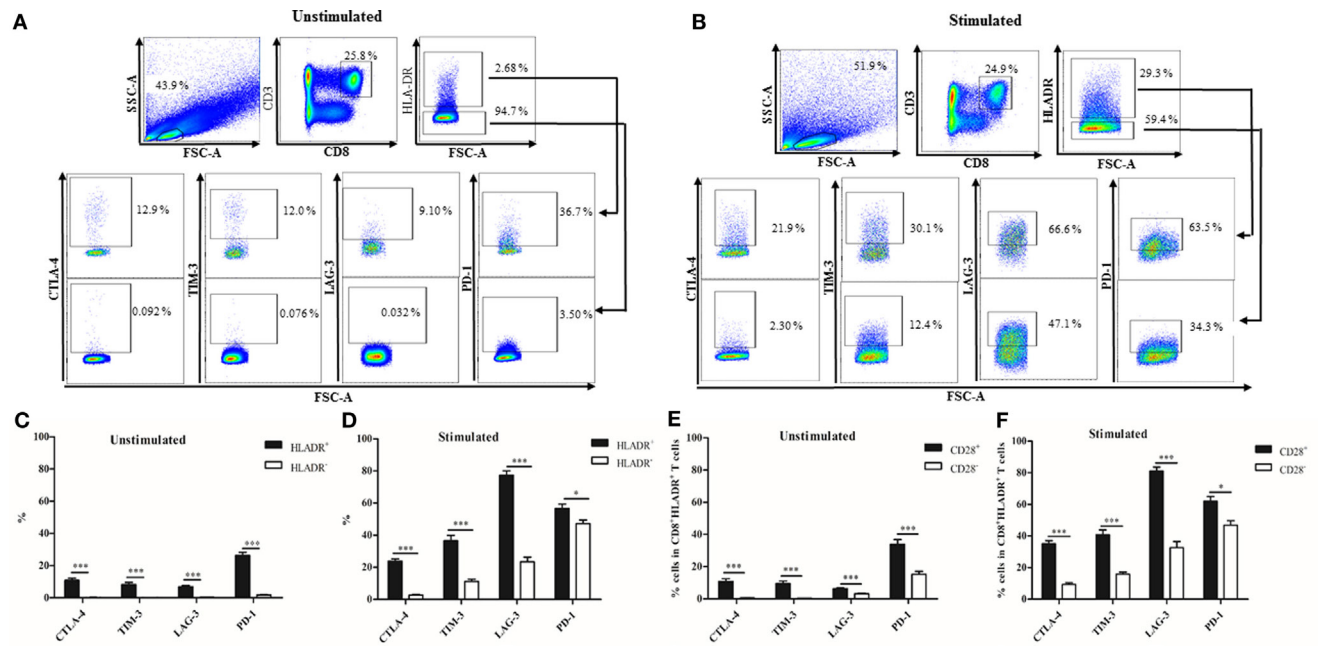

FIGURE 3 | Expression of checkpoint inhibitory molecules on CD8+human leukocyte antigen–antigen D related (HLADR)+ and on CD8+HLADR− T cells. **(A)** Representative FACS plots of inhibitory molecules in the CD8+HLADR+ (upper panel) and the CD8+HLADR− population (lower panel) using cells from a young donor. **(B)** Representative FACS plots of inhibitory molecules in the CD8+HLADR+ and the CD8+HLADR− population using cells from a young donor following stimulation with anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml) for 24 h. **(C)** Expression of checkpoint inhibitory molecules in CD8+HLADR+ vs CD8+HLADR− T cells in unstimulated cells; young donors $n = 22$ (<30 years), mean ± SEM, $^{***}p < 0.0001$. **(D)** Expression of checkpoint inhibitory molecules in CD8+HLADR+ vs CD8+HLADR− T cells following stimulation; young donors $n = 9$ (<30 years), mean ± SEM, $^{***}p < 0.0001$, $^{*}p < 0.05$. **(E)** Expression of checkpoint inhibitory molecules in CD8+HLADR+CD28+ vs CD8+HLADR+CD28− T cells in unstimulated cells; young donors $n = 22$ (<30 years), mean ± SEM, $^{***}p < 0.0001$. **(F)** Expression of checkpoint inhibitory molecules CD8+HLADR+CD28+ vs CD8+HLADR+CD28− T cells following stimulation; young donors $n = 9$ (<30 years), mean ± SEM, $^{***}p < 0.0001$, $^{*}p < 0.05$.

from the femur of patients of varying age (**Table 1**) after hip replacement surgery. We and others have recently studied the immunological memory in the human BM in depth (27–31). We were, therefore, interested whether a CD8 regulatory T cell type was also present in this organ. We compared CD8+HLADR+ T cells in BMMCs and PBMCs (**Figure 4**) and found that the proportions of this specific cell type were even higher in the BM than in the PB (**Figure 4B**). As in the PB, BM CD8+HLADR+ T cells are frequently CD28+ and do not express FOXP3 or CD25 (**Figure 4C**). In contrast to PBMCs, they contain a small proportion of CD69+ T cells (**Figure 4C**), but this is a characteristic feature of BM resting T cells and not of activation (27). The difference between CD8+HLADR+CD69+ T cells in the BM and the PB was also not significant (**Figure 4D**). As in the PB, cells expressing checkpoint inhibitory molecules were more frequent in the HLADR+ than in the HLADR− population and more frequent in HLADR+CD28+ than in HLADR+CD28− cells (**Figures 5A–C**). When CD8+HLADR+ T cells were compared in BMMCs and PBMCs there was no significant difference in the number of cells (**Figure 5D**). However, when only the CD28+ population of CD8+HLADR+ cells was analyzed, checkpoint inhibitory molecules were with the exception of LAG-3 more frequently expressed in BMMCs than in PBMCs (**Figure 5E**). As the number of samples available from the BM was relatively small and age greatly varied, no correlations with age could be made.

CD8+HLADR+ T Cells Have a Lower Expression of Checkpoint Inhibitory Molecules in Old Age

We compared CD8+HLADR+ T cells in the PB from young and elderly persons. Having found that there were more CD8+HLADR+ T cells in the PB from elderly persons than from young ones (**Figure 1**), we were now interested to define their phenotype and function. As in samples from young donors, CD8+HLADR+ T cells were more frequently CD28+CD45RA− memory-like T cells than CD28+CD45RA+ naïve (**Figures 6A,B**). Decreased percentages of CD28+CD45RA+ naïve T cells in the CD8+HLADR+ population combined with increased percentages of CD28−CD45RA+ T_{EMRA}-like cells in the old compared to the young cohort reflected age-related changes typical for the total CD8 T cell pool in old age (26). This suggests that CD8+HLADR+ T cells do not necessarily represent a separate lineage, but are subject to differentiation such as the CD8+HLADR− T cell population. As in the young population, FOXP3, CD25, and CD69 were not expressed in PB CD8+HLADR+ T cells from elderly persons (**Figures 6C,D**).

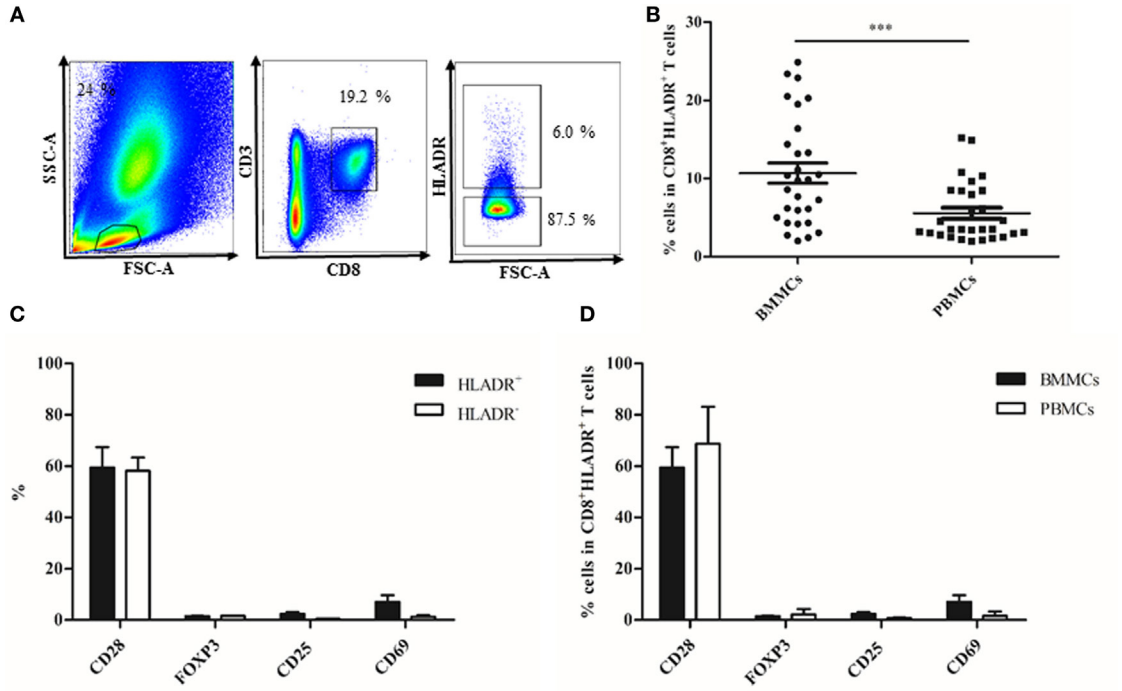

FIGURE 4 | Phenotypical characterization of CD8+human leukocyte antigen–antigen D related (HLADR)+ and CD8+HLADR− T cells in bone marrow mononuclear cells (BMMCs) and peripheral blood mononuclear cells (PBMCs) paired samples. **(A)** Representative FACS plots of the gating strategy in the bone marow. **(B)** Percentages of HLADR+ cells in CD8+ T cells in BMMCs vs PBMCs; $n = 29$; mean ± SEM, $^{***}p < 0.0001$. **(C)** Percentages of CD28, FOXP3, CD25, and CD69 in CD8+HLADR+ and CD8+HLADR− cells in BMMCs; $n = 5$; mean ± SEM. **(D)** Percentages of CD28, FOXP3, CD25, and CD69 in CD8+HLADR+ cells in BMMCs and PBMCs; $n = 5$; mean ± SEM.

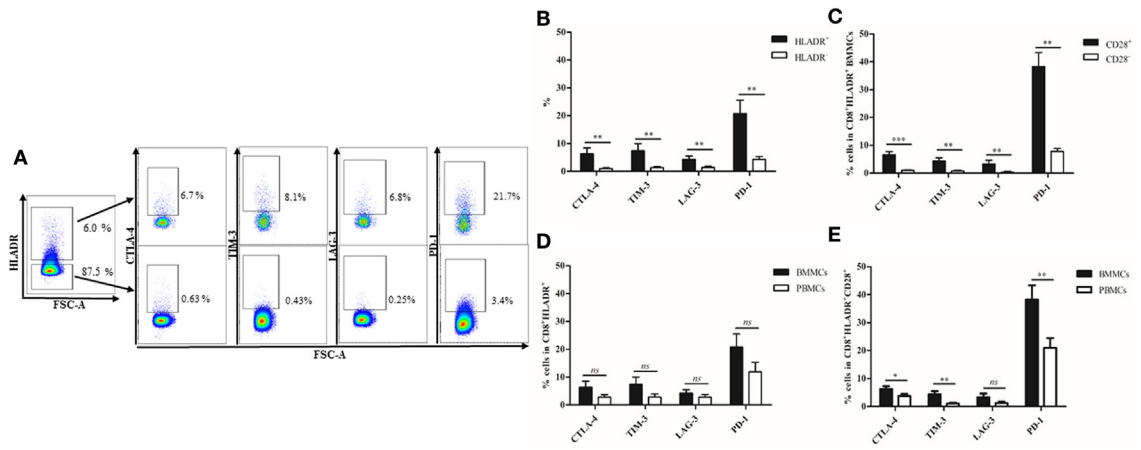

FIGURE 5 | Expression of checkpoint inhibitory molecules on CD8+human leukocyte antigen–antigen D related (HLADR)+ and on CD8+HLADR+CD28+ T cells in bone marrow mononuclear cells (BMMCs). **(A)** Representative FACS plots of inhibitory molecules in the CD8+HLADR+ (upper panel) and in CD8+HLADR− (lower panel) T cell population in BMMCs. **(B)** Expression of checkpoint inhibitory molecules in CD8+HLADR+ vs CD8+HLADR− cells in BMMCs; $n = 8$; mean ± SEM, $^{**}p < 0.001$. **(C)** Expression of checkpoint inhibitory molecules in CD8+HLADR+CD28+ vs CD8+HLADR+CD28− cells in BMMCs; $n = 8$; mean ± SEM, $^{***}p < 0.0001$, $^{**}p < 0.001$. **(D)** Expression of checkpoint inhibitory molecules in CD8+HLADR+ in BMMCs and in PBMCs; $n = 8$; mean ± SEM, ns, not significant. **(E)** Expression of checkpoint inhibitory molecules in CD8+HLADR+CD28+ in BMMCs and in PBMCs; $n = 8$; mean ± SEM, $^{**}p < 0.001$ $^{*}p < 0.05$.

When we compared the number of checkpoint inhibitory molecule positive cells in the CD8+HLADR+ T cell population from young and elderly persons, we found lower percentages of cells expressing CTLA-4+, TIM-3+, LAG-3+, and PD-1+ in the old than in the young group (**Figures 7A,B**). The mean fluorescence intensity of CTLA-4+, TIM-3+, LAG-3+, and PD-1+ (MFI) on CD8+HLADR+ T cells was also lower in the old than in the young group (**Figure 7C**). This was the case when the total

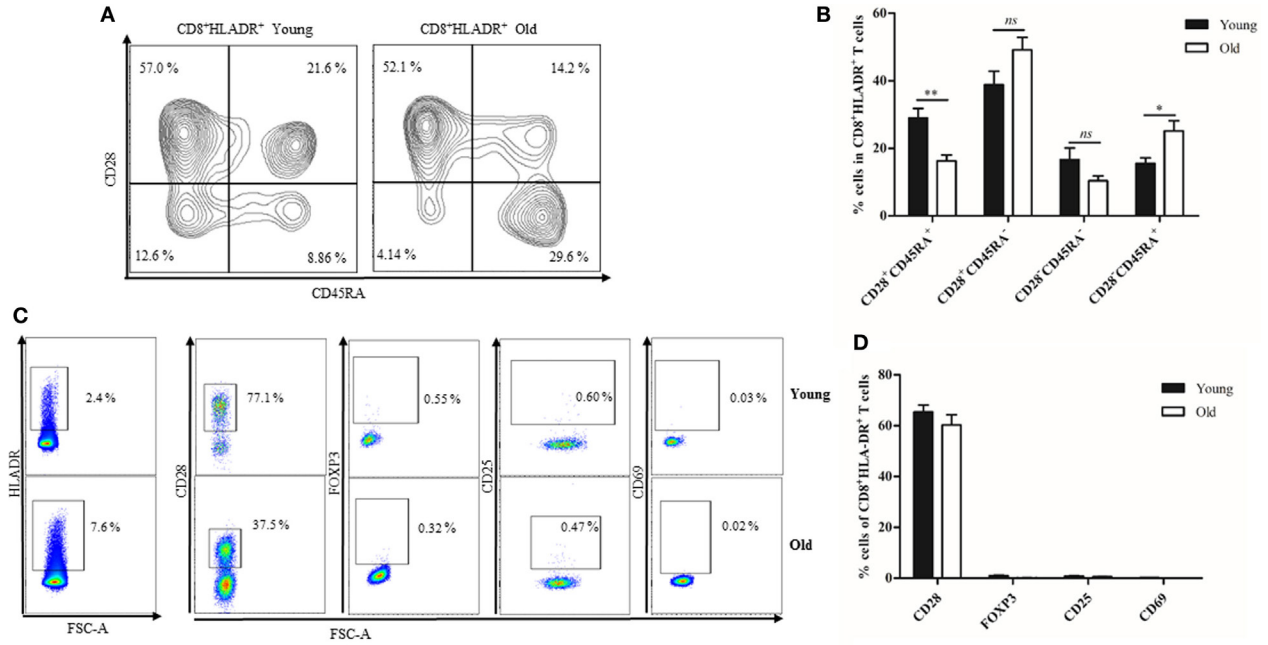

FIGURE 6 | Phenotypical characterization of CD8+human leukocyte antigen–antigen D related (HLADR)+ T cells from young and old donors. **(A)** Representative FACS plots demonstrating the expression of CD45RA and CD28 in CD8+HLADR+ cells from a young and an old donor. **(B)** Expression of CD45RA and CD28 in CD8+HLADR+ cells from young and old donors, CD28+CD45RA+, CD28+CD45RA−, CD28−CD45RA−, CD28−CD45RA+ cells are shown, young donors n = 18–21 (<30 years) and old donors n = 18–21 (≥70 years), mean ± SEM, $^{**}p < 0.001$, $^{*}p < 0.05$, ns, not significant. **(C)** Representative FACS plots of activation markers and other molecules in CD8+HLADR+ T cells from a young (upper panel) and an old donor (lower panel). **(D)** Percentages of CD28, FOXP3, CD25, and CD69 cells in the CD8+ HLADR+ population from young and old donors, young n = 18–21 (<30 years) and old donors n = 18–21 (≥70 years), mean ± SEM.

CD8+HLADR+ population was analyzed (**Figures 7B,C**) as well as in CD8+HLADR+CD28+ cells (Figures S3A,B in Supplementary Material). The number of checkpoint inhibitory molecule positive cells was low in both groups when cells were unstimulated (**Figures 7A–C**; Figures S3A,B in Supplementary Material), but increased following stimulation with anti-CD3 and anti-CD28 (**Figures 7D–F**; Figures S3C,D in Supplementary Material). As in unstimulated cells, the percentage of checkpoint inhibitory positive cells in CD8+HLADR+ and in CD8+HLADR+CD28+ cells was always higher in the young than in the old cohort following stimulation.

CD8+HLADR+ T Cells From Elderly Persons Have Reduced Suppressive Function Toward the Proliferation of Autologous PBMCs

In view of the reduced expression of checkpoint inhibitory molecules on CD8+HLADR+ T cells in old age, we were interested in the question whether this cell population also had a decreased suppressive function. We, therefore, purified CD8+HLADR+CD28+ T cells from young and elderly persons by cell sorting and co-cultured them with autologous PBMCs labeled with CFSE. In view of low cell numbers available, the co-culture experiment was only performed at a PBMC: HLADR+ T cell population ratio of 2:1 (**Figure 8**). We found that CD8+HLADR+CD28+ T cells from elderly persons had indeed lost most of their suppressive function. Whereas CD8+HLADR+CD28+ T cells from young persons had a suppressive activity of around 40%, suppression was below 20% in cells from elderly persons (**Figure 8B**). Considering that the number of cells of the different subsets of PBMCs may vary with age, we looked at the proliferation of CD4+ and CD8+ T cells within the PBMCs. We found no significant differences when we compared the suppression of CD4+ T cells vs the suppression of CD8+ T cells in both young and old persons (**Figure 8C**).

Checkpoint Inhibitory Molecules Mediate the Suppression of CD8+HLADR+CD28+ T Cells

Checkpoint inhibitory molecules are expressed at a higher level in the CD8+HLADR+ than in the CD8+HLADR− subset. They are also expressed at a higher level in young than in old donors. This may explain the higher suppressive effect of CD8+HLADR+ T cells of young donors. To check this possibility, we investigated the involvement of these molecules in mediating the suppressive effect of HLADR+ cells in neutralizing Ab experiments (**Figure 9**). We showed that the suppressive effect induced by CD8+HLADR+CD28+ Tregs at a PBMCs: suppressor cell ratio of 2:1 was distinctly inhibited by anti-CTLA-4, anti-TIM-3, anti-LAG-3 and slightly, but still significantly by anti-PD-1. The isotype control had no effect on suppression.

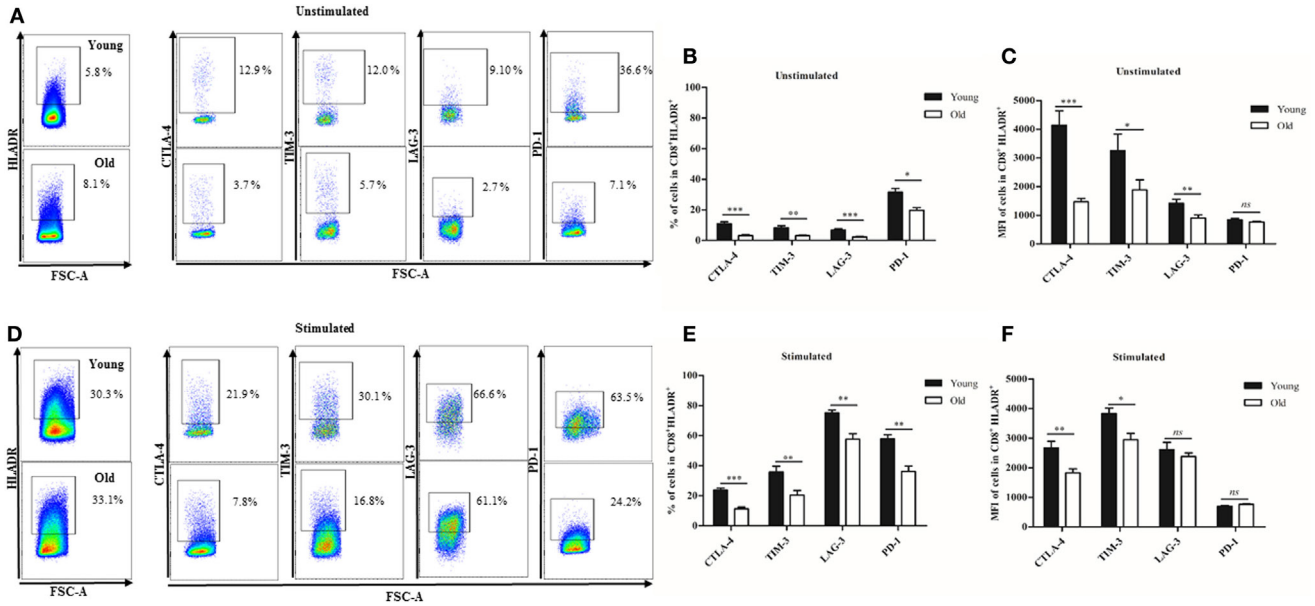

FIGURE 7 | Expression of checkpoint inhibitory molecules on CD8+human leukocyte antigen–antigen D related (HLADR)+ T cells in young and old persons. **(A)** Representative FACS plots of inhibitory molecules in the CD8+HLADR+ population from one young (upper panel) and one old (lower panel) donor before stimulation. **(B)** Expression of checkpoint inhibitory molecules (percentages) in CD8+HLADR+ cells from young and old donors before stimulation; young donors $n = 22$ (<30 years) and old donors $n = 20$ (>70 years), mean ± SEM, $^{***}p < 0.0001$, $^{**}p < 0.001$, $^{*}p < 0.05$. **(C)** Expression of checkpoint inhibitory molecules (MFI) in CD8+HLADR+ cells from young and old donors before stimulation; young donors $n = 20$ (<30 years) and old donors $n = 18$ (>70 years), mean ± SEM, $^{***}p < 0.0001$, $^{**}p < 0.001$, $^{*}p < 0.05$. **(D)** Representative FACS plots of inhibitory molecules in the CD8+HLADR+ population from one young (upper panel) and one old (lower panel) donor following stimulation with anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml) for 24 h. **(E)** Expression of checkpoint inhibitory molecules (percentages) in the CD8+HLADR+ population from young and old donors following stimulation; young donors $n = 9$ (<30 years) and old donors $n = 7$ (>70 years), mean ± SEM, $^{***}p < 0.0001$, $^{**}p < 0.001$. **(F)** Expression of checkpoint inhibitory molecules (MFI) in the CD8+HLADR+ population from young and old donors; young donors $n = 9$ (<30 years) and old donors $n = 7$ (>70 years), mean ± SEM, $^{***}p < 0.0001$, $^{**}p < 0.001$ $^{*}p < 0.05$.

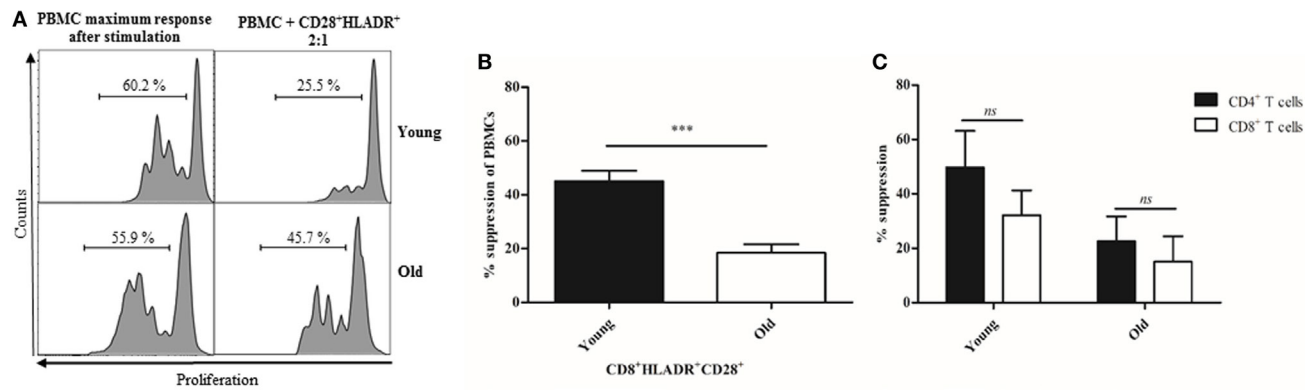

FIGURE 8 | CD8+human leukocyte antigen–antigen D related (HLADR)+CD28+ T cells suppressor properties decrease with age. **(A)** Representative histograms showing the suppressive activity of sorted CD8+HLADR+CD28+ T cells from one young and one old donor on CFSE-labeled peripheral blood mononuclear cells (PBMCs) assessed in co-culture 4 days after stimulation with anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml). **(B)** Suppressive effect of CD8+HLADR+CD28+ T cells on the proliferation of CFSE-labeled autologous PBMCs (ratio 2:1); mean ± SEM, young donors $n = 5$ (<30 years), old donors $n = 6$ (≥70 years); $^{***}p < 0.001$. **(C)** Suppressive effect of CD8+HLADR+CD28+ T cells on the proliferation of CD4+ vs CD8+ T cells within the CFSE-labeled PBMCs (ratio 2:1); mean ± SEM, young donors $n = 2$ (<30 years), old donors $n = 4$ (≥70 years).

DISCUSSION

We here show increased numbers of CD8+HLADR+ T cells in old age in a female Sardinian cohort previously genotyped and described (1) and in a smaller population of young and elderly Austrians. As the number of HLADR+ T cells has been demonstrated to be genetically linked (1), it is possible that at least in the Sardinian population the increased numbers of this specific cell

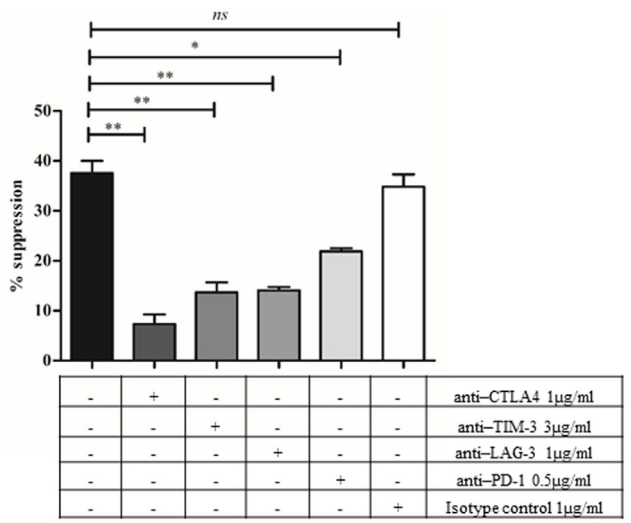

FIGURE 9 | Checkpoint inhibitory molecules are involved in the suppressive activity of CD8+human leukocyte antigen–antigen D related (HLADR)+CD28+ T cells. CFSE-labeled peripheral blood mononuclear cells (PBMCs) were analyzed following a 4 days co-culture after stimulation with anti-CD3 (1 µg/ml) and anti-CD28 (0.5 µg/ml) in the absence or presence of anti-cytotoxic T-lymphocyte-associated antigen 4 (1 µg/ml), anti-T cell immunoglobulin and mucin protein-3 (3 µg/ml), anti-lymphocyte activation gene-3 (1 µg/ml), or anti-programmed death 1 (0.5 µg/ml). Mouse IgG1 isotype Ab was used as control. PBMCs: suppressor cell ratio of 2:1. Young donors $n = 2–3$ (<30 years); mean ± SEM, $^{**}p < 0.001$, $^{*}p < 0.05$, ns, not significant.

type may be driven by the influence of certain genes. However, this is not likely to be the case in the Austrian population. We show here that CD8+HLADR+ cells most frequently have a CD28+ memory phenotype. As elderly persons have more memory than naïve cells (26), it seems plausible that the increased numbers of CD8+HLADR+ T cells in old age reflect an enlarged CD8+ memory T cell pool. In this context, it is also of interest that there are even more CD8+HLADR+ cells in the BM than in the PB, which may reflect the fact that the BM is known to be a reservoir for memory T cells (27–29, 32). CD8+HLADR+ T cells have been suggested to represent a separate lineage of T cells, as they occur also in cord blood (6). This possibility cannot be excluded, as we find CD8+HLADR+ T cells in the naïve cell population in our study. They may still be primed as naïve cells. In consequence they seem to persevere with differentiation, as CD8+HLADR+ cells occur also in memory as well as effector cell subsets. There is presently no information what the trigger for the induction of a CD8+HLADR+ phenotype is, but it may on the one hand be antigenic stimulation or also stimulation with cytokines, as suggested by the fact that high numbers are found in the BM, where BM niche cytokines such as IL-7 and IL-15 are prominent (31, 33). Experiments trying to analyze whether *in vitro* stimulation of naïve cells with various BM cytokines can induce this specific phenotype and function are presently underway.

In accordance with previous reports, we demonstrate that CD8+HLADR+ T cells can inhibit the proliferation of autologous PBMCs and can, therefore, be regarded as Tregs cells (6). As such, they may be an important cell type to maintain homeostatic equilibrium within the immune system. Suppression has previously been suggested to be due to cellular interactions mediated by CTLA-4. We now show that CD8+HLADR+ cells not only express increased amounts of CTLA-4 but also of other checkpoint inhibitory molecules such as TIM-3, LAG-3, and PD-1. It seems likely that suppression of other cells is not only mediated by one but also by a whole panel of inhibitory molecules. Our results using neutralizing Abs are in favor of this possibility. It was of interest that inhibitory molecules were stronger expressed on the CD28+ than the CD28− fraction, which may indicate that pre-stimulation *via* the antigen receptor may be one possible requirement for the induction of inhibitory molecules and their regulatory function.

In this context, it is remarkable that inhibitory molecule expression and regulatory function were decreased in CD8+HLADR+ T cells from elderly persons in spite of high cell numbers. Decreased T cell receptor signaling is known to be a characteristic feature of old age (34, 35). If inhibitory molecule levels reflect previous antigenic stimulation, checkpoint inhibitory molecule expression would be low in old age as a consequence. In how far high cell numbers could neutralize a decrease in function on a per cell basis is not clear. A similar situation is being discussed for natural killer (NK) cells (36, 37). In the case of CD8+HLADR+ T cells it seems imaginable that the synergy of a whole panel of different checkpoint inhibitory molecules on the cell surface is needed to trigger the full regulatory capacity of the cells. If these molecules are expressed at low concentrations even after antigenic stimulation, there might be no guarantee that suppressive function is maintained and decreased stimulatory activity would be the consequence.

From our data it is not yet clear toward which cell types the regulatory effect of CD8+HLADR+ T cells is directed. We can presently only show inhibition of the proliferation of autologous PBMCs as well as CD4+ and CD8+ T cells. It would be of major interest to define which cell types are target cells of the inhibitory effect and which functions other than proliferation can be influenced. This topic is difficult to study, as purified CD8+HLADR+ T cells are needed and it is hard to obtain sufficiently high numbers of pure cells after sorting. Isolation of purified cells from the BM is even more difficult. We keep trying to analyze whether BM niche cells (38), monocytes/macrophages, or dendritic cells (DCs) are affected in their stimulatory function. This would influence inflammatory processes. If CD8+HLADR+ Tregs cells do not function properly in old age, this could then support age-associated subclinical inflammation referred to as "inflamm-aging" (39, 40). As we have recently shown that "inflamm-aging" affects the BM in old age leading to increased levels of oxygen radicals, IL-15, TNF-α, and IFN-γ (31, 33), it is tempting to speculate that lack of regulatory function by CD8+HLADR+ T cells is a cause of this phenomenon.

In conclusion, we demonstrate for the first time that a decreased expression of checkpoint inhibitory molecules and consecutive lack of suppressive function of the CD8+HLADR+ T cell type is a characteristic feature of the immune system in old age. It will be of interest to learn more about the functional properties of this specific cell type in regard to inflammation and a possible imbalance between the different types of immune cells.

ETHICS STATEMENT

Cohort A: the study was approved by the Research Ethics and Bioethics Committee of the Consiglio Nazionale delle Ricerche (Italy). Written informed consent was received from participants prior to their inclusion in the study. Cohort B: the study was approved by the Ethics Committees of the Medical University of Innsbruck (Austria). Written informed consent was received from participants prior to their inclusion in the study. Cohort C: 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.

AUTHOR CONTRIBUTIONS

SY, BG-L, BW, EF, FC, and SS: study design, interpretation of data, critical appraisal, final approval of the version to be published. ML and VO: recruitment of the Sardinian cohort. KT: recruitment of Austrian probands, sample collection, and study design. SY: method design. SS: study design and sorting of cells. SY, MK, FM, and LP: experimental work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01201/full#supplementary-material.

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Involvement of MicroRNAs in the Aging-Related Decline of CD28 Expression by Human T Cells

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Loss of CD28 is a characteristic feature of T cell aging, but the underlying mechanisms of this loss are elusive. As differential expression of microRNAs (miRNAs) has been described between CD28+ and CD28− T cells, we hypothesized that altered miRNA expression contributes to the age-associated downregulation of CD28. To avoid the confounding effects of age-associated changes in the proportions of T cells at various differentiation stages *in vivo*, an experimental model system was used to study changes over time in the expression of miRNA associated with the loss of CD28 expression in monoclonal T cell populations at a lower or higher number of population doublings (PDs). This approach allows identification of age-associated miRNA expression changes in a longitudinal model. Results were validated in *ex vivo* samples. The cumulative number of PDs but not the age of the donor of the T cell clone was correlated with decreased expression of CD28. Principal component analysis of 252 expressed miRNAs showed clustering based on low and high PDs, irrespective of the age of the clone donor. Increased expression of miR-9-5p and miR-34a-5p was seen in clones at higher PDs, and miR-9-5p expression inversely correlated with CD28 expression in *ex vivo* sorted T-cells from healthy subjects. We then examined the involvement of miR-9-5p, miR-34a-5p, and the members of the miR-23a~24-2 cluster, in which all are predicted to bind to the 3′UTR of CD28, in the IL-15-induced loss of CD28 in T cells. Culture of fresh naive CD28+ T cells in the presence of IL-15 resulted in a gradual loss of CD28 expression, while the expression of miR-9-5p, miR-34a-5p, and members of the miR-23a~24-2 cluster increased. Binding of miR-9-5p, miR-34a-5p, miR-24-3p, and miR-27- 3p to the 3′UTR of CD28 was studied using luciferase reporter constructs. Functional binding to the 3′UTR was shown for miR-24-3p and miR-27a-3p. Our results indicate involvement of defined miRNAs in T cells in relation to specific characteristics of T cell aging, i.e., PD and CD28 expression.

Keywords: T cell aging, senescence, CD28, IL-15, miRNA, miR-9, miR-23a~24-2

INTRODUCTION

Full activation of naive T cells requires binding of the T cell receptor (TCR) to antigens displayed by the major histocompatibility complex on antigen-presenting cells (APCs) (known as "signal one") together with ligation of a T cell costimulatory receptor ("signal two"). The latter is archetypically mediated by CD28 on the T cell surface and CD80 or CD86 on the APC. This interaction results in complex signaling cascades which activate T cells, promote their differentiation, proliferation, and effector function, and mounts adaptive immune responses depending on clonally expanded, antigen-specific effector T cells and the subsequent generation of T cell memory (1). Costimulation *via* CD28 lowers the threshold for signaling *via* the TcR and triggers cytokine production. This allows T cells to respond to low abundance and low avidity antigens, and shapes T cell immunity by balancing the interplay between effector and regulatory T cells (1). The latter is especially important in focusing the immune response toward the pathogen, avoiding autoimmunity, and for downregulating the immune response upon pathogen clearance.

The composition and function of the T cell immune system in older people is characterized by lower proportions of naive T cells and higher proportions of memory T cells as a result of antigen exposure over the lifetime (2). Additionally, aging itself affects the characteristics of T cells within the naive and memory compartments and when these effects result in compromised functionality, these T cells can be designated "immunosenescent" (2–4). Developmentally programmed thymic involution at puberty results in an abrupt decline in the output of naive T cells, although residual thymic activity maintains the production of small numbers of such cells in most people in their 50s or 60's. The diversity of the memory T cell pool increasingly reflects pathogen exposures over the lifetime, especially its focus on maintaining immune surveillance of latent viruses, e.g., CMV, EBV, and many other pathogens (5, 6). Overall, numbers and proportions of naive T cells decline, despite partial compensation by homeostatic proliferation of these cells in the periphery, which may also contribute to their aging phenotype (7, 8). Repeated clonal expansions of memory cells on rechallenge by specific pathogens, or continuous challenges by persistent pathogens, are thought to be instrumental for the overall differences observed between T cells in younger and older individuals (9, 10). At the cellular level, T cell aging is characterized by a multitude of changes in the expression of cell surface proteins. Most notably, a gradual decline in the expression of CD28 has been reported as a characteristic feature of aged T cells, mostly but not only due to the age-associated accumulation of late-stage memory cells which do not express this coreceptor (11, 12). The exact mechanisms involved in the aging-related decline of CD28 are unknown. Dissecting the differences in CD28 expression resulting from altered proportions of naive and memory T cells with age, and the intrinsic aging process within single T cell populations is challenging. To approach this, we have employed monoclonal T cells with increasing population doublings (PDs) in culture as a longitudinal aging model to identify regulation of CD28 expression, and attempted to validate some of these in *ex vivo* sorted T-cells from healthy subjects (13, 14) Here, we report the activity of microRNAs (miRNAs) in this context.

MicroRNAs are small noncoding RNA molecules that regulate protein expression by interfering with the process of messenger RNA (mRNA) translation or by inducing mRNA degradation. miRNAs are crucially involved in T cell development, differentiation, activation, and function (15, 16). In addition, recent evidence has implicated the involvement of miRNAs in several aspects of T cell aging (15–19). However, if and how miRNAs are involved in the regulated decline of CD28 expression is unknown. High expression of the three members of the miR-23a~24-2 cluster in CD8+CD28− T cells relative to CD8+CD28+ T cells has been reported (20). Increased expression of miR-24 in CD28− T cells was associated with an increased susceptibility to cell death, which was counterbalanced by IL-15 (20). IL-15 is a homeostatic cytokine that supports survival and proliferation of naive CD28+ T cells in the absence of continuous TCR stimulation (21). Downregulation of CD28 in response to homeostatic cytokines, such as IL-15, which interact with common γ-chain receptors, has been well documented (21–23). Here, we studied the involvement of miRNAs in clonal expansion and IL-15-regulated expression of CD28 by T cells. Using T cell clones derived from healthy young and elderly donors, we observed clustering of miRNAs primarily according to the number of PDs. In addition, IL-15 induced loss of CD28 coincided with upregulation of miRNAs that interact with the 3′UTR of CD28 mRNA.

MATERIALS AND METHODS

Generation of T Cell Clones

T cell clones were generated from phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMC) by limiting dilution in the presence of IL-2 and pooled irradiated PBMC feeder cells as described previously (13, 24–26). In brief, cells to be cloned were plated at 0.45/well into 1 mm-diameter microplate wells containing 10^4 30 Gy-irradiated pooled PBMC from >20 random normal donors as feeder cells. Contents of positive wells were transferred after 1–2 weeks to 96-well 7 mm-diameter flat bottomed microtiter plate containing fresh medium and 10^5 pooled PBMC feeder cells between day 7 and 11, and to 16 mm-diameter 24-well cluster plate with 2.5×10^5 stimulators between day 12 and 16. Cultures were given fresh medium every 3 or 4 days and fresh feeder cells every 1–2 weeks thereafter. Clonal age is expressed in PD estimated by microscopic counting of the cells at each subculture and counting the number of doublings cumulatively undergone. Culture medium was the serum-free formulation X-Vivo 10 (BioWhittaker, Walkersville, MD, USA).

Three T cell clones from two healthy old and three T cell clones from one healthy young donor each at low and high PDs were selected for small RNA sequencing ($n = 12$ samples; three independent samples for each condition tested). Additional clones were used for the quantitative reverse-transcription-polymerase chain reaction (qRT-PCR) validation experiments (Table S1 in Supplementary Material). T-cell clones used were

all CD4+. CD28 expression on T cell clones with low and high numbers of PDs was assessed by standard quantitative flow cytometry and expressed as median fluorescence intensity as reported previously (24, 25).

Primary Lymphocyte Subsets

Peripheral blood mononuclear cells were freshly isolated by density gradient centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway) according to the manufacturer's protocol. Informed consent was obtained from all participants in accordance with the Declaration of Helsinki. The Medical Ethical Committee of the University Medical Center Groningen approved the study. For validation of differential miR-9-5p and miR-34a-5p expression in CD28+ versus CD28− T cells, CD3+CD28+ and CD3+CD28− cells were fluorescence-activated cell sorting (FACS) sorted from six healthy young (<30 years) and four healthy old (>60 years) subjects. For IL-15 culture experiments, CD3+CD8+CD45RO−CCR7+CD28+ T cells were FACS sorted from six healthy young (<30 years) subjects.

FACS of Human Primary Lymphocyte Subsets and Analysis of Cell Surface Markers

The following monoclonal antibodies were used: anti-CD3-e450 (OKT3), anti-CD8a-APC-e780 (OKT8) (eBioscience, Vienna, Austria), anti-CD45RO-FITC (UCHL1), anti-CCR7-PE (3D12) (BD Bioscience, Breda, Netherlands), and anti-CD28 PeCy7 (CD28.2) (Biolegend, Uithoorn, The Netherlands). Cells were sorted using a MoFlo flow cytometry cell sorter (Backman Coulter, Woerden, The Netherlands).

Expression of cell surface markers on T cells was assessed using mAbs against human CD28-PE-CY7 (CD28.2) (Biolegend), CCR7-PE (3D12), and CD45RO-FITC (UCHL1) (BD Biosciences). Cells were analyzed using a BD LSR-II Flow Cytometer and the Diva software (BD Biosciences). Data analysis was done using the Kaluza Flow Analysis Software (1.2) (Beckman Coulter).

T Cell Culture With Human Recombinant IL-15

Fluorescence-activated cell sorting-sorted CD3+CD8+CD28+CD45RO−CCR7+ (naive CD8+) T cells were suspended in RPMI medium (Lonza, Breda, The Netherlands) supplemented with 10 mg/ml gentamycin sulfate (Lonza) and 10% fetal calf serum (Thermo Scientific, Breda, The Netherlands) in a volume of 3 ml and seeded at a density of 1×10^6/ml in T25 cm flasks. A final concentration of 50 ng/ml human recombinant IL-15 (Peprotech, London, UK) was added to the cell culture at day 0 and refreshed every 5th day. On day 5, 10, and 15 of culture, cells were harvested and stained for flow cytometry analysis and/or lysed for RNA isolation. To study CD28, CD45RO, and CCR7 expression on naïve CD8+ T cells after IL-15 stimulation, CD3+CD8+CD28+CD45RO−CCR7+ T-cells were sorted as described above and stained with 10 umol/ml eF670 proliferation dye (eBioscience, Vienna, Austria). After 5, 10, and 15 days of culture in the presence of IL-15 (50 ng/ml), cells were harvested, stained, and analyzed by flow cytometer.

Culture of COS-7 Cells

COS-7 cells (African Green Monkey SV40-transformed kidney fibroblast cell line) were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Breda, The Netherlands), 200 mM L-glutamine and 10 mg/ml gentamycin sulfate (Lonza, Breda, The Netherlands) at 37°C in 5% CO_2.

RNA Isolation

Total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions. Micro Bio-SpinTM chromatography columns, supplied with Bio-Gel P-6 polyacrylamide gel matrices, were applied to maximize purity of the RNA samples (Bio-Rad laboratories). The ExperionTM RNA stdSens and HighSens analysis kits (Life Science, Bio-Rad Laboratories B.V, Veenendal, The Netherlands) were used to determine the RNA quality indicator score. The RNA concentration was measured on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Small RNA Sequencing and Data Analysis

T cell clones with the biggest difference between low and high PDs were selected for small RNA-sequencing. Samples were barcoded and sequenced with Illumina HiSEQ 2000 flowcell (Illumina). The sequence reads were analyzed using the CLC BIO Genomic Work Bench Suite 4.5 (CLC BIO, Arhus, Denmark). Reads were mapped to the mature miRNAs using miRDeep2 (27). The number of mapped reads of each sample was normalized to 1×10^6. Normalized data were imported to GeneSpring (v.11.5.1) for analysis. A total of 252 miRNAs were present in at least 3 out of 12 samples with a read count >10. Mann–Whitney *U* test was performed to identify significantly differentially expressed miRNAs. Genesis (Release 1.7.6) was used to generate heatmaps. Raw and processed data are available *via* the Gene Expresison Omninbus, accession #GSE106619.

Quantitative RT-PCR

MicroRNA and gene expression levels were determined by qRT-PCR. cDNA synthesis for miRNAs was performed with Taqman miRNA Reverse Transcription kit using a multiplex reverse transcription approach with TaqMan microRNA Assays (Life Technologies, Carlsbad, CA, USA): for miR-9-5p (000583), miR-23a-3p (000399), miR-24-3p (000402), miR-27a-3p (000408), miR-31-5p (002279), miR-34a-5p (000426), and RNU44 (001094). RNU44 served as an endogenous control.

The qPCR reaction was performed using qPCR MasterMix Plus (Eurogentec, Liege, Belgium) and mean cycle threshold (Ct) values for all genes were quantified with the ViiA™ 7 software (Life Technologies). Relative expression levels were quantified using the $2-\Delta C_t$ ($\Delta C_t = C_t$ gene $- C_t$ reference gene) method.

Cloning of 3′UTR in a Luciferase Reporter Construct, Transient Transfection, and Luciferase Reporter Assays

The CD28 3′UTR was cloned into the psiCHECK2 vector (Promega, Madison, WI, USA) in two fragments, as previously

described (28). CD28 3′UTR-1 (nt 870-2279 of ENST00000324106.8) was amplified from genomic DNA using primers containing an Xhol (5') or Notl (3') restriction site, forward: 5′-GCTCCTGC ACAGTGACTACA-3′, reverse 5′-ACCTTCTGCCTGACCACT TC-3′. CD28 3′UTR-1mut (same as above but with mutated miRNA binding sites), CD28 3′UTR-2 (nt 2534-4449), and CD28 3′UTR-2mut were ordered as minigenes (IDT, Leuven, Belgium). For both CD28 UTR-mut constructs mutations at position 2, 4, and 6 of the seed sequences were introduced at all potential miR-9-5p, -24-3p, -27-3p, and -34a-5p binding sites (based on sites indicated in **Figure 5A**). Sequences for the constructs are available on request. The inserts were sequence verified (BaseClear, Leiden, The Netherlands). COS-7 cells were transfected with 125 ng of the psiCHECK2 construct and either 50 nM hsa-miR-9-5p (PM10022), hsa-miR-24-3p (MC10737), hsa-miR-27a-3p (MC10939), hsa-miR-34a-5p (MC11030) mimics, or miRNA precursor negative control #1 (AM17110, ThermoFisher) using Saint-MIX (Synvolux products, Leiden, The Netherlands) following manufacturer's instructions. Cells were lysed 48 h after transfection and Renilla and Firefly luciferase activity was assessed using Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's protocol. For each transfection, luciferase activity was measured in duplicate with the Luminoskan Ascent Microplate Luminometer (Thermo Scientific). The Renilla over Firefly (RL/FF) luciferase ratios were calculated and the RL/FF ratio of control precursor was set to one. All luciferase measurements were performed in at least three independent experiments.

Statistical Analysis

For correlation analysis between miRNA or CD28 expression and PD the Spearman test was used. Paired samples as presented in **Figures 2E,F** were analyzed using the Wilcoxon signed-rank test and for **Figures 3** and **4B,D** (Figures S3B–D,H–J in Supplementary Material) and **Figure 5** using the Friedman test with *post hoc* Dunnett's multiple comparison test. Results obtained from luciferase assay were analyzed using the paired *T*-test. Statistical analyses were performed with GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

PDs of T Cells Associates With Differential Expression of CD28 and miRNAs

For all 16 T cell clones, a passage at a lower number of PDs (≤40, median 29) and a passage at a high number of PDs (>40, median 56) was used (Table S1 in Supplementary Material). The Mean fluorescence intensity (MFI) of CD28 showed an inverse correlation with the number of PDs (**Figure 1**), which is in line with previous work (**Figure 1**) (14). No changes in CD28 expression were observed in the different age groups in which clones were grouped (data not shown). Small RNA sequencing was performed on the highest and lowest PD passage of 6 T cell clones. The T-cell clones used for this analysis were selected based on PD passages at the lowest and highest end of the PD spectrum.

Principal component analysis of the 252 miRNAs detected in at least 3 of 12 samples revealed a perfect separation of the T cell clones in the first component based on PD (**Figure 2A**). No clustering was observed according to the age of the donor. Ten miRNAs were significantly differentially expressed between T cell clones with a low and a high number of PDs (**Figure 2B**). Five of these ten miRNAs were selected for validation based on having high expression levels and a more than 1.5-fold change in expression levels (see Table S2 in Supplementary Material). Validation was done by qRT-PCR on the 6 T cell clones that had been included for small RNA sequencing complemented with 10 additional T cell clones, also harvested at (intermediate) low and a high PD, giving a total of 32 samples (Table S1 in Supplementary Material). We observed a significant correlation for both miR-9-5p and miR-34a-5p levels with the number of PDs of the T cell clones (**Figures 2C,D**) and not for the other three miRNAs (data not shown).

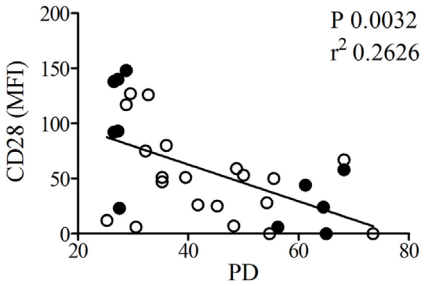

FIGURE 1 | The population doublings (PDs) of T cell clones inversely correlate with CD28 expression. CD4+ T cell clones used for small RNA sequencing (filled symbols) with high and low PD and additional T cell clones (open symbols) were fluorescence-activated cell sorting analyzed for expression of CD28. Shown is the relation between CD28 expression based on mean fluorescence intensity (MFI) and PD of the T-cell clones. For one of the clones no MFI data are available.

Independent Validation in Primary T Cell Subsets

To further validate the association between miR-9-5p and miR-34a-5p with aged T cells and CD28 loss, we sorted CD3+ CD28+ and CD3+CD28− T cells from peripheral blood of six healthy young (<30 years) and four healthy old (>60 years) subjects. In line with the results obtained from the high and low PD T cell clones, qRT-PCR analysis revealed significantly higher levels of miR-9-5p in the CD3+CD28− T cell population as compared to the CD3+CD28+ T cells (**Figure 2E**). No significant differences were observed for miR-34a-5p (**Figure 2F**). Of note, lower relative expression levels of both miR-9-5p and miR-34a-5p were observed in the primary sorted T-cells, compared to the T-cell clones.

Upregulation of miRNAs by IL-15 in Naïve CD8+ T Cells

Regulation of CD28 expression in CD8+ T cells has been described to occur downstream of IL-15. This prompted us to investigate whether IL-15 regulated the expression levels of miR-9-5p and miR-34a-5p. We also included miR-23a-3p, miR-24-3p,

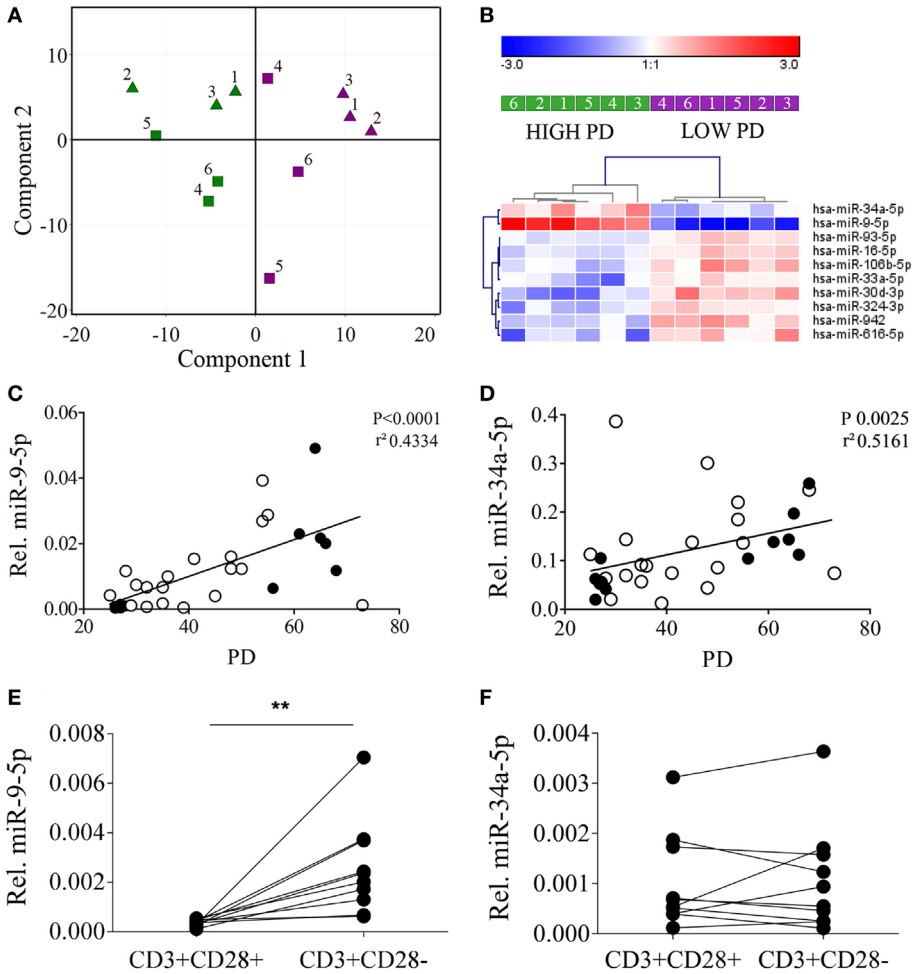

FIGURE 2 | MicroRNAs (miRNA) expression analysis in CD4+ T cell clones reveals primarily clusters according to the proliferative history of the T cell clones and partly correlates with the expression of CD28. **(A)** Principal component analysis identifies low population doublings (PDs) (purple samples) versus high PDs (green samples) as the primary identifier of biological variation for miRNA expression. Triangles indicate young and squares old donors and numbers correspond to the T cell clone numbers indicated in Table S1 in Supplementary Material. **(B)** Hierarchical clustering of the T cell clones according to low and high PDs based on the 10 most significantly differentially expressed miRNAs. Correlation between **(C)** miR-9-5p and **(D)** miR-34a-5p and the PDs of the T cell clones used in the analysis. Filled symbols denote T cell clones used in the small RNA-sequencing samples and open symbols denote additional T cell clones used to validate data from small RNA-sequencing. Significant higher expression of **(E)** miR-9-5p but not **(F)** miR-34-5p in fluorescence-activated cell sorting-sorted CD3+CD28− T cells versus CD3+CD28+ T cells. Expression levels of the miRNAs relative to the expression of RNU44 is shown. Significance (**$P \leq 0.01$) is depicted.

and miR-27a-3p all belonging to the miR-23a~24-2 cluster, in this analysis as differentially expression in CD28+ versus CD28− T cells has been previously described (20).

To this end, we first sorted naive CD8+CD28+ T cells and cultured them for 15 days in the presence of IL-15 (Figure S1 in Supplementary Material). Culturing naive CD8+CD28+ T cells in the presence of IL-15 resulted in a shift to a memory phenotype as shown by a gain of CD45RO expression and a concomitant decrease in the expression of CCR7 (**Figures 3A,B**) and CD3 (data not shown). In line with the literature (21–23), we observed a significant downregulation of CD28 expression by naive T cells in response to IL-15. The percentage of CD28+ T cells decreased to 36% after 15 days culture in the presence of IL-15 (**Figure 3C**). Next to the decrease in the percentage of CD28+ T cells, also the expression of CD28 per cell was measured by the MFI decreased significantly (**Figure 3D**).

Association Between miRNA Binding to the 3′UTR of CD28 and CD28 Expression

Next we studied whether IL-15-induced downregulation of CD28 was directly related to cell division. Analysis of sorted naïve T-cells stained with a proliferation dye revealed a progressive downregulation of CD28, both in terms of percentage positive cells and expression level, directly related to the number of cell divisions. Cells that did not divide retained CD28 expression. Similarly, cell division also correlated with acquiring a memory phenotype, as denoted by a loss of CCR7 and gain of CD45RO

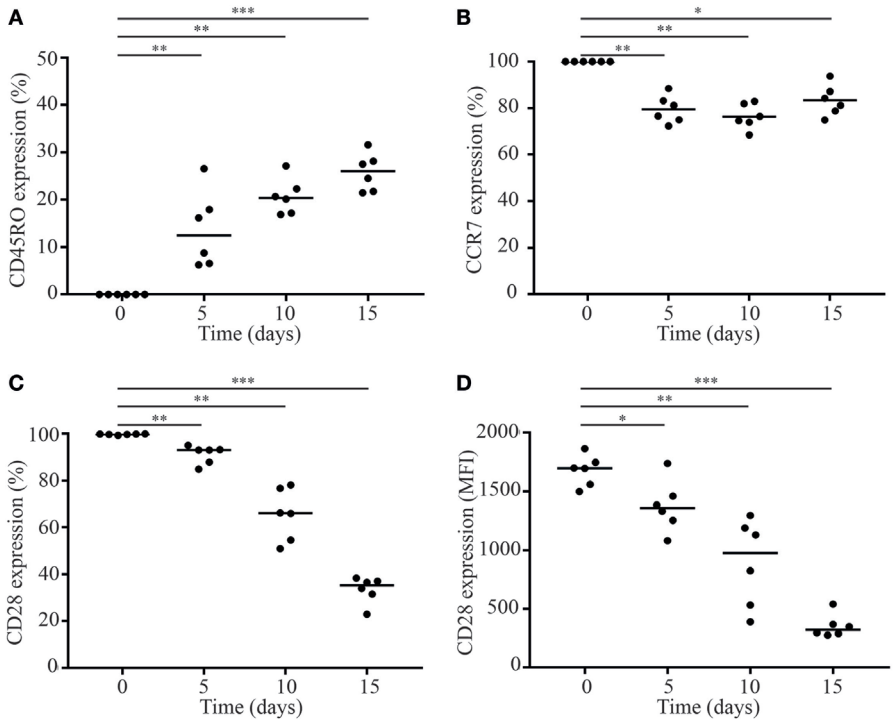

FIGURE 3 | IL-15 induces loss of CD28 by naive CD8+ T cells. Fluorescence-activated cell sorting-sorted naive CD8+CD45RO−CCR7+CD28+ T cells were cultured in the presence of IL-15 (50 ng/ml). Directly after sorting and after 5, 10, and 15 days of culture, expression of **(A)** CD45RO, **(B)** CCR7, and **(C,D)** CD28 was assessed. MFI = median fluorescence intensity. Significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) is depicted. $N = 6$.

expression. These differences were most pronounced at day 15, but were also seen after 5 and 10 days stimulation with IL-15 (Figures S2 and S3 in Supplementary Material).

Expression levels of the five selected miRNAs were assessed directly after sorting cells and after 5, 10, and 15 days of culture in the presence of IL-15. Expression of miR-9-5p, miR-34a-5p, miR-23a-3p, miR-24-3p, and miR-27a-3p increased over the 15 days of culture with similar kinetics for miR-34a-3p (**Figure 4B**) and the members of the miR-23a~24-2 family (**Figures 4C–E**). MiR-9-5p levels increased already at day 5, although to a lower extend (**Figure 4A**). Absolute expression levels significantly differed between the miRNAs with miR-24-3p having the highest and miR-9-5p the lowest expression levels. As a control, we tested the expression of a randomly selected unrelated miRNA (miR-31-5p), which did not significantly change as a result of stimulation with IL-15 (**Figure 4F**).

Next, we identified putative miRNA binding sites in the 3′UTR of the CD28 mRNA. Using the Targetscan miRNA binding site prediction algorithm as well as manual searches for 6-, 7-, and 8-mer seed binding sites we identified two binding sites for miR-9-5p, miR-23a-3p, and miR-34a-5p and three binding sites for miR-24-3p and miR-27a-3p (**Figure 5A**). The presence of binding sites for these IL-15 responsive miRNAs in the 3′UTR of the CD28 transcript suggests a direct regulation. Two consecutive regions covering the 3′UTR of CD28 were cloned in a luciferase reporter construct for analysis to assess direct binding of miR-9-5p, miR-34a-5p, and the two most abundant members of the miR-23a~24-2 family (miR-24-3p and miR-27a-3p). In addition, we also generated CD28 3′UTR constructs in which the binding sites of the four miRNAs were mutated. COS-7 cells were transiently transfected with these constructs and specific miRNAs or control mimics. Luciferase assays using the wild-type (WT) CD28 3′UTR regions indicated binding of miR-9-5p, miR-24-3p, and miR-27a-3p to CD28-UTR-1 and of miR-24-3p to CD28-UTR-2 (**Figures 5A,B**). Comparison of WT to mutated constructs indicated that the relative R/F ratio of miR-27a-3p alone and in combination with miR-24-3p was reduced, albeit not significant (p-value = 0.11, p-value = 0.058, **Figure 5C**). For CD28-UTR-2 a reduced relative R/F ratio was observed for miR-24-3p and miR-34a-5p (p-value = 0.0062, p-value = 0.012, **Figure 5C**). Together these data suggest that miR-24-3p and miR-27a-3p are the most important miRNAs for direct regulation of CD28 expression.

DISCUSSION

Loss of CD28 is regarded as a hallmark of T cell aging (11, 14). To study T cell aging, various *in vitro* models have been applied, among which are T cell clones and IL-15 treatment of naive CD28+ T cells (21–24). We showed an inverse correlation between both miR-9-5p levels and miR-34a-5p, and CD28 expression in T cell clones with low and high PDs, in aged primary T cells and in IL-15 exposed naive T cells. In the latter model, we also showed IL-15 induced upregulation of the members of the miR-23a~24-2 cluster. Luciferase reporter assays showed

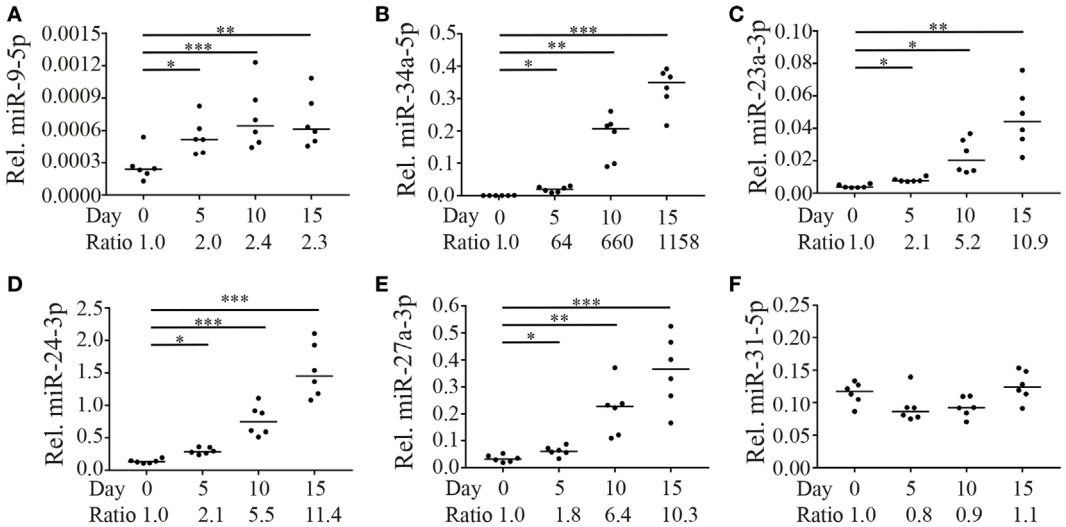

FIGURE 4 | Expression of miR-9-5p, miR-34a-5p, and miR-23a~24-2 cluster in CD8+ naive T cells is regulated by IL-15. Fluorescence-activated cell sorting-sorted naive CD8+CD45RO−CCR7+CD28+ T cells were cultured in the presence of IL-15 (50 ng/ml). Directly after sorting and after 5, 10, and 15 days of culture, expression levels of **(A)** miR-9-5p, **(B)** miR-34a-5p, **(C)** miR-23a-3p, **(D)** miR-24-3p, **(E)** miR-27a-3p (all three part of the miR-23a~24-2 cluster), and **(F)** as a non-IL-15-responsive control, miR-31-5p were assessed by RT-qPCR. Expression levels of the microRNAs (miRNAs) relative to the expression of RNU44 are shown. Significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) is depicted. $N = 6$.

targeting of the 3′UTR of the CD28 transcript by miR-24 and miR-27a, indicating miRNA dependent regulation of CD28 expression upon T cell aging.

The negative correlation between the number of PDs and the expression level of CD28 in T cell clones is consistent with their previously reported senescent phenotype (13, 14). We showed a clear PD-associated difference in miRNA expression levels in T cell clones, which was independent of the age of the T cell donor. These observations are consistent with previous findings showing an overall immune-biological similarity between T cell clones from centenarian and young adults (26, 29) and confirm that centenarian-derived T cell clones are, *per se*, not functionally compromised. We identified 10 miRNAs with a significant differential expression pattern in T cell clones with low and high number of PDs and confirmed an association with the number of PDs for miR-9-5p and miR-34a-5p. For miR-9-5p, we validated higher miR-9-5p levels in primary CD28− T cells as compared to CD28+ T cells isolated from both young and old subjects.

In a second model system used in this study, we assessed the kinetics of miR-9-5p and miR-34a expression in naive CD28+ T cells in response to IL-15. IL-15 expression increases with age and maintains survival of effector CD8+ T-cells. Increased expression of IL-15 in aged individuals has been implicated in the loss of CD28 expression by T-cells (6). In line with literature findings, culture of naïve CD8+ T cells with IL-15 induced a memory phenotype and concomitant gradual downregulation of CD28 in the proliferating cell fraction. We show that IL-15 mediated downregulation of CD28 by naïve CD8+CD28+ T cells is associated with a concomitant upregulation of miR-9-5p, miR-34a-5p, and the three members of the miR-23a~24-2 cluster. This confirms the previously reported high expression of the members of the miR-23a~24-2 cluster in CD28− T cells compared to CD28+ T cells (20). It remains to be established whether IL-15 induced miRNA expression involves other cytokines besides IL-15, such as TNF-a, which has also been implicated in the regulation of CD28 expression (30, 31).

Aging-related changes in expression of cell surface receptors are not restricted to CD28. Specifically, CD3γ regulated modulation of the TcR/CD3 complex has been reported in relation to aging (32). Downregulation of CD28 expression in our study was not seen as a result of stimulation by common γ-chain interacting cytokines *per se*, as culturing T cells in the presence of IL-4 did not induce downregulation of CD28 expression (data not shown). However, implication of the epigenome in the aging immune signature of memory CD8 T cells, involving silencing of the IL-7R gene and IL-7 signaling, has been described previously (33, 34).

As a mechanism to explain IL-15 induced loss of CD28 expression, we propose involvement of miRNAs targeting of the 3′UTR of the CD28 transcript. Indeed, several potential binding sites of miR-9-5p, miR-34a-5p, and miR-23a~24-2 cluster members are present in the 3′UTR of CD28. Using a luciferase reporter assay we confirmed binding of miR-24-3p and miR-27a-3p to the 3′UTR of the CD28 transcript, thus providing evidence for their functional involvement in the regulation of CD28 expression upon induction by IL-15.

We initiated our search for aging associated miRNAs with CD4+ T-cell clones. This allowed us to analyze both high and low PD samples of the same T cell clone. Because CD28 expression is regulated more profoundly in CD8+ T cells and previous studies have been conducted specifically using CD8+ T cells, we subsequently used sorted naïve CD8+ T cells to study IL-15 stimulation induced regulation of miRNAs and CD28 expression. We confirmed differential expression for miR-9-5p and miR-34a-5p. Between the various experimental settings, we noted considerable differences

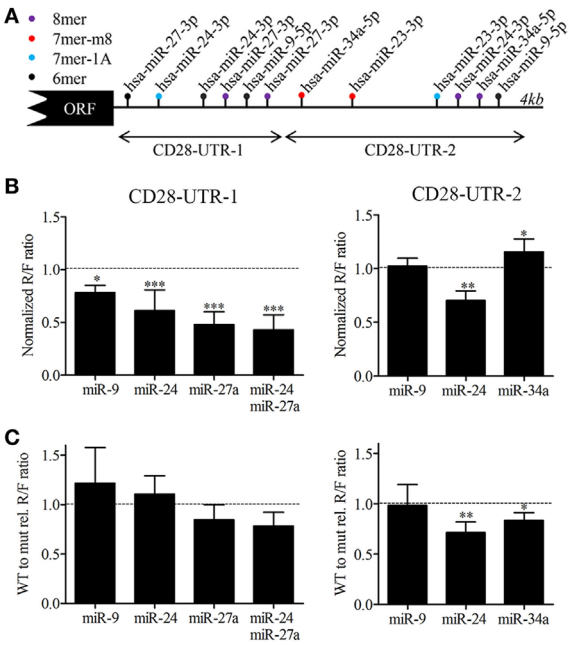

FIGURE 5 | MicroRNAs (miRNAs) binding site analysis of the CD28 3′UTR. **(A)** Schematic overview of the predicted miRNA binding sites for miR-9-5p, miR-34a-5p, and members of the miR-23a~miR-24-2 cluster in the 3′UTR of CD28 (ENST00000324106.8). Binding sites were identified using the Targetscan prediction algorithm (release 7.1) in combination with a manual search for 6mers. The arrow indicates the two regions that were cloned in the luciferase reporter constructs. 8mer: exact match to positions 2–8 of the mature miRNA followed by an A, 7mer-m8: exact match to positions 2–8 of the mature miRNA, 7mer-A1: exact match to positions 2–7 of the mature miRNA followed by an A and 6mer: exact match to positions 2–7 of the mature miRNA. **(B)** Binding analysis of miR-9-5p, miR-24-3p, and miR-27a-3p to the proximal part of the CD28 3′UTR (CD28-UTR-1, left) and of miR-9-5p, miR-24-3p, and miR-34a-5p to the distal part (CD28-UTR-2 right). Cos-7 cells were transfected with psi-Check-2 construct harboring each fragment of the 3′UTR of CD28 **(A)** in combination with control precursor, or the miRNA(s) as indicated. The Renilla (R) over Firefly (F) luciferase ratio set to one for the control precursor transfected cells is shown. **(C)** The normalized R/F ratio of the wild-type CD28 UTR region relative (rel) to the normalized R/F ratio of the same region with mutated binding sites for the tested miRNAs. Significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) is depicted. Each luciferase experiment was measured in duplicate and each experiment was performed at a minimum of three independent experiments.

in the expression level of the miRNAs studied. Expression of miRNAs is cell type dependent and might, as such, explain differences observed between the various experimental settings.

We used a luciferase reporter system to verify functional binding of miR-9-5p miR-24-3p, miR-27a-3p and miR-34a-5p to the 3′UTR of CD28. Two approaches were followed, the first using WT 3′UTR constructs in combination with miRNA precursor overexpression and the second using 3′UTR constructs in which seed sequences of the miRNAs had been mutated. The combined analysis indicated that miR-24-3p and miR-27a-3p was the most effective in binding to the 3′UTR of CD28. It should be noted that results were obtained using COS-7 cells and it remains to be determined whether these results can be translated to T-cells. However, transducing T-cells directly with these constructs is not only technically complicated but also induces unpredictable activation-associated physiological changes.

The aging-related decline in CD28 expression is observed in human T cells but not in rodents. Notably, two of three miR-27a binding sites are missing in the 3′UTR of mouse CD28. On the other hand, the 3′UTR of mouse CD28 has one additional miR-24 binding site compared to the 3′UTR of human CD28. Such differences, but also other differences including higher or lower miRNA or target gene levels can explain differences in miRNA-mediated CD28 regulation between humans and rodents.

In conclusion, our results provide evidence for involvement of miRNAs in the process of replication-associated aging and IL-15-mediated regulation of CD28 expression. The data support a positive feedback loop in which IL-15 induces loss of CD28 and induction of miR-9 as well as the members of the miR-23a~24-2 cluster during T cell aging. The induced loss of CD28 and associated senescent phenotype of aged T cells may be stabilized or further enhanced by induction of the IL-15-induced miRNAs.

ETHICS STATEMENT

Informed consent was obtained from all participants in accordance with the Declaration of Helsinki. The study was approved by the Medical Ethical Committee (METC) of the University Medical Center Groningen.

AUTHOR CONTRIBUTIONS

NT and GD performed experiments, prepared the data, and were involved in writing. PH supervised experiments and revised the manuscript. MT, PJ, and DJ performed experiments. EB, GP, and KK supervised the project and revised the manuscript. GP provided the T cell clones. AvdB, JK, AB, and B-JK initiated and supervised the project and the experiments and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01400/full#supplementary-material.

FIGURE S1 | Sorting strategy of CD8+CD45RO−CCR7+CD28+T cells.

FIGURE S2 | Loss of CD28 expression after 15 days culture with IL-15. Fluorescence-activated cell sorting-sorted naïve CD8+CD45RO−CCR7+CD28+ T cells were stained with proliferation dye to study CD28 expression after proliferation upon IL-15 stimulation (50 ng/ml). Proliferation of the naïve CD8 T cells was assessed after 15 days of culture. **(A)** Gate setting for the identification of the population doublings (PDs). Within the different PDs, **(B,E)** expression of CD28 per generation, **(C)** CD28 expression per cell per generation, **(D,F)** CD45RO, and **(F)** CCR7 expression was assessed. MFI = median fluorescence intensity. Significance (*$p < 0.05$, **$p < 0.01$) is depicted. $N = 3$.

FIGURE S3 | Loss of CD28 expression after 5 and 10 days culture with IL-15. Fluorescence-activated cell sorting-sorted naïve CD8+CD45RO−CCR7+ CD28+ T cells were stained with proliferation dye to study CD28 expression after proliferation upon IL-15 stimulation (50 ng/ml). Proliferation of the naïve CD8 T cells was assessed after **(A–F)** 5 and **(G–L)** 10 days of culture. **(A,G)** Gate setting for the identification of the population doublings (PDs). Within the different PDs **(B,E,H,K)** expression of CD28 per generation, **(C,I)** CD28 expression per cell per generation, **(D,F,J,L)** CD45RO and **(F,L)** CCR7 expression was assessed. MFI = median fluorescence intensity. Significance (*$p < 0.05$, **p **<** 0.01) is depicted. N **=** 3.

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ORIGINAL RESEARCH
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Effect of Age on NK Cell Compartment in Chronic Myeloid Leukemia Patients Treated With Tyrosine Kinase Inhibitors

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Natural killer (NK) cells are a very important component of the innate immune response involved in the lysis of virus infected and tumor cells. Aging has a profound impact in the frequency, phenotype and function of NK cells. Chronic Myeloid Leukemia (CML) is caused by the BCR-ABL gene formation encoding aberrant oncoprotein tyrosine kinase. Treatment with tyrosine kinase inhibitors (TKIs) induces durable deep molecular response. The response to treatment and life expectancy is lower in older patients with chronic phase of CML than in younger patients. In this work we analyse NK cells from TKI-treated CML patients and healthy controls stratified according to age. We have analyzed the expression of NK receptors, activation markers, NK cell differentiation in $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets and the expression of CD107a and IFN-γ in NK cells stimulated with K562. Whereas significant differences on the phenotype and function of NK cells were found between middle-aged (35–65 years old) and elderly (older than 65) healthy individuals, NK cells from TKI-treated CML patients do not show significant differences related with age in most parameters studied, indicating that age is not a limitation of the NK cell recovery after treatment with TKI. Our results also revealed differences in the expression of NK receptors, activation markers and functional assays in NK cells from TKI-treated CML patients compared with age-matched healthy controls. These results highlight the relevance of NK cells in TKI-treated patients and the need of an extensive analysis of the effect of aging on NK cell phenotype and function in these patients in order to define new NK-cell based strategies directed to control CML progression and achieve long-term disease remission after TKI cessation.

Keywords: aging, CML, NK receptors, activation markers, differentiation markers, cytokines, NK cell subsets, tyrosine kinase inhibitors

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INTRODUCTION

Natural Killer cells (NK) are innate lymphoid cells (ILCs) that represent ∼15% of peripheral blood lymphocytes (PBLs). NK cells share many features with ILC1 although they are developmentally distinct (1). NK cells can be classified in two major subpopulations according to CD56 expression. CD56^{bright} NK cells are less differentiated subpopulation that represents <10% of peripheral blood NK cells and have an immune-modulatory role with high production of cytokines and chemokines. CD56^{dim} NK cells, a more differentiated subpopulation that represents about 90% of NK cells, are mainly cytotoxic and interferon-gamma (IFN-γ) producers after direct contact with target cells (2, 3). A model of differentiation from immature CD56^{bright} that leads to more mature CD56^{dim} NK cells in the periphery has been proposed (4). Another subpopulation of NK cells, that do not express CD56 but express other NK receptors, expanded in healthy old and HIV-1 or hepatitis C infected individuals (5, 6), has been defined. NK cell function depends on a balance between activating and inhibitory signals triggered by activating and inhibitory receptors (7).

Chronic Myeloid Leukemia (CML) is an aging-associated disease (approximately half of cases are diagnosed in people older than 65) caused by reciprocal translocation between chromosomes 9 and 22 that give rise to the Philadelphia chromosome (Ph) and the BCR-ABL gene formation that encodes an oncoprotein tyrosine kinase with an aberrant activity in the hematopoietic stem cells (8, 9). Age has been included as a poor prognostic factor for survival in CML (10, 11). About half of the patients diagnosed with CML are between 60 and 65 years old (12–14) and the response to disease and life expectancy is lower in older patients with chronic phase of CML than in younger patients (15, 16). However, elderly CML patients are underrepresented in clinical studies having a reduced access to investigational therapies and median age of CML patients in cancer registries and patients included in clinical trials differs by 10–20 years (13, 14), supporting the interest to study immune parameters in elderly CML patients.

Immunosenescence is defined as age-associated dysregulation and dysfunction of the immune system characterized by impaired protective immunity and decreased efficacy of vaccines (17–20). These changes mainly affect the adaptive immune response (21) although consistent findings reveal that innate immune response is also affected (22, 23). In addition to age, situations of chronic activation of the human immune system, such as viral infections, autoimmune diseases and cancer, are involved in the development of immunosenescence (24–27).

Age-related alterations in frequency, distribution, phenotype, and function of NK cell subsets have been described, including an increased expression of CD57 (considered a marker of 'memory-like' NK) and a decreased expression of Natural Cytotoxicity Receptors (NCRs) and other NK activating receptors (22, 23, 28–31), CD69 (32), and CD94/NKG2A, and an increase of killer Ig-like receptors (KIR) (33–35) in older individuals.

Several studies have also found a decrease in the frequency and function of NK cells in CML patients at the time of diagnosis, with a progressive functional deterioration during all phases of the disease (36–39). It has been described a decreased expression of NKG2A, NKp30, and NKp46 at the time of diagnosis and changes in the NKG2C and KIR receptors (40, 41). Patients with CML also show a decrease in NKG2D expression, that mediates NK anti-CML response through its ligands MICA/B, when compared with healthy controls (42). NK cells from acute myeloid leukemia patients also have a downregulated expression of activating receptors NKp30 and NKp46 (27, 43–45) and DNAM-1 (27, 46), likely as a consequence of the interaction with their ligands in leukemic blasts.

The standard treatment for CML patients is based in the use of tyrosine kinase inhibitors (TKIs) such as imatinib, and more potent second-generation nilotinib and dasatinib, that have improved CML poor prognosis (47, 48). TKIs have a direct effect inhibiting the BCR-ABL1 kinase activity to induce a durable deep molecular response, a prelude to successful treatment-free remission that occurs in ∼50% of all CML patients who cease TKI therapy (48–51). In addition to their direct anti-kinase activity, TKIs contribute to the restoration of immune cell function leading to the efficient immunological control of CML (41). Recent studies show the impact of TKIs on NK cells, finding that the expression of NK activating receptors is restored to normal levels compared to their low level at the time of diagnosis (40, 41).

Considering that both aging and CML induce changes in phenotype and function of NK cells, in this work we have studied the expression of several markers (CD11b, CD27, CD57, CD69, HLA-DR, NKG2A, NKG2C, NKG2D, NKp30, NKp44, NKp46, and NKp80) in NK cell subsets, and CD107a and IFN-γ in K562 stimulated NK cells, from CML patients and healthy controls, stratified according to age in middle-aged and elderly donors.

MATERIALS AND METHODS

Study Subjects

A total of 80 individuals were included in the study, 38 CML patients treated with first-line TKIs Imatinib, and 42 healthy controls, stratified in two groups according to age: middle-aged (35–65 years) and old-age (over 65 years) (**Table 1**). All the participants in the study were CMV-seropositive (Non-reactive IgM and reactive IgG, data not shown). Controls were excluded of the study if they had infection at the time of sample collection, suffered or had suffered cancer or autoimmune diseases, were under immunosuppressive drugs or calcium channel blockers. The Ethical Committees of the Faculty of Medicine of the University of Coimbra and the Coimbra Hospital and University Centre (Portugal) and the Ethics Committee of the Reina Sofia University Hospital of Cordoba (Spain) approved this study and all volunteers agreed and signed informed consent to participate.

Procedures of Sample Collection and Processing

Peripheral blood samples from all individuals were obtained in heparinized tubes. Flow cytometry studies were performed on freshly-obtained cells. After antibody staining, BD FACS Lysing Solution (BD Biosciences, San Jose, CA, USA) was used for lysis of red blood cells. Subsequently, cells were washed and resuspended in Dulbecco's Phosphate Buffered Saline (PBS)

TABLE 1 | Demographics characteristics of individuals ($n = 80$).

Group name	No.	Sex (male: female)	Mean (SD)[a]	p[b]
Middle age (Control)	19	8:11	51 (7.85)	0.924
Middle age (CML*)	19	12:7	51 (8.97)	
OLD (Control)	23	10:13	74 (6.17)	0.814
OLD (CML*)	19	12:7	75 (5.71)	

[a]*Average age (Standard Deviation) of the group.*
[b]*p-value for the comparison of means between controls and CML, within the same age group (t-Student test).* *TKI-treated CML patients.*

pH 7.4 (Ambion, Austin, TX, USA) for later acquisition on the cytometer. Cell suspensions were acquired in a BD FACS Canto II cytometer (BD Biosciences, San Jose, CA, USA). These procedures were performed according to the manufacturer protocols.

Flow Cytometric Analysis and Monoclonal Antibodies

Fresh blood was used for the analysis of the surface receptors by flow cytometry in different tubes. The following mouse anti-human conjugated monoclonal antibodies (mAbs) were used: anti-CD3 V500 (clone UCHT1, BD Horizon), anti-CD14 V500 (clone M5E2, BD Horizon), anti-CD19 V500 (clone HIB19, BD Horizon), anti-CD3 APC-H7 (clone SK7, BD Biosciences), anti-CD56 PerCP-Cy5.5 (clone HCD56, BD Pharmingen), anti-CD11b V450 (clone ICRF44, BD Horizon), anti-CD27 FITC (clone 0323, Biolegend), anti-CD57 Pacific Blue (clone HNK-1, Biolegend), anti-CD69 FITC (clone FN50, Biolegend), anti-HLA-DR V500 (clone G46-6, BD Pharmingen), anti-NKp30 Alexa Fluor 647 (clone P30-15, Biolegend), anti-NKp44 Alexa Fluor 647 (clone P44-8, Biolegend), anti-NKp46 PE (clone 9E2, Biolegend), anti-NKp80 PE (clone SD12, Biolegend), anti-NKG2A PE (clone 131411, R&D Systems), anti-NKG2C APC (clone 134591 R&D Systems), anti-NKG2D APC (clone 1D11, Biolegend). The expression of HLA-DR, NKp30, NKp44, NKp46, NKp80, NKG2D, (measured as Median Fluorescence Intensity, MFI) and the expression of CD11b, CD27, CD57, CD69, NKG2A, and NKG2C (measured as relative frequency) were determined in the different NK cell subpopulations and analyzed by multiparametric flow cytometry. The data were analyzed using FlowJo v10 (Tree Star, Inc.) from PBLs, selecting singlets. NK cells ($CD56^+$ and $CD3^-/CD14^-/CD19^-$) were selected and two subpopulations of NK cells were described, in relation to their expression of CD56, as $CD56^{bright}$ and $CD56^{dim}$ (**Figure S1A**). Isotype matched antibodies, labeled with the appropriate fluorochromes, were used as negative controls. Representative histograms for the NK cell markers, and the isotype and fluorochrome of the antibodies used in the studies, are shown in **Figure S1B**.

NK Cell Stimulation With K562, Degranulation Assay, and IFN-γ Staining

Effector peripheral blood mononuclear cells were isolated from heparinized blood samples by a standard density gradient procedure (Ficoll-Paque PLUS, Merck KGaA, Darmstadt, Germany) and counted to adjust the concentration to 50×10^6cells/mL. Target cells (K562 cell line) were prepared; viability determined and counted to adjust the concentration to 1×10^5cells/mL. Then, effector and target cells were mixed in a 25:1 effector-to-target ratio into 12 × 75 mm tubes with anti-CD107a PE (clone H4A3, BD Pharmingen®) antibody and brefeldin A (Merck, 10 μg/mL). Tubes were incubated in a humidified CO_2 incubator in the water reservoir at the bottom for 4 h. At the end of incubation cells were washed and resuspended in 100 μL of 1xPBS (phosphate-buffered saline) and the extracellular antibodies were added, anti-CD56 APC (clone B159, BD Pharmingen®) and anti-CD3V500 (clone UCTH1, BD Horizon®). After 15 min of incubation at RT in the dark, suspensions were treated with Fix and Perm A solution (Invitrogen®) for 15 min, in the dark at room temperature. Cells were centrifuged at 453 g for 5 min and the supernatant discarded. Next, cells were incubated with Fix and Perm B solution (Invitrogen®) and the intracellular antibody anti-IFN-γ PE-Cy7 (clone B27, BD Horizon®) for 20 min, in the dark at room temperature. After centrifugation at 453 g for 5 min and supernatant discarded cells were resuspended in 1x PBS and acquired in the flow cytometer (BD FACS Canto II).

Statistical Analysis

Shapiro-Wilk test was used for checking the normal distribution of the data. We used Kruskall-Wallis (non-parametric) test for multiple comparison and Mann-Whitney *U*-test (non-parametric) was used to compare specific groups. All tests were performed using statistical software package SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Values $p < 0.05$ were considered significant. The results were shown as median with interquartile range and the graphics were performed using GraphPad Prism software version 6.0 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Expression of Activating and Inhibitory Receptors on NK Cells From TKI-Treated CML Patients

We studied the expression of activating and inhibitory receptors on $CD56^{dim}$ and $CD56^{bright}$ NK cells in healthy donors and CML patients, stratified in middle age and old age. The expression of Natural Killer Group 2 (NKG2) receptors was measured as the percentage of positive cells or as MFI measured in the total of cells (**Figure 1A**). Our results showed a significant decrease in the expression of the inhibitory receptor NKG2A on $CD56^{dim}$ NK cells in middle-aged CML patients compared with middle-aged healthy donors and a decrease in the percentage of $NKG2A^+CD56^{bright}$ NK cells in old CML patients compared with old healthy donors. Age-associated changes in the expression of NKG2A were only observed in healthy donors showing an increase with age in the percentage of $NKG2A^+CD56^{bright}$ NK cells. In contrast, NKG2C receptor expression was not influenced by CML or age. Regarding the activating receptor NKG2D, we found a significant decrease in

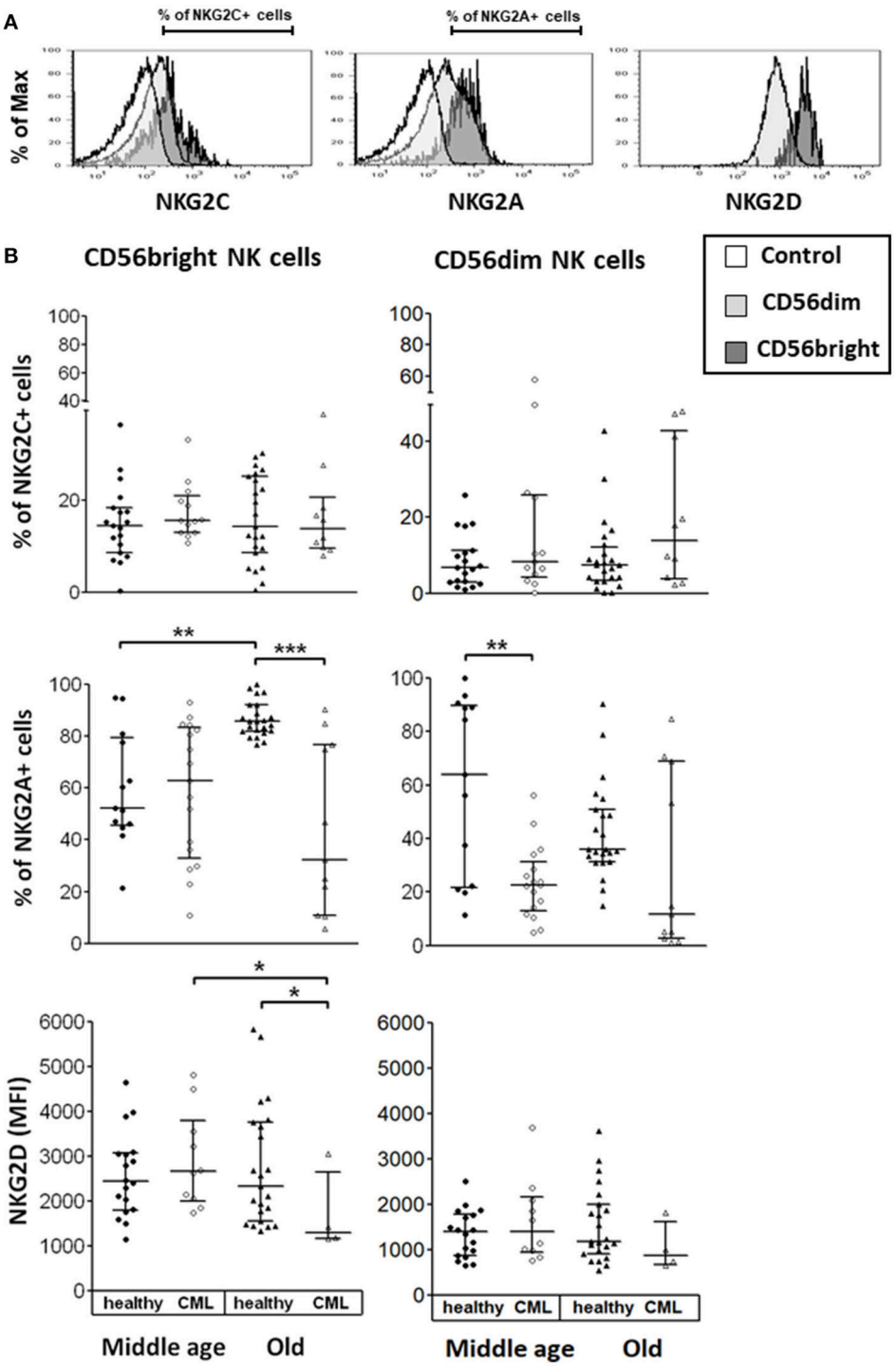

FIGURE 1 | Expression of Natural Killer Group 2 (NKG2) receptors on NK cell subpopulations. **(A)** Representative histograms for each marker are shown (the non-shaded area represents the control, the shaded area of light gray, the CD56^{dim} cells, the shaded one of gray, the CD56^{bright} cells). The percentage of cells expressing NKG2C, NKG2A, and NKG2D as (MFI) measured in the total of cells, was determined on the surface of each subset by multiparametric flow cytometry. **(B)** Expression of activating receptors (NKG2C and NKG2D) and of inhibitory receptor (NKG2A) on CD56^{bright} and CD56^{dim} NK subsets from healthy individuals and TKI-treated CML patients, stratified according to age (middle-aged 35–65 years and old >65 years). Number of donors: NKG2C middle-aged healthy $n = 19$, middle-aged CML $n = 13$, old healthy $n = 23$, and old CML $n = 10$; NKG2D middle-aged healthy $n = 18$, middle-aged CML $n = 10$, old healthy $n = 23$, and old CML $n = 4$; NKG2A middle-aged healthy $n = 13$, middle-aged CML $n = 17$, old healthy $n = 23$, and old CML $n = 11$. The results, expressed as median with interquartile range, were considered significant at $p < 0.05$. *P*-values were determined comparing middle age with old and healthy with TKI-treated CML patients. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

the MFI of NKG2D on CD56^{bright} NK cells in old CML patients compared with old healthy donors, and a decrease with age in CML patients **(Figure 1B)**.

We have also studied the expression of NCRs on NK cell subpopulations. Results were expressed as MFI measured in the total number of cells **(Figure 2A)**. We have found that the expression of NKp30 and NKp80 (not a NCR) on CD56^{bright} NK cells and NKp80 on CD56^{dim} NK cells was lower in old CML patients compared with old healthy donors. NKp80 expression on CD56^{bright} cells and NKp46 expression on CD56^{dim} NK cells were significantly decreased in middle-aged CML patients compared with middle-aged healthy donors. The analysis of age-associated changes in the expression of NCRs in CML patients showed a decrease in NKp30 expression on CD56^{bright} NK cells. No significant differences associated with age (middle-aged vs. old age) were observed in healthy donors **(Figure 2B)**.

Expression of Activation Markers of NK Cells From TKI-Treated CML Patients

In this study, we have analyzed the expression of several activation markers (HLA-DR, CD69, and NKp44) on the surface of NK cell subsets in resting conditions. CD56^{bright} NK cells in elderly CML patients showed a decreased expression of HLA-DR (measured as MFI) and a reduction in the percentage of CD69 and NKp44 positive cells compared with elderly healthy donors. An age-associated increase in the expression of these three markers was observed on CD56^{bright} NK cells in elderly healthy donors compared with middle-aged healthy donors. In contrast, in CML patients a decrease of CD69 expression on CD56^{bright} NK cells was associated with age **(Figure 3A)**.

Regarding the expression of these activation markers on the CD56^{dim} NK cell subset, our results showed a decreased expression of these markers in elderly CML patients, as well as a decreased expression of CD69 and HLA-DR in middle-aged CML patients compared with age-matched healthy donors. Nevertheless, it is interesting to highlight the increase observed in the percentage of NKp44^{+} cells in middle-aged CML patients compared with middle-aged healthy donors. We also found an age-associated increase in the expression of HLA-DR and NKp44 in healthy individuals and a decrease of CD69 and NKp44 expression in CD56^{dim} NK cells from elderly CML patients compared with CD56^{dim} NK cells from middle-aged CML patients **(Figure 3B)**.

Analysis of NK Cell Differentiation Markers on NK Cells From TKI-Treated CML Patients

The analysis of NK cell subsets in healthy donors showed a decreased percentage of CD56^{bright} that correlated with an increased percentage of CD56^{dim} NK cells in elderly donors compared with middle-aged healthy donors. In contrast, no statistical significant differences in NK cell subset distribution was observed in CML patients according to age. Percentage of total NK cells was not influenced by CML or age (**Figure 4A**). The expression of CD57 was increased on CD56^{dim} NK cells from CML patients compared with age-matched healthy donors. Moreover, in healthy individuals, CD57 expression on both CD56^{bright} and CD56^{dim} NK cells increased with age (**Figure 4B**). The co-expression of CD11b and CD27 markers was also analyzed in each NK cell subset **(Figure 4C)**. Whereas, a high percentage of CD56^{bright} NK cells were CD11b^{+}CD27^{+}, the majority of CD56^{dim} NK cells from peripheral blood were CD11b^{+}CD27^{-}. Our results revealed a decrease of CD11b^{+}CD27^{+}CD56^{dim} NK cells associated with CML in middle-aged individuals and a decrease of this cell subset related to age in healthy donors (**Figure 4C**). Not significant differences were found in CD56^{bright} NK cells.

CD107a Expression and IFN-γ Production in NK Cells From TKI-Treated CML Patients Activated With K562

We analyzed the percentage of NK cells expressing CD107a or IFN-γ after stimulation with the K562 cell line. Representative analysis of middle-aged and old controls and TKI-treated CML patients are shown **Figures 5A,C**. The results did not show significant differences on CD107a expression and IFN-γ production in K562 stimulated NK cells between middle-aged healthy donors and middle-aged CML patients. NK cells from healthy elderly donors have higher expression of CD107a or IFN-γ compared with middle-aged healthy donors. In a similar way the percentage of NK cells expressing CD107a or IFN-γ was higher in healthy elderly donors than in TKI-treated CML old patients. On the contrary no significant age-associated differences on CD107a expression and IFN-γ production were observed in K562 stimulated NK cells from CML patients (**Figures 5B,D**).

As shown in **Figure S2**, the comparison of cytokine production in healthy controls vs. TKI-treated CML patients shows that the expression of IFN-γ by unstimulated NK cells is higher in middle-aged TKI-treated CML patients and lower in elderly patients compared with their age-matched controls, whereas the expression of IL-10 is higher in TKI-treated CML patients from both age groups compared with their respective controls**.**

DISCUSSION

A decrease in the frequency and function of NK cells in CML patients at the time of diagnosis has been demonstrated, with a progressive functional deterioration during disease progression to advanced and blast crisis phase (36–39, 52). In addition, NK cells from CML patients at diagnosis show a reduced expression of activating and inhibitory NK receptors compared to healthy donors (40, 53). NK cells play an important role in the control of CML not only during TKI treatment (40, 41) but also after TKI cessation (54, 55).

It has been shown that life expectancy is lower in older than in younger patients with chronic phase of CML and that aging is a poor prognostic factor for survival and response to treatment in CML (10, 11, 15, 16). Cumulative evidences support that aging affects NK cell subsets, phenotype and

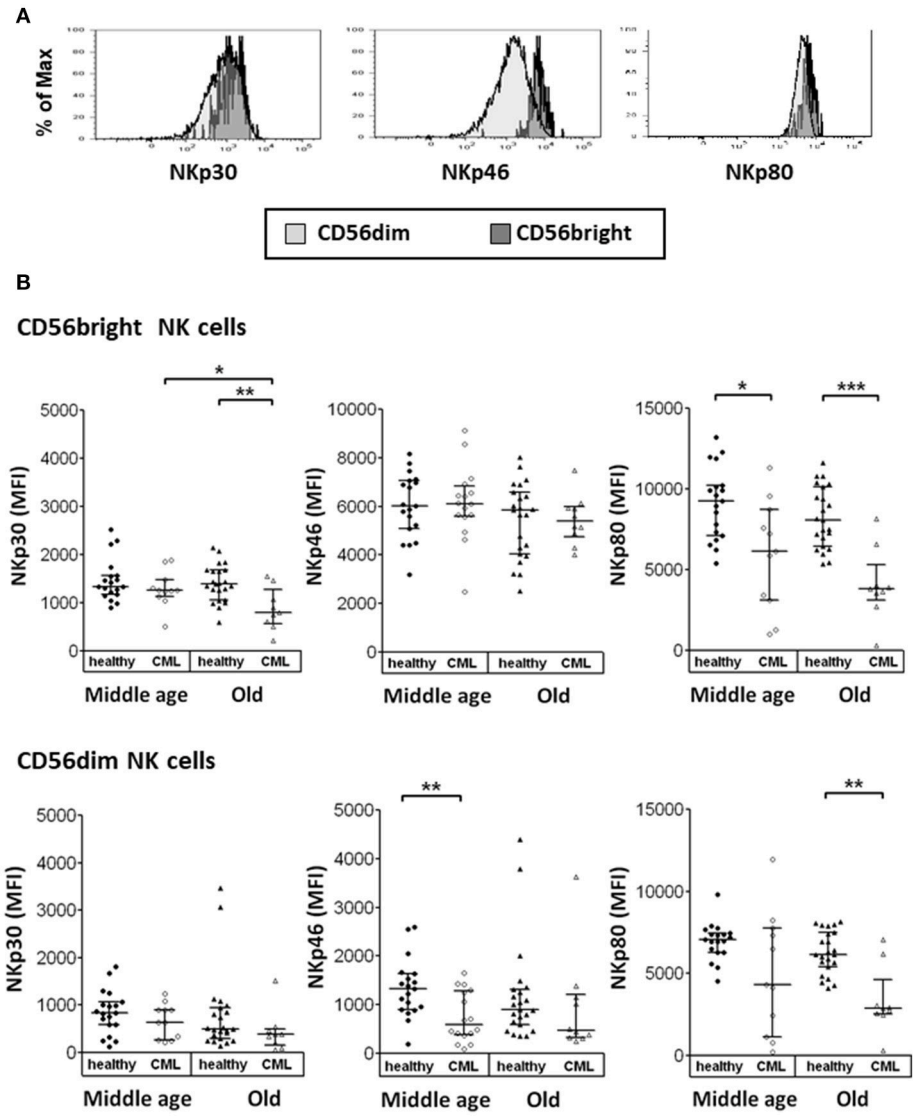

FIGURE 2 | Expression of Natural Cytotoxicity Receptors (NCRs) on CD56^{bright} and CD56^{dim} NK cells. **(A)** Representative histograms for NKp30, NKp46, and NKp80 are shown (the shaded area of light gray, the CD56^{dim} cells, the shaded one of gray, the CD56^{bright} cells). Results were expressed as Median Fluorescence Intensity (MFI), measured in the total of cells. **(B)** Expression of activating receptors NKp30, NKp46, and NKp80 on CD56^{bright} and CD56^{dim} NK subsets from healthy individuals and TKI-treated CML patients, stratified according to age (middle-aged 35–65 years and old >65 years). Number of donors: NKp30 and NKp80 middle-aged healthy $n = 19$, middle-aged CML $n = 11$, old healthy $n = 23$, and old CML $n = 9$; NKp46 middle-aged healthy $n = 19$, middle-aged CML $n = 16$, old healthy $n = 23$ and old CML $n = 10$. The results were expressed as median with interquartile range. *P*-values were determined comparing middle age with old and healthy with TKI-treated CML patients and were considered significant at $p < 0.05$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

function (22, 23, 56), including the expression of activating and inhibitory NK cell receptors (28–30, 57). The analysis of NK cells from middle-aged and elderly healthy donors, summarized in **Figure 6A**, is in line with previous data on the differences between NK cells from young and old healthy donors. Thus, there are significant differences in the frequency of NK cells subsets with different maturation stages. The percentages of more immature CD56^{bright} NK cells (2, 3), and CD11b^{+}/CD27^{+} NK cells, that represent a minor subset of immunoregulatory NK cells described as an intermediate differentiation stage (58), are lower in elderly than in middle-aged healthy donors, whereas the percentage of mature highly cytotoxic CD56^{dim}CD57^{+} NK cells is higher in the elderly (**Figure 6A**), confirming the age-associated shaping of NK cell subsets (22, 30). The expression of NK cell activation markers HLA-DR, CD69, and NKp44 is also higher in NK cells from elderly healthy donors, likely as a consequence of low grade age-associated inflammation (inflamm-aging) (59). Inflamm-aging is also consistent with the observation that NK cells from old healthy donors show higher expression of CD107a or IFN-γ in response to K562

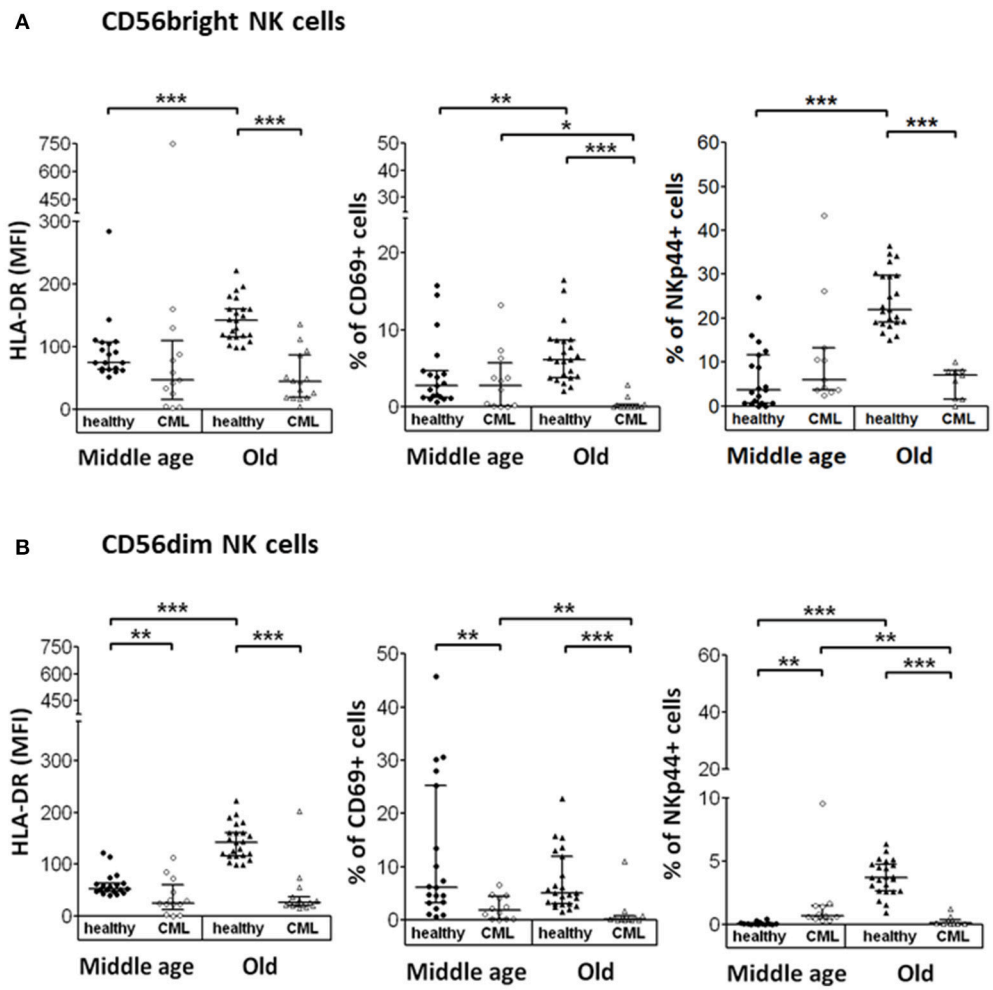

FIGURE 3 | Expression of activation markers on CD56^{bright} and CD56^{dim} NK cell subpopulations. Determination of activation markers expression (HLA-DR, CD69, and NKp44) on the surface of CD56^{bright} and CD56^{dim} NK cells from the different study groups. NK cells were not stimulated. **(A)** Expression of HLA-DR (measured as MFI), CD69 and NKp44 (in percentage) on CD56^{bright} NK cells. **(B)** Expression of HLA-DR (MFI) and percentage of CD69 and NKp44 on CD56^{dim} NK cells. Number of donors: HLA-DR middle-aged healthy $n = 19$, middle-aged CML $n = 13$, old healthy $n = 23$ and old CML $n = 15$; CD69 middle-aged healthy $n = 19$, middle-aged CML $n = 12$, old healthy $n = 23$, and old CML $n = 15$; NKp44 middle-aged healthy $n = 19$, middle-aged CML $n = 11$, old healthy $n = 23$, and old CML $n = 9$. The results, expressed as median with interquartile range, were considered significant at $p < 0.05$. *P*-values were determined comparing middle age with old and healthy with TKI-treated CML patients. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

stimulation than NK cells from middle-aged donors. The effect of aging on NK cell cytotoxicity and IFN-γ has been extensively analyzed in discrepant results have been found among different groups probably due to different selection age ranges, technical procedures, and health status of the individuals studied although it is generally accepted that the total number and cytotoxic function of NK cell are preserved or increased in healthy aging compared with young and middle-aged individuals (22, 23, 56).

The study of NK cells in middle-aged TKI-treated CML patients shows that the percentage of CD56^{bright} NK cells is similar to the percentage found in heathy middle-aged individuals, whereas the minor population of CD56^{dim} NK cells co-expressing CD11b and CD27 is dramatically decreased **(Figure 6B)**. In addition, CD56^{dim} NK cells from TKI-treated CML patients have higher expression of CD57, a marker of highly differentiated NK cells (22, 30), and NKp44 indicating that NK cells from TKI-treated CML patients are highly differentiated activated NK cells, as it has been recently suggested (40, 54). However, there is a lower expression of NKp46, NKG2A, CD69, and HLA-DR on CD56^{dim} NK cells and of NKp80 on CD56^{bright} NK cells **(Figure 6B)**. The comparison of NK cells from old TKI-treated CML patients with those from old healthy individuals shows a lower expression of NK receptors NKp44, NKp80, CD69, and HLA-DR in both CD56^{bright} and CD56^{dim} NK cells and also a reduced expression of NKp30, NKG2D, and NKG2A in CD56^{bright} NK cells **(Figure 6C)**. Thus, despite the well-established observations that CML patients

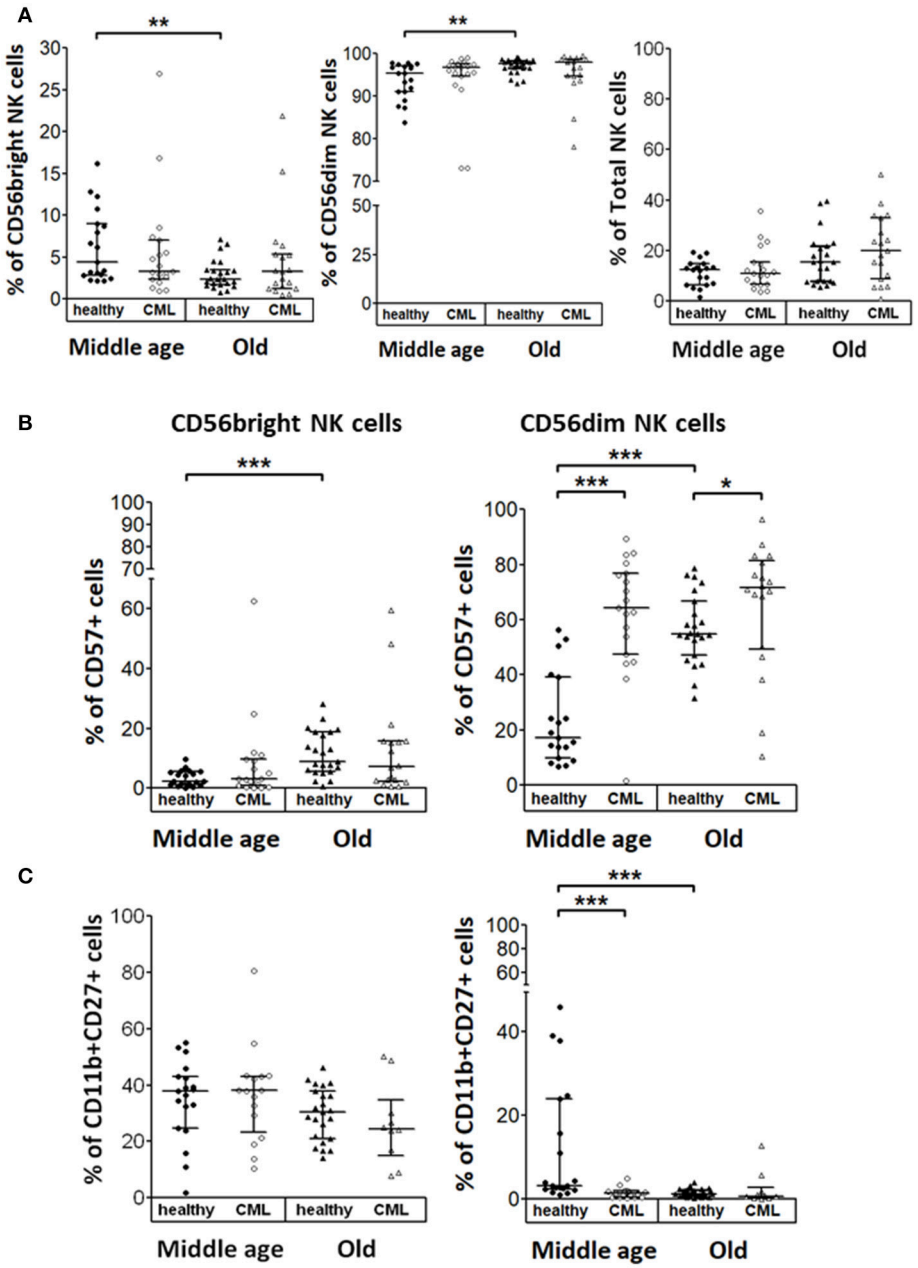

FIGURE 4 | NK cell subsets in healthy and TKI-treated CML individuals stratified by age. **(A)** Percentage of CD56^{bright} and CD56^{dim} NK cells referred to the total of CD56^{+} NK cells and NK cells referred of total Lymphocytes from healthy donors and TKI-treated CML patients. **(B)** Percentage of NK cells expressing CD57 determined in CD56^{bright} and CD56^{dim} NK cell subsets from both healthy and TKI-treated CML individuals. **(C)** Percentage of CD11b and CD27 positive cells in CD56^{bright} and CD56^{dim} NK cell subsets from both healthy and TKI-treated CML individuals. Number of donors: CD56^{bright}, CD56^{dim}, and NK cells, middle-aged healthy $n = 19$, middle-aged CML $n = 19$, old healthy $n = 23$, and old CML $n = 19$; CD57 middle-aged healthy $n = 19$, middle-aged CML $n = 19$, old healthy $n = 23$, and old CML $n = 18$; CD11b^{+}CD27^{+} middle-aged healthy $n = 19$, middle-aged CML $n = 16$, old healthy $n = 23$, and old CML $n = 10$. Graphics show the median with interquartile range. Kruskal-Wallis test was used for multiple comparisons and the Mann-Whitney *U*-test for the comparison of specific groups. The differences were considered significant in a range of $p < 0.05$. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

have a decreased expression of NK receptors at diagnosis (36–39), our results confirm that NK cells from TKI-treatment CML patients can express NK activating receptors (40, 41) such as NKp30, NKp46, and NKp80, although this expression is heterogeneous and in some cases their levels are lower than those found in age-matched healthy controls. NKp30 and NKp46 are NCRs involved in NK cells cytotoxicity after interaction with their ligands on target cells (60). NKp80, is

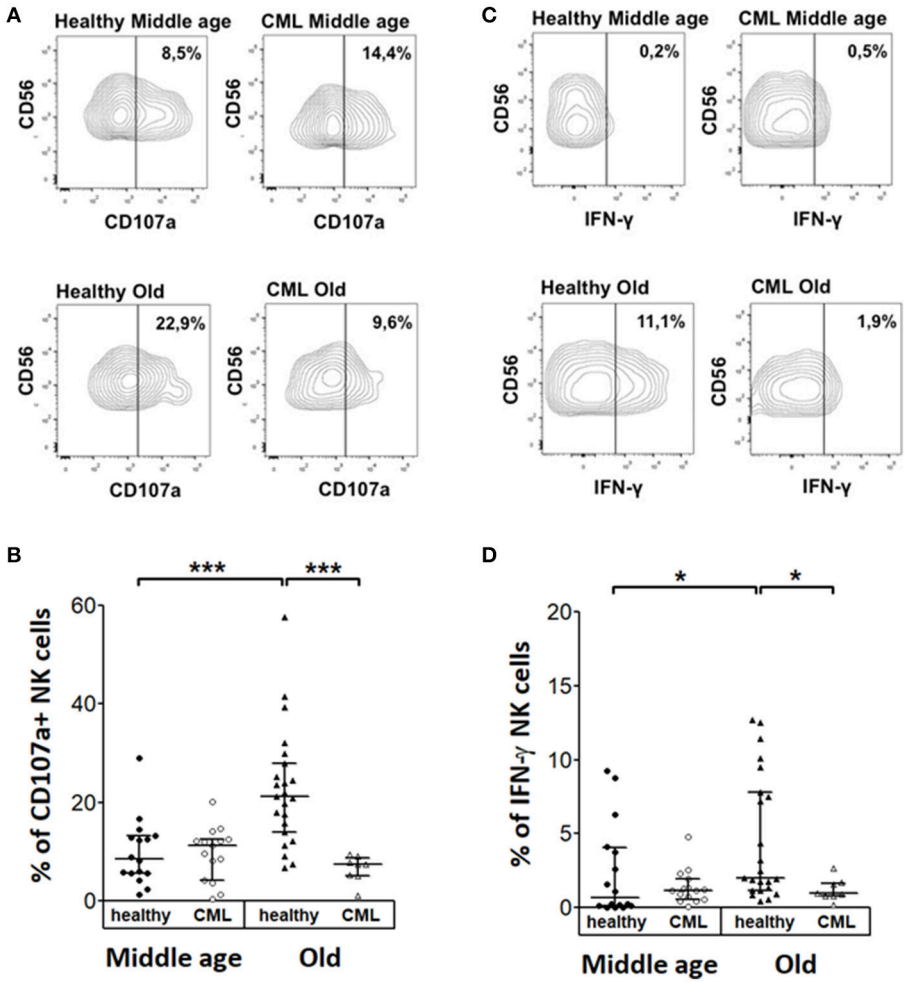

FIGURE 5 | Cytokine expression and degranulation in stimulated NK cells. Analysis of CD107a degranulation assay and IFN-γ expression by Natural killer cells following stimulation with K562 target cells. **(A)** Representative dotplots and **(B)** percentage of CD107a in NK cells from healthy donors and TKI-treated CML patients according to age and CML. **(C)** Representative dotplots and **(D)** IFN-γ expression in NK cells from healthy donors and TKI-treated CML patients according to age and CML (middle-aged healthy $n = 16$, middle-aged CML $n = 15$, old healthy $n = 23$, and old CML $n = 8$). Numbers in **(A,C)** represent the percentage of positive cells referred to the $CD56^{+}$ NK cells. The results in **(B,D)** were shown as median with interquartile range. Values of $p < 0.05$ were considered significant. *P*-values were determined comparing middle age with old and healthy with TKI-treated CML patients. $^{*}p < 0.05$; $^{***}p < 0.001$.

an activating C-type lectin-like receptor expressed on NK cells that interact with its ligand activation-induced C-type lectin (AICL) expressed on myeloid cells, including myeloid leukemia cells (61). It has been suggested that after TKI treatment NK cells are involved in the control of CML blasts (40, 41), thus the lower expression of these receptors compared with healthy controls can be the consequence of the interaction of NK cells with their ligands expressed on leukemic blasts, as suggested for NK cells from AML patients (44–46). Downregulation of NKG2D after its interactions with MICA/B ligand is a well-defined phenomenon in different tumors (62, 63). However, the expression of NKG2D is well preserved in all NK cell subsets from TKI treated CML patients, with the exception of $CD56^{bright}$ from elderly patients, confirming recent studies showing that the downregulated expression of NKG2D at the time of CML diagnosis, is restored to normal levels after TKI treatment (40, 41). The comparison of NK cell response to K562 in healthy controls vs. TKI-treated CML patients did not show significant differences on CD107a expression in K562 stimulated NK cells when middle-aged healthy donors were compared with middle-aged or old CML patients **(Figures 6B,C)** supporting previous findings that TKI treatment is associated with immune system re-activation and restoration of NK cell immune surveillance in CML patients (40, 41). On the contrary the observation that the percentage of NK cells expressing CD107a in TKI-treated CML old patients was lower than in healthy elderly donors, together with the lower expression of NK receptors in old TKI-treated CML patients compared with healthy elderly healthy donors **(Figure 6C)**, support that the alterations on NK cells observed in healthy elderly donors likely associated

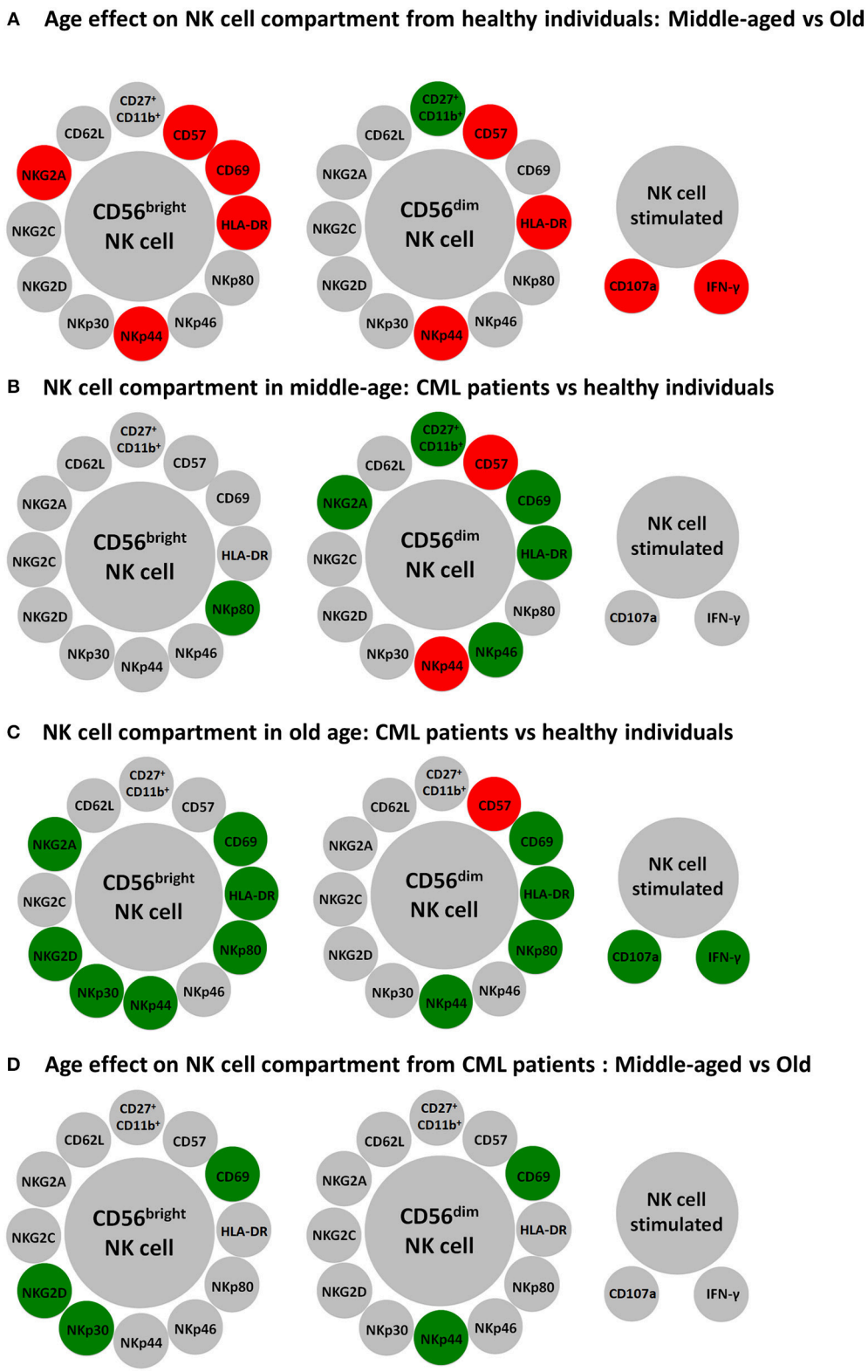

FIGURE 6 | Schematic representation of major differences in resting and K562 stimulated NK cells according to age and CML. Circles represent NK cell subpopulations expressing the indicated membrane markers in CD56^{bright} and CD56^{dim} resting NK cells and the expression of CD107a and IFN-γ in K562 stimulated NK cells. Red color was used for increased expression and green color for decreased expression of the membrane markers in NK cells from healthy old compared with healthy middle-aged individuals **(A)**, from CML patients compared with age-matched healthy donors **(B,C)**, or in old CML patients compared with middle-aged CML patients **(D)**.

with chronic virus infection, such as CMV, and inflamm-aging are not observed in old TKI-treated CML patients. The high variability observed in IFN-γ production by NK cells in response to K562 in healthy donors and the low response observed in most CML patients, represent a limitation of the study that precludes to obtain a conclusion on the significance of cytokine production in the disease control. The NK cell functional capacity and cytokine production during TKI-treatment and after TKI cessation in CML patients requires further analysis to discriminate a possible role in the long-term elimination of CML blasts (54, 55).

The analysis of the possible effect of age on NK cells from TKI-treated CML patients shows a lower expression of activation markers NKp44 and CD69 in elderly compared with middle-aged TKI-treated CML patients whereas no significant differences related with age are found in the other parameters studied, including CD107a expression and IFN-γ production in K562 stimulated NK cells from TKI-treated CML patients **(Figure 6D)**, indicating that age is not a limitation of the NK cell recovery after treatment with TKI.

In conclusion, despite the deleterious effect of aging in CML prognosis, our results showing that activating NK cell receptors can be expressed both in middle-aged and elderly TKI-treated CML patients highlight the interest to extensively analyse the effect of aging on NK cell phenotype and function in these patients. The possibility of enhancing NK cell activity by using cytokines and immunomodulating agents open new perspectives for the design of novel clinical trials aiming effective long-term treatment-free remission after TKI cessation in CML patients.

AUTHOR CONTRIBUTIONS

PR-S, NL-S, CC, RS, RT, and MS-R: research study design; PR-S, NL-S, JSA, PC, and VA: experiments conduction and data acquisition; LR and PF-T: clinical data and patient management; PR-S, NL-S, RT, JSA, CC, and CA: data analysis; PR-S, CA, and MS-R: reagents providing; PR-S, NL-S, CC, RT, and RS: manuscript writing; All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02587/full#supplementary-material

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