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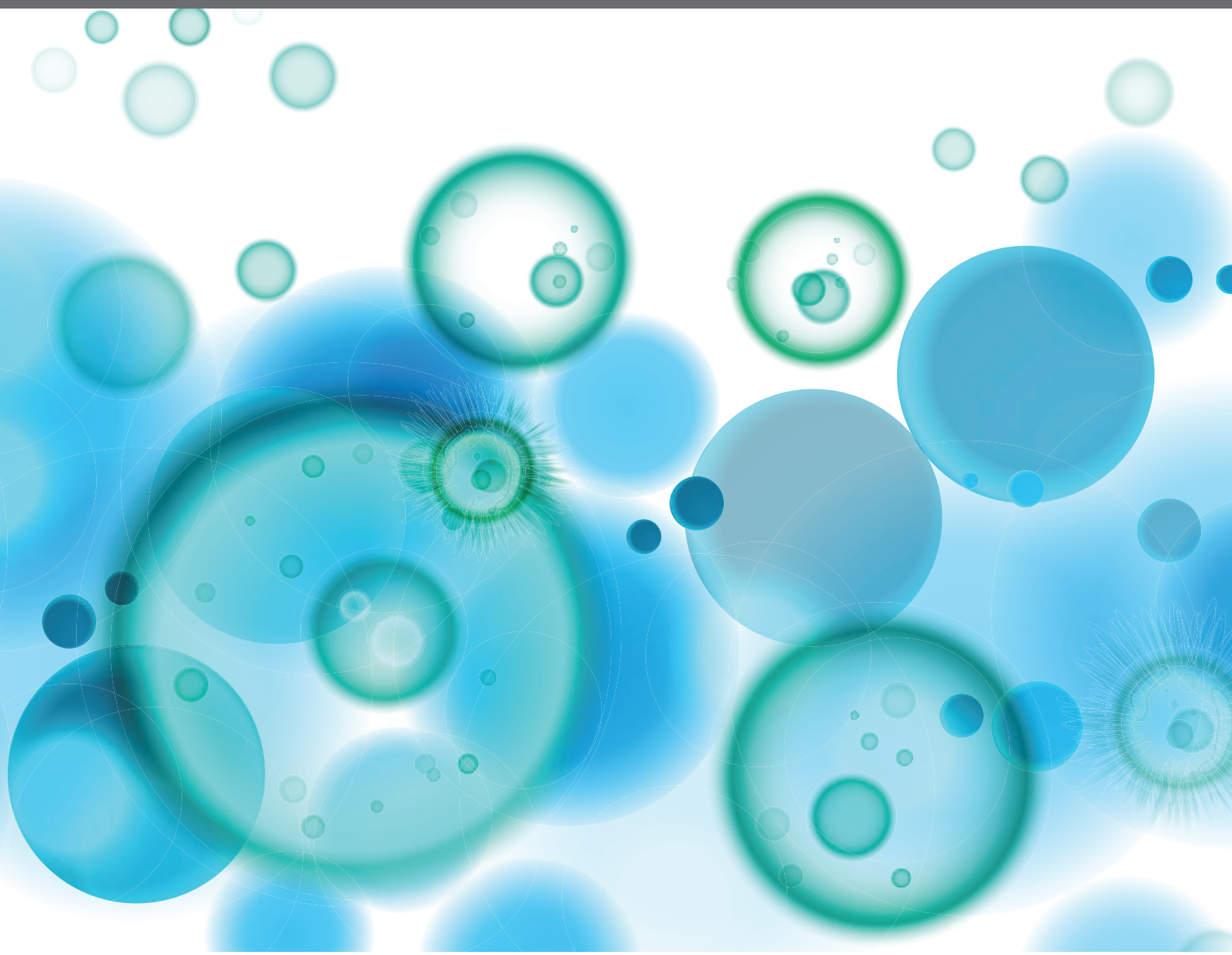

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IMMUNOSENESCENCE AND CLINICAL CONSEQUENCES

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GDF15 Plasma Level Is Inversely Associated With Level of Physical Activity and Correlates With Markers of Inflammation and Muscle Weakness

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Growth differentiation factor 15 (GDF15) is a stress molecule produced in response to mitochondrial, metabolic and inflammatory stress with a number of beneficial effects on metabolism. However, at the level of skeletal muscle it is still unclear whether GDF15 is beneficial or detrimental. The aim of the study was to analyse the levels of circulating GDF15 in people of different age, characterized by different level of physical activity and to seek for correlation with hematological parameters related to inflammation. The plasma concentration of GDF15 was determined in a total of 228 subjects in the age range from 18 to 83 years. These subjects were recruited and divided into three different groups based on the level of physical activity: inactive patients with lower limb mobility impairment, active subjects represented by amateur endurance cyclists, and healthy controls taken from the general population. Cyclists were sampled before and after a strenuous physical bout (long distance cycling race). The plasma levels of GDF15 increase with age and are inversely associated with active lifestyle. In particular, at any age, circulating GDF15 is significantly higher in inactive patients and significantly lower in active people, such as cyclists before the race, with respect to control subjects. However, the strenuous physical exercise causes in cyclists a dramatic increase of GDF15 plasma levels, that after the race are similar to that of patients. Moreover, GDF15 plasma levels significantly correlate with quadriceps torque in patients and with the number of total leukocytes, neutrophils and lymphocytes in both cyclists (before and after race) and patients. Taken together, our data indicate that GDF15 is associated with decreased muscle performance and increased inflammation.

Keywords: GDF15, physical activity, sedentarity, inflammation, skeletal muscle, healthy aging

INTRODUCTION

Growth differentiation factor 15 (GDF15), also known as macrophage inhibitory cytokine 1 (MIC-1), is a stress responsive member of the transforming growth factor-β (TGF-β) cytokine superfamily, discovered in 1997 (1). GDF15 modulates appetite and energy metabolism possibly by regulating mitochondrial functions, such as mitochondrial biogenesis, thermogenesis, and fatty

acid metabolism (2). Interestingly, mice overexpressing human GDF15 display increased life span (3). However, the role of GDF15 in promoting health or disease is still debated. There are in fact several evidences indicating that GDF15 levels are associated to progression of many diseases, such as cardiovascular diseases, insulin resistance and type 2 diabetes, neurodegeneration, renal chronic disease and cancer, but also to the limitation of the damage caused by stress and injuries (4–8). Accordingly, GDF15 has recently emerged as a potential biomarker for the aging process and many age-related diseases (2, 9–11). As far as muscle atrophy and sarcopenia, there is debate on whether GDF15 is to be considered protective or detrimental. Recent data from animal models showed that GDF15 is able to induce muscle fiber apoptosis (12, 13), but also the ablation of GDF15 resulted in an amplified skeletal muscle post exercise stress response, with a bigger increase of markers of muscle stress (Atf3, Atf6, and Xbp1s) (14). In humans, circulating GDF15 levels are significantly higher in subjects with sarcopenia or muscle atrophy (15–17) with respect to healthy subjects of comparable age. Recent studies demonstrate that GDF15 levels are negatively correlated with skeletal muscle mass index, hand-grip strength, muscle cross-sectional area and thickness (15, 18). Moreover, the loss of muscle mass observed in cachectic patients is mediated at least in part by the activity of GDF15 (19). On the other hand, it is known that physical exercise can effectively combat muscle atrophy, but is characterized by an increase in the level of circulating GDF15 (20–22). This could be explained by the fact that, as recently proposed, skeletal muscle is not the primary source of GDF15 (22). Finally, it is known that GDF15 is a stress molecule that is produced in response to mitochondrial, metabolic and inflammatory stresses (2, 23). To this regard, it is worth noting that a chronic state of low-grade inflammation, termed inflammaging, characterizes old people and is at the basis of many age-related diseases (24–26).

The aim of this study is to analyse the levels of circulating GDF15 in people of different age characterized by different levels of physical activity and to seek for correlation with hematological parameters related to inflammation. To this purpose, we studied three groups of subjects: 1. patients with chronic lower limb mobility impairment as a model of physical inactivity; 2. a group of amateur endurance cyclists as a model of physical activity; 3. and, age-matched subjects recruited from the general population, not actively exercising. Cyclists were sampled before and after a strenuous physical bout (a 130-km long distance road cycling race with a total uphill gradient of 1,871 m).

METHODS

Subjects

In the present study, a total of 228 subjects in the age range from 18 to 83 years were recruited and divided into three different groups based on the level of physical activity: patients with lower limb mobility impairment (hereinafter patients), cyclists and controls. Patients suffered of coxarthrosis or hip dysplasia, causing them to be chronically unable to walk or exercising, and were therefore considered a model for prolonged physical inactivity. All subjects were further divided according to their age into the following groups: young, adult, late adult, old (**Table 1**). All subjects were enrolled in Italy in the framework of the following projects: the EU project MYOAGE for patients and controls, the "Novecolli Life" project promoted by Italian National Transplant Center for cyclists. The study protocols were approved by the Ethical Committee of Istituto Ortopedico Rizzoli, Bologna, Italy (ethical clearance no. 10823 issued on April 26, 2010) and by the Ethical Committees of the Italian Institute of Health (ethical clearance prot.no. 14/420 issued on March 7, 2014), respectively. All subjects signed an informed consent before entering the study. Age (>18 years) and ability to provide informed consent were inclusion criteria. Exclusion criteria were the presence of chronic kidney or liver diseases, unstable cardiovascular pathology, bleeding disorders, diabetes, neuromuscular disorders, systemic infections, major psychological problems, malignant neoplasia and/or a current therapy with immune suppressor drugs (like cyclosporine, methotrexate, glucocorticoids, etc.) or anticoagulant drugs, history of alcohol or drug abuse.

TABLE 1 | Experimental sample description.

	Cyclists	Controls	Patients
***n*° Young**	**10** (2F, 8M)	**15** (9F, 6M)	**15** (5F, 10M)
Age range	18–39 yrs	18–39 yrs	24–39 yrs
(average, ±SD)	(30.3, ± 6.0)	(29.1, ± 7.6)	(33.0, ± 4.8)
***n*° Adult**	**32** (4F, 28M)	**21** (5F, 16M)	**20** (8F, 12M)
Age range	40–60 yrs	41–60 yrs	43–60 yrs
(average, ±SD)	(49.8, ± 5.8)	(51.7, ± 6.5)	(51.4, ± 5.6)
***n*° Late adult**	**5** (0F, 5M)	**32** (14F, 18M)	**17** (12F, 5M)
Age range	61–71 yrs	63–71 yrs	61–70 yrs
(average, ±SD)	(64.6, ± 6.0)	(67.7, ± 2.8)	(65.3, ± 2.5)
***n*° Old**	——	**43** (13F, 30M)	**12** (7F, 5M)
Age range		72–82 yrs	73–83 yrs
(average, ±SD)		(75.6, ± 3.1)	(79.6, ± 3.5)

Subjects are divided in four groups (in bold) according their age: Young, Adult, Late adult, Old. Each age group includes subjects with different levels of physical activity: Cyclists, Controls, Patients. The number (n°) of the subjects for each groups are reported in bold.

The Race

Briefly, the race, known as "Nove Colli," is a long-distance cycling road race that takes place in Romagna (Forlì-Cesena and Rimini, Italy). The characteristics of the route were: length, 130 km; total uphill gradient, 1,871 m; uphill riding, 50 km over 4 hills; downhill riding, 46 km; flat terrain, 34 km; maximum riding time allowed, 7.5 h. For further details see Mosconi et al. (27, 28).

Sampling and Data Collection

For patients and controls, blood was drawn in the morning after overnight fasting. All samples were processed immediately to collect plasma. For cyclists, the collection of venous blood (30 mL) samples was done at three different times: time 1 (T1), the day before the race, time 2 (T2), immediately after crossing the finish line, and time 3 (T3), 18–24 h after competing.

Plasma was obtained within 4 h from venipuncture by centrifugation at 2,000 g for 20 min at 4°C, rapidly frozen and stored at −80°C.

Blood cells and creatinine were measured by standard biochemical assays. White blood cell counts for cyclists (both T1 and T2) and patients divided for age group are

presented in **Supplementary Table 1**. For cyclists, both at T1 and T2, three markers of cellular inflammation were calculated (**Supplementary Table 2**) as described below. The neutrophil-lymphocyte ratio (NLR) was calculated on the basis of absolute neutrophil (N; $\times 10^3/microL$) and lymphocyte (L; $\times 10^3/microL$) blood counts, using the formula: NLR = N/L. The platelet-lymphocyte ratio (PLR) was calculated on the basis of peripheral platelet (P; $\times 10^3/microLiter$) and lymphocyte (L; $\times 10^3/microL$) blood counts, using the formula: PLR = P/L. The systemic immune-inflammation index (SII) was calculated on the basis of peripheral platelet (P), neutrophil (N), and lymphocyte (L) blood counts, using the following formula: SII = P * N/L. All the inflammatory markers are ratios thus do not have a unit (29, 30).

Estimated glomerular filtration rate (eGFR) was calculated according to CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation based on serum creatinine, age, sex and ethnicity. (31). Body mass index (BMI) was calculated as weight in kilograms divided by the square of the height in meters (kg/m2). For patients, maximal quadriceps torque and *vastus lateralis* thickness were measured by using a Handifor® dynamometer and portable ultrasound (Mylab25, Esaote), respectively, as reported in (32).

GDF15 concentration was determined in plasma samples by ELISA assay using commercial kits, R&D (DGD150), according to the manufacturer's instructions. All the samples were measured in duplicate and the analyses were performed in a blind setup.

Statistical Analysis

The data were analyzed with non-parametric tests since they did not follow a normal distribution. In particular, the comparisons among cyclists, controls and patients in the different age groups

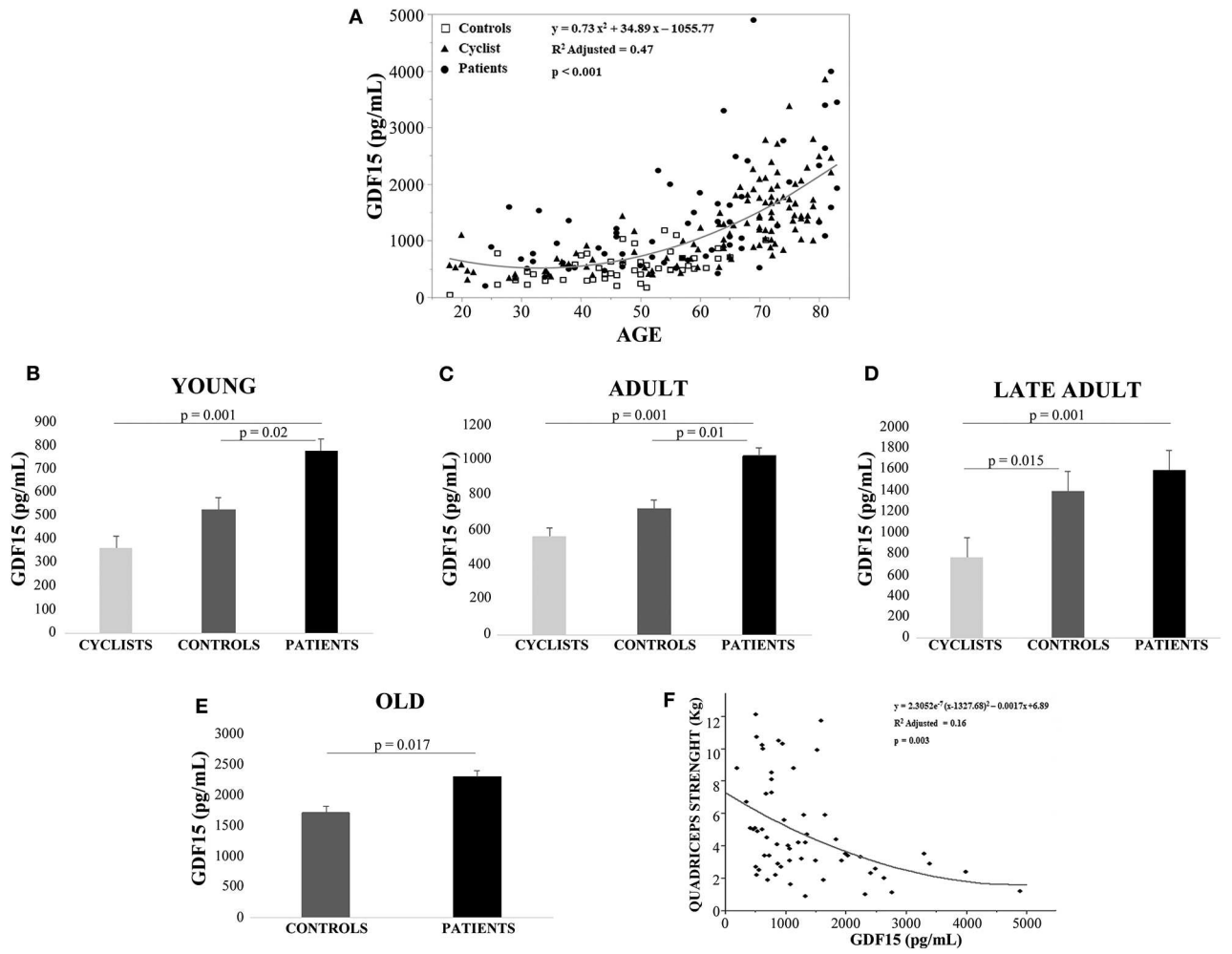

FIGURE 1 | Plasma levels of GDF15 increase with age and are negatively associated with active lifestyle. **(A)** Regression analysis of circulating GDF15 and age in cyclists, controls and patients. **(B–D)** Circulating plasma levels of GDF15 in young cyclists (T1), controls and patients **(B)**; in adult cyclists (T1), controls and patients **(C)**; in late adult cyclists (T1), controls and patients **(D)**. Data are expressed as mean ± SE and *p*-values refer to Kruskal-Wallis test. **(E)** Circulating plasma levels of GDF15 in old controls and patients, data are expressed as mean ± SE and *p*-values refer to Mann-Whitney test. **(F)** Regression analysis of circulating GDF15 vs. quadriceps maximal torque normalized for age and *vastus lateralis* muscle thickness in patients.

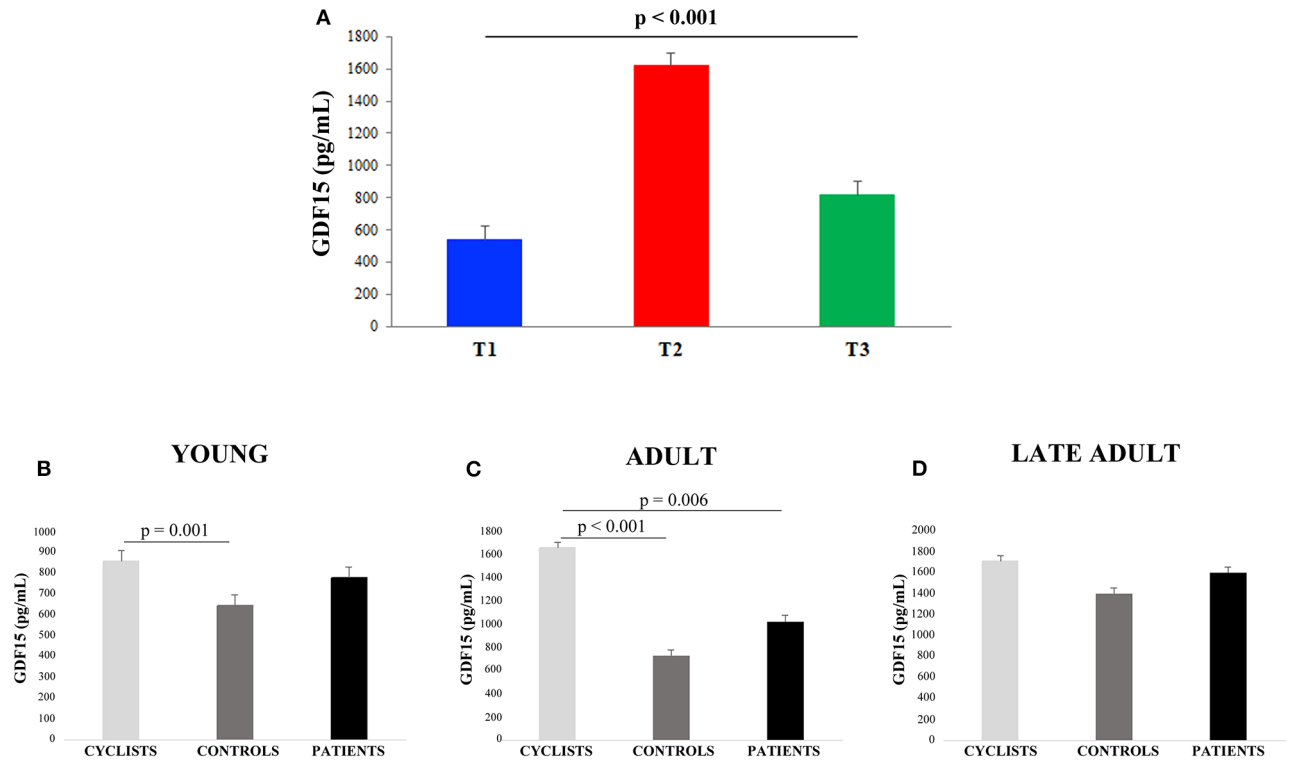

FIGURE 2 | GDF15 plasma levels in cyclists before and after the race. **(A)** The differences among plasma GDF15 levels before the race (T1), immediately after the race (T2), and after 18–24 h from the race (T3) were analyzed. Data are expressed as mean ± SE *p*-values refer to Friedman test. **(B–D)** Circulating plasma levels of GDF15 in young cyclists (T2), controls and patients **(B)**; in adult cyclists (T2), controls and patients **(C)**; in late adult cyclists (T2), controls and patients **(D)**. Data are expressed as mean ± SE and *p*-values refer to Kruskal-Wallis test.

(young, adult, late adult) were performed by using Kruskal-Wallis test with a Steel *post-hoc* test, while the comparison between patients and controls in old group was performed by Mann-Withney test. To compare the GDF15 levels in cyclists at different times of the race (T1, T2, T3) we performed a Friedman test. The relationship among GDF15 levels and age, white blood cells, hematological markers of cellular inflammation (NLR, PLR, SII), creatinine, eGFR, and quadriceps torque were calculated by Spearman rank correlation test and regression analysis. Significance was accepted as $p < 0.05$. Data are expressed as mean ± SE. All data were analyzed using the SPSS 25.0 for Windows software (SPSS Inc.; Chicago, IL, USA).

RESULTS

Plasma Levels of GDF15 Increase With Age and Are Negatively Associated With Active Lifestyle

Linear regression analysis showed that GDF15 plasma levels were significantly associated with age for all the 228 subjects (**Figure 1A**). Spearman rank correlation coefficient and *p*-value are: $\rho = 0.741$, $p < 0.0001$. This age-related increase of plasma GDF15 was evident irrespective to the level of physical activity of the subjects, confirming previous data on GDF15 and age (10). However, when the subjects were subdivided on the basis of their age and level of physical activity (as described in Materials and Methods Section), the plasma levels of GDF15 were significantly higher in inactive patients and significantly lower in active people such as cyclists with respect to control subjects, in young, adult and late adult people (**Figures 1B–D**). For ages over 72 years, only patients and controls were available but also in this case the same trend was observed (**Figure 1E**). The relationship between physical activity and GDF15 was also analyzed considering BMI as a covariate, and the results remained the same (data not shown). Therefore, it seems that the level of physical activity determines the plasma levels of GDF15 at any age. For patients, the values of maximal quadriceps torque normalized on *vastus lateralis* muscle thickness were available, and these values, considering age as a covariate, resulted inversely correlated to GDF15 plasma levels; Spearman rank correlation coefficient and *p*-value are: $\rho = -0.449$ and $p < 0.0001$ (**Figure 1F**). Similar results were obtained when quadriceps torque was normalized on total BMI (data not shown). However, as already reported, a strenuous physical exercise (in our case, the long-distance cycling race) is able to cause a dramatic increase of plasma GDF15, as evidenced by the difference between T2 (immediately after the race) and T1 (before the race) (**Figure 2A**). After 18–24 h from the race (T3), the levels of GDF15 tend to return at baseline level, even if these levels remain significantly higher than T1 (**Figure 2A**). We also compared the levels of GDF15 at T2 with those observed in controls and patients of similar

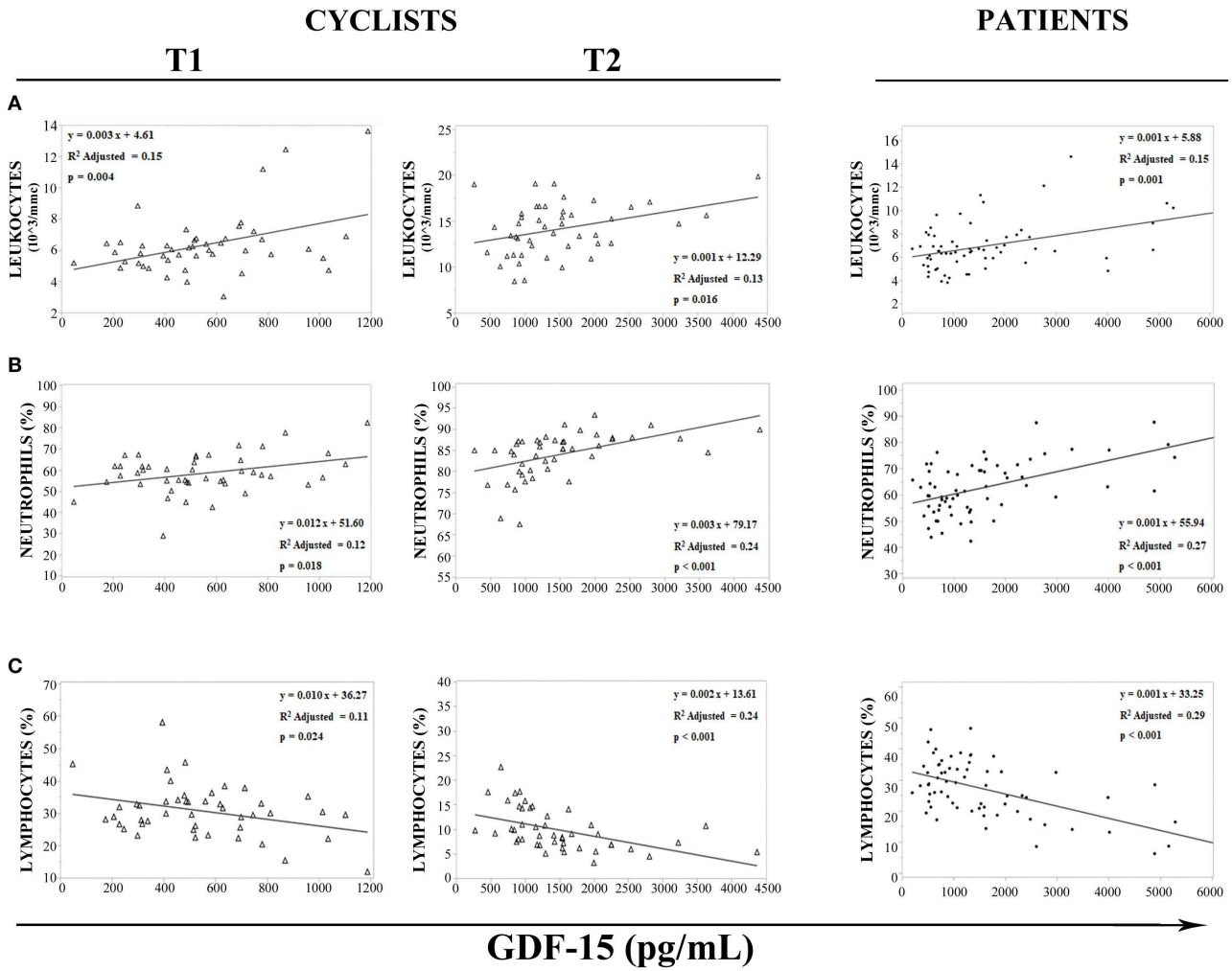

FIGURE 3 | Regression analysis of GDF15 plasma levels with hematological parameters. **(A–C)** Regression analysis of circulating GDF15 levels and leukocytes **(A)**, neutrophils **(B)**, lymphocytes **(C)**, in cyclists before the race (T1), immediately after the race (T2), and in patients.

age. In the group of young subjects GDF15 levels resulted significantly higher with respect to controls and similar to patients (**Figure 2B**), while in the adult group the GDF15 levels resulted significantly higher with respect to controls and patients (**Figure 2C**). In the group of late adults, no difference was present (**Figure 2D**).

Relationship Among Plasma Levels of GDF15 With Hematological Parameters Related to the Inflammatory Response and Renal Function

It is known that GDF15 is responsive to mitochondrial stress and inflammation, two conditions that apply under strenuous physical exercise (33). We then sought for associations with hematological parameters associated to an inflammatory response. In cyclists, before (T1) and immediately after (T2) the race, regression analysis has shown an association between white blood cells and GDF15 plasma levels (**Figures 3A–C**). Moreover, GDF15 plasma levels significantly and positively correlated with the number of total leukocytes (T1: $\rho = 0.311$ and $p = 0.036$, T2: $\rho = 0.343$ and $p = 0.019$) (**Figure 3A**) and neutrophils (T1: $\rho = 0.346$ and $p = 0.018$, T2: $\rho = 0.621$ and $p < 0.0001$) (**Figure 3B**), and negatively with lymphocytes (T1: $\rho = -0.332$ and $p = 0.024$, T2: $\rho = -0.613$) (**Figure 3C**). Interestingly, the same associations were found for patients (**Figures 3A–C**) and in this case the Spearman rank correlation coefficients were similar to those observed for cyclists' T2 (leukocytes: $\rho = 0.388$ and $p = 0.001$; neutrophils: $\rho = 0.519$ and $p < 0.0001$; lymphocytes: $\rho = -0.536$ and $p < 0.0001$). Therefore, it seems that a strenuous physical exercise produces a transient state of stress that, as far as GDF15 and inflammatory parameters, is similar to that present in patients.

Recently, several studies have proposed NLR, PLR and SII as white blood-cell-based inflammatory markers. The levels of these inflammatory indices are in fact elevated in individuals with chronic inflammation and several age-related pathologies (29, 30). To evaluate whether strenuous exercise influences

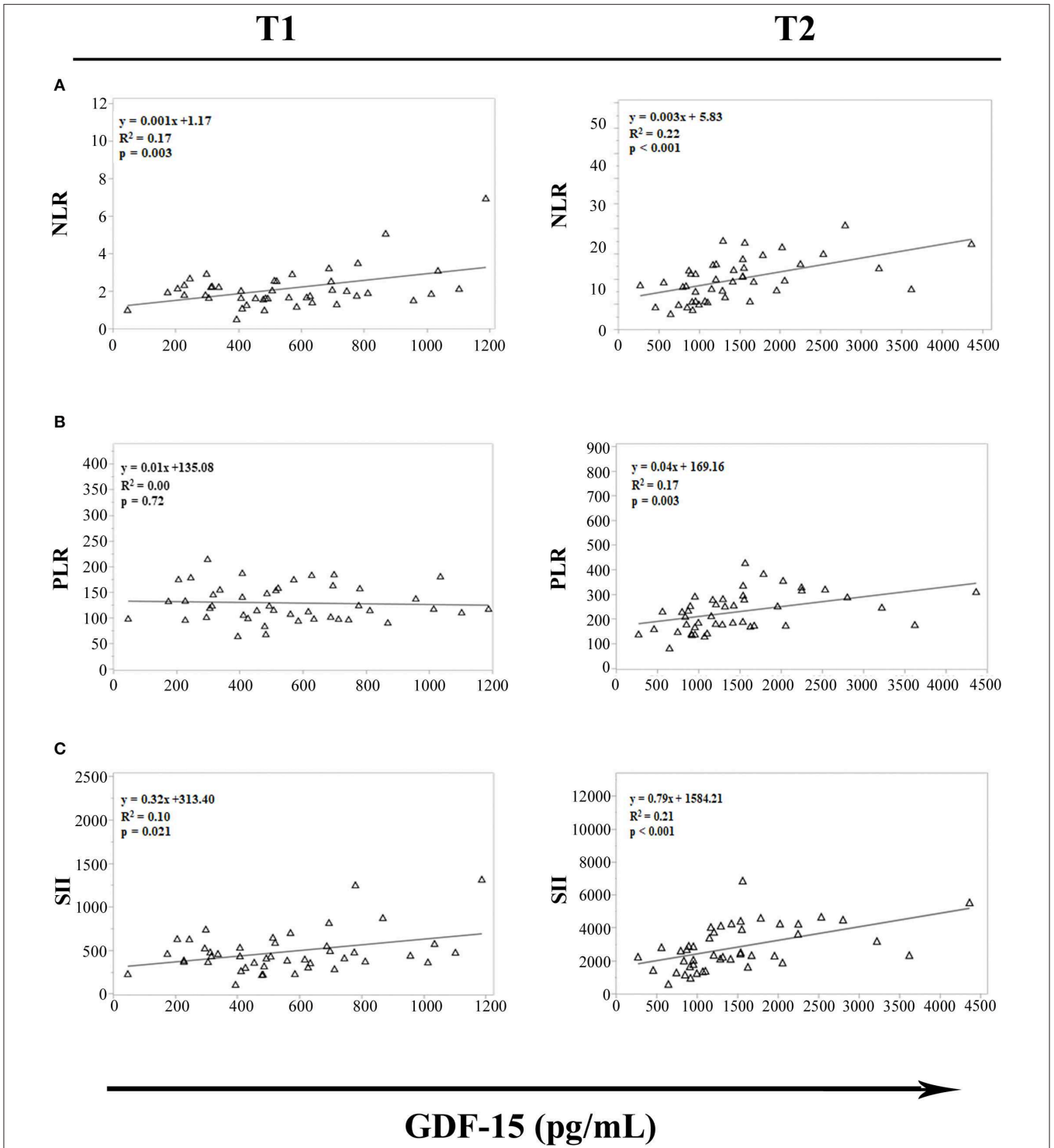

FIGURE 4 | Regression analysis of GDF15 plasma levels with hematological markers of cellular inflammation in cyclists before (T1) and immediately after (T2) the race. **(A)** Regression analysis of circulating GDF15 levels and neutrophil/lymphocyte ratio (NLR); **(B)** regression analysis of circulating GDF15 levels and platelet/lymphocyte ratio (PLR); **(C)** regression analysis of circulating GDF15 levels and systemic immune-inflammation index (SII).

the relationship between GDF15 plasma levels and these inflammatory markers, a regression analysis in cyclists at T1 and T2 was performed. We observed significant association between GDF15 and NLR, and GDF15 and SII, both at T1 and T2 (**Figures 4A,C**); while the association between GDF15 and PLR is present only at T2 (**Figure 4B**). Interestingly, these associations

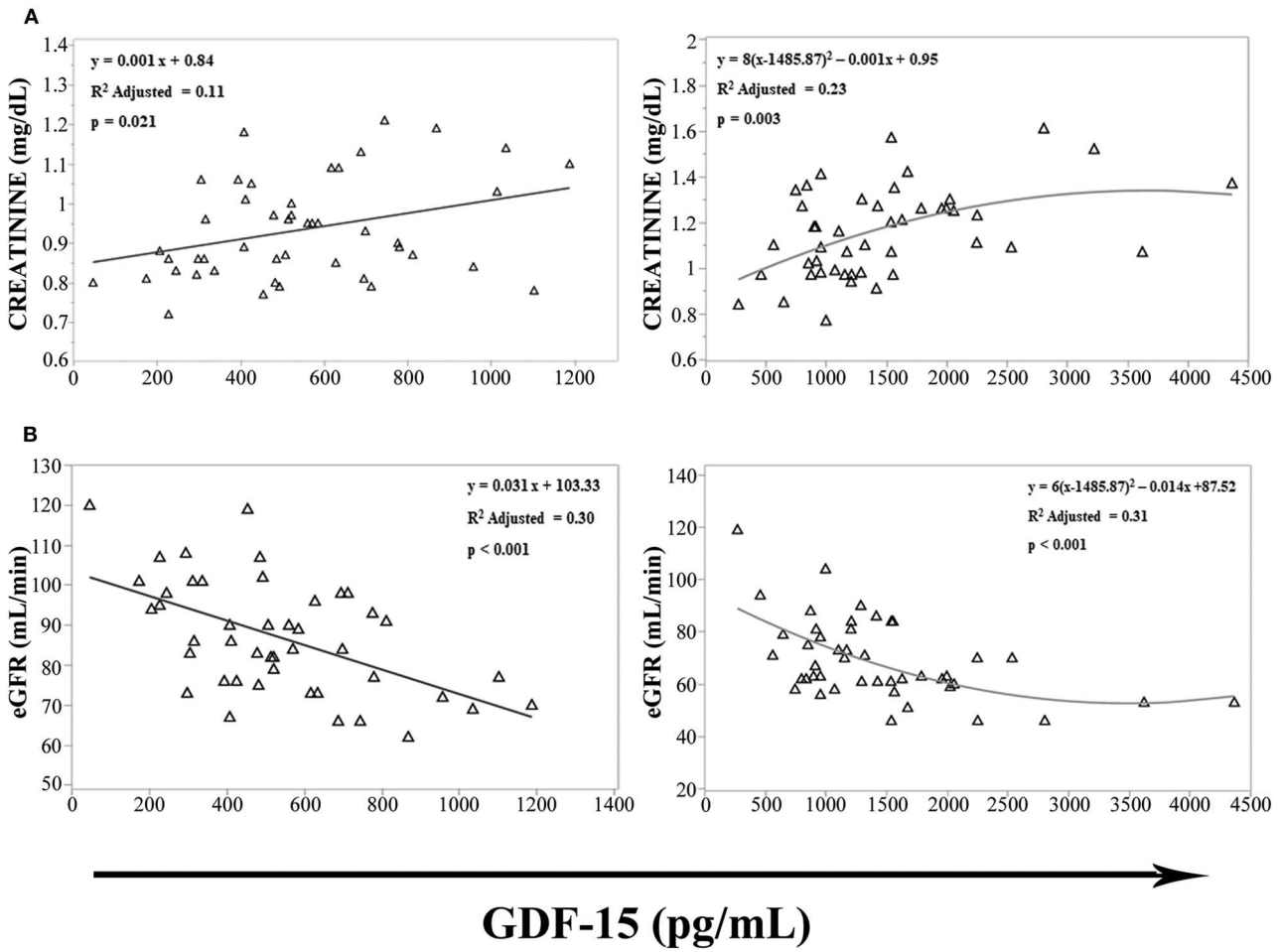

FIGURE 5 | Regression analysis of GDF15 plasma levels with estimated glomerular filtration rate (eGFR) and creatinine. **(A,B)** Regression analysis of circulating GDF15 and eGFR **(A)**, creatinine **(B)** in cyclists before the race (T1), immediately after the race (T2).

are stronger at T2, as also confirmed by Spearman's correlation analysis. In this case in fact, only at T2 the levels of GDF15 significantly correlate with these markers (NLR: $\rho = 0.616$ and $p < 0.0001$; PLR: $\rho = 0.567$ and $p < 0.0001$; SII: $\rho = 0.580$ and $p < 0.0001$).

It has been recently reported that upon metformin treatment, GDF15 is increased in distal intestine and kidney (34), and an intense sport exercise induces acute renal stress (35), with an increase in creatinine levels and a decline of estimated glomerular filtration rate (eGFR), as indices of renal function (36). We then evaluated in cyclists at T1 and T2 the possible relationship among GDF15 levels, creatinine, and eGFR. We observed that GDF15 levels were associated with creatinine (**Figure 5A**) and eGFR (**Figure 5B**). Furthermore, according to the Spearman's correlation analysis, GDF15 plasma levels, both at T1 and T2, positively correlated with creatinine (T1: $\rho = 0.316$ and $p = 0.032$, T2: $\rho = 0.422$ and $p = 0.003$), and negatively with eGFR (T1: $\rho = -0.506$ and $p < 0.0001$, T2: $\rho = -0.486$ and $p = 0.001$). These results suggest that the elevation of GDF15 observed during a strenuous physical exercise is associated with renal stress.

DISCUSSION

GDF15 is a cytokine that was considered expressed only by a limited number of tissues, such as liver, lung and kidney, as well as by the placental trophoblast (37). More recently it has been demonstrated that GDF15 is responsive to mitochondrial stresses (38), and, according to the theory that aging is associated with increased mitochondrial dysfunction, elevated circulating levels of GDF15 are found in elderly people and centenarians (9–11). As it is known that strenuous exercise induces GDF15 expression (20–22), it has been hypothesized that also skeletal muscle can be a source of this cytokine. Accordingly, GDF15 is

expressed in muscles from mouse models of aging and inactivity (12, 14). However, this idea has been recently challenged, as the concentrations of GDF15 during a physical exercise resulted similar in arterial and venous blood across the exercised leg (22). Our results are in favor of the idea that skeletal muscle is not the primary source of GDF15, as its basal levels are lower in actively exercising people like cyclists with respect to age-matched controls. However, this does not exclude at all that skeletal muscles can produce a little amount of GDF15. Moreover, after a bout of strenuous physical activity, the levels of GDF15 are correlated with markers of kidney injury, suggesting that, according to literature data (34), the elevation of GDF15 may be a response to an injury to other organs, including the kidney.

Whether GDF15 is beneficial or detrimental for skeletal muscle is still debated. It has been previously reported that GDF15 causes anorexia/cachexia via its impact on energy metabolism (18) and, accordingly, it is found inversely associated with muscle mass (39). Moreover, even though its receptor, GDNF family receptor α-like (GFRAL), has not been found expressed in muscle (40) GDF15 is able to induce muscle fiber apoptosis via phosphorylation of STAT3 (13). On the other side, as mentioned, the GDF15 knockout determines the elevation of markers of muscle stress (Atf3, Atf6, and Xbp1s) upon exercise (14). Our data suggest that GDF15 is inversely associated with muscle health, as it is elevated in patients with lower limb mobility impairment and inversely associated with their quadriceps maximal torque. It is at present unclear whether this association is causal or not. In order to reconcile our data with those demonstrating a beneficial role for GDF15, we can hypothesize that transient peaks of GDF15 are stimulatory/homeostatic, whereas long-lasting elevated systemic levels can turn detrimental. This could be the case of patients with lower limb mobility impairment.

Another alternative possibility is that GDF15 acts in synergy or in opposition with other factors. Interestingly, some samples from cyclists used in this study were previously assayed for the expression of inflammatory mediators such as IL-6, TNF-α, and IFN-γ that resulted dramatically increased after the race (33). To this regard, it is worth noting that GDF15 is also responsive to inflammation, mostly *via* p53 (41). It has indeed been shown that GDF15 is a direct target gene of p53. Recently, it has been demonstrated that GDF15 is necessary for tolerance to inflammation induced by viral or bacterial infections (23). It has also a clear anti-inflammatory activity in experimental models of liver injury and myocardial infarction (42, 43). In particular, GDF15 attenuates the LPS-induced production of classical pro-inflammatory cytokines such as TNF-a, IL-1β and IL-6 in Kupffer cells (42), and is able to inhibit the chemokine-activated leukocyte arrest on the myocardial endothelium of infarcted heart (43). Here we show that GDF15 levels are clearly associated with hematological parameters related to inflammation, *i.e.*, increased number of leukocytes (in particular neutrophils) and decreased number of lymphocytes, in both cyclists and patients. Thus, it is possible that the net effect of GDF15 on muscle health depends on the fine interaction with inflammatory mediators.

Given its responsiveness to inflammation and reported anti-inflammatory effects, the elevated levels of GDF15 can be interpreted as an automatic mechanism to blunt the detrimental effects of inflammation (acute like a strenuous bout of physical activity, or chronic like that present in inactive patients). It has been previously reported that a chronic, subclinical inflammation (inflammaging) is a typical feature of old people. Therefore, it is tempting to speculate that GDF15 is elevated in the elderly at least in part as a consequence of inflammaging, and that GDF15 could be added to the list of anti-inflammaging mediators. Elderly people are also characterized by a loss of muscle mass and power (44), and we have reported that GDF15 levels are very high in old people and centenarians (10). Therefore, due to its wasting activity on muscle, it is thus conceivable that the elevated levels of GDF15 in elderly people and particularly in the oldest old can be a *trait-d'union* between inflammaging and the observed loss of muscle mass and power.

Finally, it can be hypothesized that people with less inflammaging have consequently a lower production of GDF15. A corollary of this hypothesis is that the positive effects of GDF15 are likely not enough to overcome the detrimental ones brought by inflammaging. These considerations possibly indicate GDF15 as a target for future pharmacological or life-style interventions to implement healthy aging and longevity, whose goal would be to obtain the beneficial effects of GDF15 avoiding the detrimental ones. Future studies are needed in this perspective.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The study protocols were approved by the Ethical Committee of Istituto Ortopedico Rizzoli, Bologna, Italy (ethical clearance no. 10823 issued on April 26, 2010) and by the Ethical Committees of the Italian Institute of Health (ethical clearance prot.no. 14/420 issued on March 7, 2014), respectively. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MCo: patients' enrollment, data generation and collection, statistical analysis, and writing of the manuscript. MM: analysis of GDF15 and manuscript revision. GM: cyclists' enrollment and manuscript revision. AC: analysis of GDF15. MCa and VT: clinical data collection of cyclists. AS: manuscript revision. CF: critical discussion. SS: analysis of the data and writing of the manuscript. 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.2020.00915/full#supplementary-material

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Obesity Accelerates Age Defects in Mouse and Human B Cells

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Obesity, similar to aging, is associated with chronic low-grade systemic inflammation, known as inflammaging, and represents a significantly higher risk for developing chronic diseases typical of old age. Immune cells are recruited to the obese adipose tissue (AT) by chemotactic molecules secreted by non-immune and immune cells in the AT, both contributing to the release of several pro-inflammatory mediators that fuel local and systemic inflammation, to the refractory response of immune cells to further *in vivo* and *in vitro* stimulation and to the induction of autoimmune B cells with potentially pathogenic repertoires. In terms of molecular mechanisms involved, leptin, an adipokine secreted primarily by adipocytes, has been proposed to be involved in the reduced generation of protective antibodies, and in the increased generation of autoimmune antibodies, further supporting the concept that obesity accelerates age defects. Leptin has also been shown to induce intrinsic B cell inflammation and B cell immunosenescence. The results presented in this review highlight the importance of weight reduction programs to improve immunity and reduce the risk for developing chronic diseases in obese and older individuals.

Keywords: aging, obesity, B cells, inflammation, antibody responses

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INTRODUCTION

Obesity, defined as body-mass index (BMI) $\geq$ 30 kg/m^2 by CDC and WHO, is an increasing health concern that affects young (1) and older adults (2), and has reached pandemic proportions. Individuals with obesity are at higher risk for developing chronic diseases typical of old age such as cardiovascular disease (3), Type-2 Diabetes Mellitus (T2DM) (4–6), cancer (7), psoriasis (8), atherosclerosis (9), inflammatory bowel disease (10). Obesity-induced metabolic changes cause tissue dysfunction, disruption of the integrity of lymphoid tissues, and decreased leukocyte development and function, all leading to reduced protective immunity. One of the reasons is because obesity, similar to aging, is an inflammatory condition associated with chronic low-grade systemic inflammation, inflammaging (11), which is negatively associated with a functional immune system, healthspan and longevity in both mice and humans (12). All immune cells contribute to the inflammatory status of obese individuals, and increased frequencies of pro-inflammatory macrophages (13, 14), T cells (15, 16), and B cells (17, 18) have been reported. Decreased frequencies of regulatory B cells have also been shown in the blood of individuals with obesity (19).

THE OBESE ADIPOSE TISSUE (AT)

Obesity is characterized by increased mass of the AT. The AT is a storage of nutrients and an active endocrine and immunological tissue. The AT is composed of adipocytes and a mixture of mesenchymal, endothelial and immune cells, known as the Stromal Vascular Fraction (SVF) (17, 20). Under conditions of over-nutrition, the AT changes from a condition of insulin sensitivity (IS) to a condition of insulin resistance (IR) that is occurring in parallel with the expansion of adipocyte mass, remodeling of extracellular matrix components (collagens, elastins and the associated blood vasculature) and increased secretion of pro-inflammatory mediators (cytokines, chemokines, adipokines, leukotrienes), involved in the recruitment of immune cells to the AT. Chronic inflammation in the AT contributes to inflammaging and leads to increased IR in obesity (21). IR also increases with age (22, 23), and is associated with high serum levels of glucose and free fatty acids (FFAs), and markers of metabolic inflammation, metaflammation (24), that fuels inflammaging and promotes aging, diseases and death.

Altered innate and adaptive immune responses occur in the AT under conditions of over-nutrition (17, 20). Mouse (25, 26) and human (27, 28) results have shown that immune cells are recruited to the obese AT by chemotactic molecules secreted by non-immune and immune cells in the AT, generating a positive feedback loop in which a large number of pro-inflammatory mediators are secreted, contributing to local and systemic inflammation. The obese AT of mice (25, 26) and humans (28, 29) also secretes antibodies that have been shown to be pathogenic (25) in mouse studies. These antibodies are IgG2c, a subclass associated with murine autoimmunity (26). These antibodies are specific for AT-derived "self" proteins and nucleic acids, including dsDNA, found increased in the plasma of elderly individuals, suggesting that obesity may drive the secretion of autoimmune antibodies during aging. This may occur even in elderly lean individuals, due to the deposition of fat on internal organs which is known to increase during aging. An age-associated increase in ectopic deposit of triglycerides in several tissues (liver, muscle, heart, pancreas, kidney) (30–34) and blood vessels (35) has indeed been reported and the word "TOFI" (thin-outside-fat-inside) has been coined to identify lean individuals with abnormal abdominal adiposity and inflammaging. Moreover, age-associated changes in abundance, distribution and cellular composition of the AT have been reported and shown to accelerate the onset of age-associated diseases (36, 37). Computational tomography scans have shown that with age subcutaneous AT (SAT) mass decreases, whereas visceral AT (VAT) mass increases (38). SAT and VAT are biologically distinct in secretion of pro-inflammatory mediators, with VAT being more inflammatory. Furthermore, secretion of adipokines by cells in the AT is regulated by nutrients, and these responses are increased with aging (39).

Senescent cells accumulate in the AT of aging mice and humans (40). Senescent cells are characterized by the irreversible arrest of cell proliferation due to different types of stress, and by the secretion of factors that constitute the senescence-associated secretory phenotype (SASP), consisting of soluble pro-inflammatory molecules, soluble receptors, growth factors and extracellular matrix macromolecules (41). The age-dependent accumulation of senescent cells is a favorable environment for the development of inflammatory-based age-associated diseases and for this reason several strategies have been developed to decrease accumulation of senescent cells in tissues and suppress the SASP with the aim to delay the onset of age-associated diseases (42, 43).

EFFECTS OF OBESITY ON MOUSE B CELLS

Obesity, similar to aging, impairs several aspects of B cell biology. In mice fed a high-fat diet (HFD), early B cell development is characterized by decreased frequencies of B cell subsets in the bone marrow (BM) and reduced expression of early lymphoid commitment markers such as the B cell transcription factor PAX5 (44). Mechanistic experiments using co-cultures of BM cells with the OP9 stromal cell line have shown that BM adipocytes secrete soluble factors that drive the development of myeloid-derived suppressor cells (MDSCs) (45, 46). MDSC inhibition of B lymphopoiesis is mediated by MDSC-derived IL-1β and the inflammatory molecule complex called calprotectin, suggesting that these may be therapeutic targets for the restoration of B lymphoiesis in obesity and aging.

Splenic B cell function is also affected by HFD. Initial studies have indicated that mice fed HFD secrete more pro-inflammatory cytokines (IL-6/TNF-α) than B cells from mice fed normal-fat diet (NFD), thus contributing to the higher levels of systemic inflammation observed in mice fed HFD (47) and in aged mice (48). B cells from HFD mice, in turn, induce changes in the AT and promote adipocyte hyperthropy, hyperglycemia and IR and induce T cell and macrophage inflammation (25). Mice lacking B cells (μMT mice) (49) have reduced IR and glucose intolerance.

Splenic B cells from obese mice have been shown to be pathogenic, as demonstrated by adoptive transfer experiments in which B cells from HFD mice, transferred into B^{null} mice, induce IR and glucose intolerance only if recipients are on HFD, suggesting that the development and/or maintenance of pathogenic B cells requires exposure to HFD (25). B cells from HFD mice influence the function of T cells and macrophages and induce secretion of IFN-γ and TNF-α, respectively, two crucial cytokines involved in the establishment of IR. IgG antibodies isolated from the serum of HFD, but not NFD, mice are mediators of IR and glucose intolerance and induce FcγR-mediated activation of macrophages and consequent TNF-α secretion. B cell depletion using anti-CD20 antibodies decreases obesity-induced glucose abnormalities and ameliorates metabolic disease. All these results were among the first to show the fundamental role of B cells in the pathogenesis of obesity-associated IR.

While the spontaneous secretion of pathogenic IgG antibodies increases in the spleen of HFD mice (25), as well as in the spleen of aged mice (26), the secretion of protective IgG antibodies decreases (44, 50). It has been shown that even mice fed a

Western Diet (that provides a moderate but lower quantity of fat than the HFD), showed significantly lower influenza-specific titers as compared to NFD mice after infection with the influenza virus A/Puerto Rico/8/34 (44). In the same study, it was shown that mice fed HFD together with DHA (docosahexaenoic acid), an essential FA with immunostimulatory function, whose serum levels are low in obesity, had improved influenza-specific antibody responses, suggesting that DHA may be used as a therapeutic strategy to increase humoral immunity.

Also, mucosal B cells from HFD mice regulate obesity-induced IR (51). IgA secreting B cells, as well as secreted IgA antibodies, are significantly reduced in the colon of HFD vs. NFD mice, similar to what has been observed in the colon of aged mice (52). IgA deficiency, specifically in intestinal B cells, deteriorates glucose homeostasis in HFD but not NFD mice, further confirming that the negative regulation of glucose metabolism needs exposure to HFD. IgA antibodies control host-microbiome homeostasis and provide a barrier for microbial and/or ingested antigens that may translocate from the gut into the blood, inducing inflammatory responses. IgA antibodies also regulate lipid absorption from the gut.

The characterization of potentially pathogenic B cell repertoires, performed using high-throughput Ig sequencing from several tissues of mice fed HFD and NFD, has shown that HFD significantly changes the biochemical properties of Ig heavy-chain complementarity-determining region-3 (CDRH3) sequences, with IgA antibodies being characterized by shorter and highly hydrophobic CDRH3 (53). HFD is also associated with higher frequencies of unmutated IgA. These changes occur in B cells from the gut and the AT, suggesting the possibility of a gastrointestinal-AT immune axis shaped by HFD. Surprisingly, similar gene rearrangements were found in B cells from the gut, AT and peritoneal cavity of several individual mice, suggesting that affinity maturation may have occurred in these tissues in a similar antigen-specific way.

B cells infiltrate the AT under obesity conditions (25, 26, 54), recruited by several chemotactic signals including those generated by the interaction of the leukotriene B4 with its receptor. Inhibition of this interaction has been shown to reduce B cell recruitment and activation and to mitigate the contribution of B cells to local inflammation and IR (55). AT-associated B cells are highly inflammatory and secrete several pro-inflammatory mediators (cytokines, chemokines, adipokines). It has recently been shown that aging further increases the expansion of these AT resident B cells, through the activation of the NLRP3 inflammasome, a major regulator of inflammaging and age-associated metabolic disorders, likely due to AT-associated metabolic and mitochondria dysfunction and increased production of mitochondrial reactive oxygen species (54). Our studies on mice fed HFD have confirmed the above findings and have shown that the increased size of the AT, increased infiltration of immune cells and increased secretion of pro-inflammatory mediators induce a powerful feed-forward loop of inflammation, both locally and systemically, that are responsible for the refractory response of immune cells to further *in vivo* and *in vitro* stimulation. In particular, we have shown that the AT directly impairs B cell function by changing the composition of the B cell pool and inducing higher frequencies of pro-inflammatory B cells (26), and similar results have been observed in old mice (56).

EFFECTS OF OBESITY ON HUMAN B CELLS

Studies on B cell development in the human BM have shown that soluble factors secreted by the adipocytes inhibit early stages of B lymphopoiesis, with the inhibition occurring at the common lymphoid progenitor to pre/pro-B cell stage (57), suggesting that the age-related decline in B lymphopoiesis is due at least in part to an increase in BM adipocytes, and an increase in adipocyte-derived factors (IL-1β) that directly inhibit B lymphopoiesis.

Obesity decreases B cell function in humans as well, and it is associated with impaired B cell responses to infections and vaccines (58–60). Our results in humans have demonstrated that obesity-associated defects in class switch recombination (CSR) and somatic hypermutation (SHM), two processes necessary for the generation of class switched high affinity secondary antibodies (61), are due to reduced expression of activation-induced cytidine deaminase (AID), the enzyme of CSR and SHM, and E47, encoded by the E2A gene, a key transcription factor regulating AID (62). Both AID and E47 are decreased in B cells isolated from the blood of obese young and elderly individuals as compared to lean controls. Importantly, the response of elderly lean individuals was not different from that of young obese individuals, supporting the hypothesis that obesity accelerates age defects in B cells. At least one mechanisms involved in the decrease of AID/E47 in B cells from obese vs. lean individuals was the decreased expression of phosphorylated-AMPK (59), upstream of phosphorylated-p38 MAPK, crucial for E47 activation, as previously shown in murine B cells (63). Another mechanism was associated with the increased expression of the inflammatory micro-RNA (miR)-155 and miR-16 in unstimulated B cells from obese vs. lean individuals, with miR-155 binding the 3'-untranslated region (3'-UTR) of AID mRNA and miR-16 binding the 3'-UTR of E47 mRNA, inducing their degradation (59). These results recapitulate what we have initially shown in our studies on the effects of aging on B cell function in which both AID and E47 were found decreased in mitogen-stimulated B cells from elderly as compared to young individuals (64).

Leptin has been proposed to be at least one molecular mechanism involved in dysfunctional B cell function in individuals with obesity. Leptin is an adipokine secreted primarily by the adipocytes (65) with endocrine and immune functions, whose serum concentration correlates with the amount of body fat and BMI (66). Leptin increases the secretion of pro-inflammatory cytokines by immune cells, and *ob/ob* mice that are leptin-deficient have reduced secretion of Th1 cytokines and increased secretion of Th2 cytokines (67).

Leptin levels in the serum of young obese individuals are comparable to those in the serum of elderly lean individuals (68), and we have recently demonstrated that incubation of B cells from young lean individuals with leptin decreases class switch and influenza vaccine-specific IgG antibodies, similar

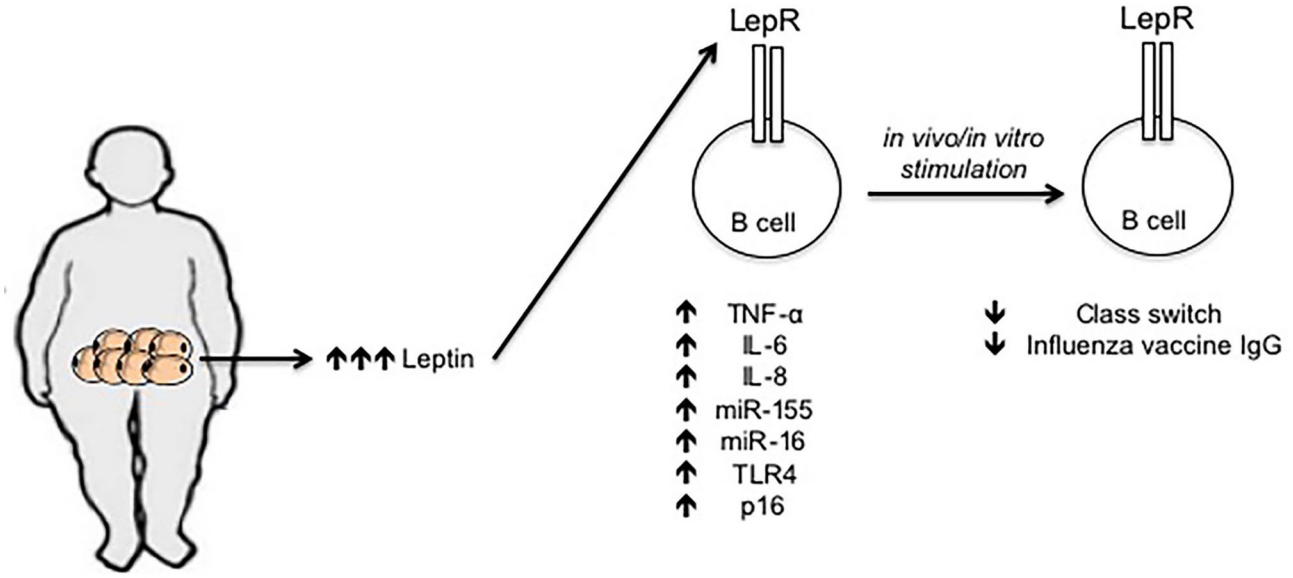

FIGURE 1 | Effects of leptin on human B cell function. Leptin is secreted by adipocytes in the AT. Leptin concentration increases locally and systemically under obesity conditions. Leptin interacts with its receptor on the surface of B cells and increases mRNA expression of several markers of inflammation and cellular senescence, such as TNF-α, IL-6, IL-8, miR-155, miR-16, TLR4, and p16. The expression of these mediators in unstimulated B cells is negatively associated with the response of the same B cells after *in vivo/in vitro* stimulation, e.g., with the influenza vaccine.

to the levels observed in B cells from young obese and from elderly lean individuals, further supporting the concept that obesity accelerates age defects. Leptin also increases the frequencies of pro-inflammatory B cells and induces intrinsic B cell inflammation, measured by mRNA expression of several pro-inflammatory markers associated with immunosenescence, the expression of which before stimulation negatively correlates with the response of the same B cells after stimulation (68). Previously published data have also shown that leptin activates human peripheral blood B cells to secrete the pro-inflammatory cytokines TNF-α and IL-6 (69, 70). **Figure 1** summarizes our recently published results on the effects of leptin on B cell function.

Obesity increases blood frequencies of the subset of B cells called late memory, tissue-like or double negative (DN) B cells (CD19+CD27-IgD-), that represents the most inflammatory B cell subset, also increased in the blood of elderly individuals (71, 72) and of autoimmune patients (73–75). DN B cells do not proliferate and do no make antibodies to "new" antigens, but they secrete antibodies specific for autoantigens known to increase with age and autoimmune diseases, such as the "self" antigens dsDNA and Malondealdehyde, a product of lipid peroxidation and a marker of oxidative stress (76). DN B cells that secrete anti-"self" antibodies are characterized by the membrane phenotype CD95+CD21-CD11c+, and by the spontaneous expression of the transcription factor T-bet (29, 72), two features of human B cells present not only in patients with autoimmune diseases but also in individuals with chronic inflammatory conditions, including aging and obesity (77, 78).

Previously published results have indicated that the plasma of obese individuals with IR contains autoantibodies specific for intracellular proteins, ubiquitously expressed in tissues including pancreas, nervous tissues, muscle, AT, as well as in immune cells (25), suggesting the release of "self" antigens under obesity conditions in insulin target tissues. More recently, we have shown that the human obese AT contributes to increased secretion of adipocyte-specific IgG antibodies and this occurs without any stimulation, likely because the ongoing process of cell death in the obese AT leads to the release of "self" antigens, that are almost exclusively intracellular or cell-associated, able to chronically stimulate B cells (28). Adipocyte-specific IgGs secreted in the obese AT are significantly correlated with those present in the plasma (79).

DN B cells are the cells secreting anti- "self" antibodies in the human obese AT. DN B cells are significantly increased in frequencies in the SVF of the human obese AT. Autoimmune antibody secretion occurs after a metabolic adaption that allows DN B cells to activate oxidative phosphorylation, aerobic glycolysis and fatty acid oxidation, as well as pathways that mitigate stress and cell death, leading to a better survival and function in the hostile pro-inflammatory environment of the obese AT (29). Under these conditions, metabolic reprogramming represents a significant advantage, allowing cells to adapt and survive even when they encounter metabolically restrictive conditions, such as hypoxia, nutrient deprivation and exposure to inflammatory stimuli, as it happens during obesity and aging.

CONCLUSIONS

The mechanisms for the down-regulation of mouse and human B cell responses by obesity and aging are in large part overlapping. Obesity accelerates inflammaging and induces metabolic, physiological, and functional changes in immune cells that lead to defective humoral immunity. The results in this review highlight the importance to prevent obesity as a way to improve immunity and reduce the risk for developing chronic diseases typical of old age.

AUTHOR CONTRIBUTIONS

DF wrote the mini review. DF and BB were involved in funding acquisition, reviewing, and editing the manuscript.

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Hip Fracture Leads to Transitory Immune Imprint in Older Patients

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Background: Hip fracture (HF) is common in the geriatric population and is associated with a poor vital and functional prognosis which could be impacted by immunological changes. The objective here is to decipher immune changes occurring in the 1st days following HF and determine how phenotype, function, and regulation of innate and adaptive compartments adapt during acute stress event.

Methods: We included HF patients, aged over 75 years. For each patient, blood samples were taken at five different timepoints: four in the perioperative period (day 0 to hospital discharge) and one at long term (6–12 months). Phenotypical and functional analysis were performed longitudinally on fresh blood or cryopreserved PBMCs. Clinical data were prospectively collected.

Results: One-hundred HF patients and 60 age-matched controls were included. Innate compartment exhibits pro-inflammatory phenotypes (hyperleukocytosis, increase of CD14+ CD16+ proportion and CCR2 expression), maintaining its ability to produce pro-inflammatory cytokines. Adaptive compartment extends toward a transitory immunosuppressive profile (leucopenia) associated with an active T-cell proliferation. Furthermore, increases of LAG-3 and PD-1 and a decrease of 2-B4 expression are observed on T-cells, reinforcing their transitory suppressive status. Of note, these immune changes are transitory and sequential but may participate to a regulation loop necessary for homeostatic immune control at long term.

Conclusion: HF is associated with several transitory immunological changes including pro-inflammatory phenotype in innate compartment and immunosuppressive profile in adaptive compartment. A comprehensive assessment of immune mechanisms implicated in the patient's prognosis after HF could pave the way to develop new immune therapeutics strategies.

Keywords: acute stress, immune response, aging, inflammation, regulation loop

INTRODUCTION

Worldwide, 1.6 million of patients suffer of hip fracture (HF) each year, notably in the aged population (1). This frequent pathology is associated with a poor prognosis with high mortality rate (20–30% of one-year mortality) and a decrease of functional autonomy (2–4). Main factors associated with death are not directly due to the HF and/or its treatment but are represented by comorbidities decompensations (cardio-vascular events) and secondary infections (5). The fall, and consequently the HF generates an important acute stress that impacts organism and could induce immunological changes in this context (6, 7).

Immunosenescence, defined as the impact of age on the immune system, is characterized by phenotypical and functional changes that affect innate and adaptive compartments. Briefly, phagocytosis and chimiotactism of innate cells (monocytes, macrophages, or neutrophils) are decreased. The pool of naïve T cells decreases due to the thymic involution (8) and there is a shrinking of TCR repertoire (9). Furthermore, older patients present an elevated level of pro-inflammatory cytokines coined "Inflam-aging" (10).

Several studies have shown immunological changes after HF. Neutrophils exhibit functional alterations with a defect in phagocytosis ability and superoxide production (6). Conventional monocytes switch toward inflammatory phenotype with an increased production of tumor necrosis factor alpha (TNF-α) (11).

Finally, HF prognosis has been associated with an increase of pro-inflammatory cytokines (IL-6, TNF-α) and few biomarkers have been described (c-reactive protein, procalcitonin) (7, 12). In a previous study, we observed that pre-operative rate of neopterin (a molecule secreted by myeloid lineage under IFN-γ stimulation) was strongly associated with long-term mortality (13). The objective of this longitudinal study is to decipher immune changes occurring in the 1st days following HF and determine how phenotype, function, and regulation of innate and adaptive compartments adapt during acute stress event.

MATERIALS AND METHODS

Patients Cohort

We included hip fracture patients, aged over 75 years admitted between 2013–2015 and 2017–2018 in emergency department of Pitié Salpêtrière hospital. Patients with metastatic fracture, history of cancer, autoimmune disease, and/or immunosuppressive treatment were excluded. For each patient, five blood sample were taken at different timepoints: in pre-operative period (Pre), 24 h after surgery (Post), between day 3 and 5 of hospitalization usually (Hosp), then at patient hospital discharge (Discharge) and finally at long term post-surgery (6–12 months; Long term). Healthy individuals matched for age were included in geriatric department and a unique blood sample was collected. One milliliter of fresh blood was immediately used for cell count and innate phenotyping. PBMCs (isolated by density gradient centrifugation) and plasma were cryopreserved until use.

Clinical data including age, sex, comorbidity scale (Cumulative Illness Rating Scale, CIRS), functional status and frailty scale {Activity of daily living [ADL (14)], Instrumental activity of daily living [IADL (15)], Clinical Frailty Scale (CFS) (16)[1]} at admission and functional and vital status at long term (6–12 months) were prospectively collected.

This study was approved by the ethics committee (CPP Pitié-Salpêtrière, Paris, France). All participants included were informed and gave their consent. The database was recorded to the French National Commission for Computing and Liberty (CNIL, Paris, France).

Flow Cytometry Analysis

Staining on Fresh Cells

The percentages and absolute counts of lymphocyte subpopulations were determined in whole blood using CytoStat tetra-CHROME reagents (panel 1: CD45-FITC/CD56-PE/CD19-ECD and CD3-PC5; panel 2: CD45-FITC/CD4-RD1/CD8-ECD and CD3/PC5; Beckman Coulter, Hialeah, Florida). Sample acquisition with Flow-Count Fluorospheres was performed on a FC500 flow cytometer (Beckman Coulter).

Cell Counts

Innate phenotyping

Directly conjugated antibodies were obtained from the following vendors: BD Biosciences (San Jose, CA): CD3 (FITC), CD14 (BV605), HLADR (PE-CF594), CD16 (APC-H7); R&D systems: CCR2 (APC); eBioscience (San Diego, CA): CX3CR1 (PE), CD62L (PerCP-eF710). Staining for innate cell surface markers was performed with 100 µl of fresh blood incubated 15 min with antibodies. The blood was then lysed with BD FACS lysing, resuspended with PBS 1X (BD Biosciences) and immediately analyzed by flow cytometry.

Staining on Frozen Cells

Immune checkpoint phenotype

Directly conjugated antibodies were obtained from the following vendors: BD Biosciences (San Jose, CA): CCR7 (PC7), CD45-RA (V450), CD4 (HV500), HLA-DR (BV650), CD8 (APC-Cy7); BioLegend (San Diego, CA): PD1 (BV711), LAG3 (FITC), CD244/2B4 (PE). Staining was performed on cryopreserved PBMCs with standard method.

Homeostatic proliferation assay and activation status

Directly conjugated antibodies were obtained from the following vendors: BD Biosciences (San Jose, CA): CD4 (APC-cyanin7), CCR7 (PE-Cy7), CD38 (APC), and Ki67 (FITC); Beckman Coulter: CD45RA (ECD), Caltag (Burlingame, CA): CD8 (Alexa405); Dako (Glostrup, Denmark): CD3 (Cascade Yellow); BioLegend (San Diego, CA): CD27 (AlexaFluor700). Cell surface marker stainings were performed by addition of the respective antibodies for 15 min at room temperature. After incubation, cells were washed in PBS and then permeabilized with Perm/fix kit (eBiosciences) before the addition of Ki67 antibody. Stainings

[1] Available online at: https://www.dal.ca/sites/gmr/our-tools/clinical-frailty-scale.html.

were analyzed on an LSR2 flow cytometer (Becton Dickinson) with appropriate isotype controls and color compensation.

CMV responsiveness

To assess functional capacity of HCMV specific CD8+ or CD4+ T cells, PBMC were stimulated with 15 amino acid long synthetic peptides (5 μM) overlapping by 10 amino acids and spanning the two HCMV proteins, pp65. After 1 h, the secretion inhibitor brefeldin A (5 μg/mL; Sigma-Aldrich) was added and the incubation was continued overnight at 37°C in a 5% CO_2 atm. Cytofix/Cytoperm™ (BD Biosciences) was used to fix/permeabilize the cells prior to staining for intracellular IFN-γ and TNF-α. The limit of detection for cytokine secretion was 0.01% in CD8+ or CD4+ T cell populations. "FunkyCellsBoolean Dataminer" software (www.FunkyCells.com), provided by Dr. Martin Larsen (INSERM U1135, Paris, France), was used to determine the Polyfunctionality Index in response to pp65 stimulation (17).

Monocyte function

PBMCs (1×106/well) were stimulated overnight at 37°C with LPS 10μg/ml (*E. coli* serotype O55:B5; Alexis Biochemicals). After 1 h, 5μg/ml of brefeldin (Sigma-Aldrich) was added. After 12 hours of stimulation, cell surface staining was performed with CD14-BV605, HLA-DR BV650, CD16-APCH7, CD3-AF700 (BD Biosciences San Jose,CA). After washes in PBS, cells were fixed in Cytofix/Cytoperm buffer (BD Biosciences), for 30 min at 4°C, washed in PermWash buffer (BD Biosciences, and then stained for intra-cellular markers: IL8-BV510, TGFβ-PE-CF594, TNFα-APC (BD Biosciences San Jose, CA); IL10-AF488 (R&D systems); IL1β-PE (eBioscience, San Diego,CA); IL6-PECy7 (Biolegend, San Diego, CA) for 30 min at 4°C. After wash, PBMCs were resuspended in PBS, before their flow cytometry acquisition on LSR2 flow cytometer. Data were analyzed using FlowJo v9 (Tree Star, Inc.) and DIVA softwares (BD Biosciences).

Statistical Analysis

Data are expressed as frequencies and percentages for categorical variables and as medians and interquartile ranges (IQR) for continuous variables. Bivariable associations were evaluated with the use of Mann–Whitney *U*-test for continuous variables. All analyses were performed at a two-sided alpha level of 5%. A *P*-value of <0.05 was considered to significant. All analyses were performed with SPSS software, version 20, and Graph Pad Prism, version 5.

RESULTS

Patients Characteristics

One hundred patients with HF and 60 age-matched controls were included in this study. Main clinical characteristics are reported in **Table 1**. Patients with HF were 87 years old [83–92] and 27% were men. As expected, patients exhibit characteristics of geriatric population: they were comorbid (the median CIRS = 9 [7–13], polymedicated for 63% of them, and mildly frailed (a median CFS = 5 [4–6]. At 12-months, 19 of them were deceased. Patients and age-matched controls were comparable in terms of global comorbidities (CIRS) and functional autonomy (ADL and IADL) despite a higher clinical frailty score for HF.

TABLE 1 | Patients characteristics.

Variable	Hip fracture group ($n = 110$)	Control group ($n = 60$)	*p*-value
Age (years)	87 [83–92]	84 [80–89]	0.05
Male sex	30 (27.3%)	17 (28.3%)	0.9
Comorbidities			
CIRS	9 [7–13]	9 [6–12]	0.3
Dementia	46 (41.8%)	35 (58.3%)	0.02
Hypertension	74 (67.3%)	33 (55%)	0.1
Diabetes	16 (14.5%)	7 (11.7%)	0.6
Chronic cardiac failure	21 (19.1%)	5 (8.3%)	0.06
Chronic renal failure	67 (60.9%)	42 (70%)	0.2
COPD	8 (7.3%)	5 (8.3%)	0.8
Polymedication (≥5 drugs)	70 (63.6%)	37 (61.7%)	0.8
Frailty/functional autonomy			
CFS	5 [4–6]	4 [4–5]	0.03
ADL	6 [3.5–6]	6 [5–6]	0.2
IADL	2 [0.75–4]	3 [1–4]	0.3
Ability to walk	103 (93.6%)	59 (98.3%)	0.5
Type of fracture			
Intertrochanteric	55 (50%)		
Femoral neck	55 (50%)		
Number of post-operative complication	2 [1–4]		
Long term outcomes			
6-months mortality	17 (15.5%)		
12-months mortality	19 (17.3%)		
ADL at M6 ($n = 48$)	3 [2–6]		
IADL at M6 ($n = 48$)	1 [0–3.25]		
Ability to walk at M6 ($n = 68$)	55 (80.9%)		

Data are median (25th−75th interquartile), or number (percentage). CIRS, Cumulative Illness Rating Scale; COPD, Chronic Obstructive Pulmonary Disease; CFS, Clinical Frailty Scale; ADL, Activity of Daily Living; IADL, Instrumental Activity of Daily Living.

Immunophenotyping and Function

Innate Compartment

The total neutrophils and monocytes counts transitory increase in the pre-operative period comparatively to controls (Neutrophils 8.9 × 10^6/mm^3 [7–10.9] vs. 3.9 × 10^6/mm^3 [3–4.9], $p < 0.0001$; Monocytes 0.77 × 10^6/mm^3 [0.57–0.94] vs. 0.58 × 10^6/mm^3 [0.43–0.72], $p < 0.0001$) before cell counts normalization and return to baseline at long term

(**Figures 1A,E**), suggesting an immediate mobilization of the innate compartments.

Neutrophils subsets were gated on size and structure as well as the combination of CD16+ and CD62L (**Figure 1B**) in order to differentiate inflammatory neutrophils ($CD16+CD62L^{high}$; **Figure 1C**) from anti-inflammatory neutrophils ($CD16+CD62L^{low}$; **Figure 1D**). Their respective proportions were not significantly different from the control group (**Figures 1C,D**). Gating strategy of monocytes was represented in the **Figure 1F** and relies on the combination of size, structure, HLA-DR, CD14, and CD16 expression. The proportion of intermediary monocytes (CD14+CD16+), known as "inflammatory" monocytes (18), increased during hospitalization before return to baseline at long term (**Figure 1H**). On the contrary, the proportions of conventional and non-conventional monocytes decreased in post-operative period and during hospitalization comparatively to control group and pre-operative period respectively (**Figures 1G,I**). Concerning CCR2 and CX3CR1 chemokines, known to be differentially expressed according to cell subsets, we observed that the expression of CCR2 within conventional monocytes increased significantly after HF to be maximal in the post-operative period (MFI 849 [637–1,211] vs. 442 [248–728], $p = 0.002$) (**Figures 1J,L**) and this trend is the same whatever the monocytes subset (**Supplementary Figures 1A,B**). Inversely, the expression of CX3CR1 transitory decreased after HF to be minimal at the post-operative timepoint (MFI 10400 [3,120–28,896] vs. 31,427 [22,395–37,612], $p = 0.01$; **Figures 1K,M**) with the same trends for the other monocytes subset (**Supplementary Figures 1C,D**). These results suggest an increased turnover of monocyte/macrophage precursors in the bone marrow (decrease of CX3CR1 expression) (19) and an elevated monocyte migration from bone marrow to inflammatory site (increase of CCR2 expression).

Overall, these results show a transitory activation of the innate compartment after HF, followed by a normalization of the different phenomenons leading to a homeostatic return at long term. To evaluate monocytes function after HF, we analyzed their pro-inflammatory cytokines production under LPS stimulation. At each time points, monocytes were able to secrete IL-1β, IL-6, IL-8, and TNF-α without significant difference compared to age-matched controls (**Figure 2**). Therefore, despite the hyper-inflammatory context of acute HF, functionality of monocytes to induce pro-inflammatory signals in response to acute stress is preserved after HF.

Adaptive Compartment

Contrary to the innate cell subsets, we observed a significant and transitory lymphopenia after HF compared to control group (post-operative $0.94 \times 10^6/mm^3$ [0.66–1.37] vs. control $1.59 \times 10^6/mm^3$ [1.13–1.9], $p < 0.0001$; **Figure 3A**). This lymphopenia is significantly observed on T cells (CD3+, both for CD4+ and CD8+ cells; **Figures 3B–D**), on B cells (CD19+) (**Figure 3E**) and on NK cells (CD3–CD56+) (**Figure 3F**). Of note, all subsets seem to be differentially affected: the lymphopenia is more sustained for the CD4+ proportionally to the CD8+ compartment; B cells recover faster during hospitalization whereas NK cells are mobilized at early timepoints post-fracture (**Supplementary Figure 2**).

Altogether, these results suggest a transitory lymphosuppressive profile within adaptive compartment after HF.

To evaluate if this lymphopenic state led to compensatory mechanisms such as lymphopenia-induced proliferation through homeostatic signals, we measured their *ex vivo* proliferation capacity (Ki67 level) on naïve and memory T cell compartments. Whatever the subsets followed, we observed that T-cells isolated from elderly patients suffering from HF were proliferated during their stay to hospital in order to counteract the existing and persistent lymphopenia (**Figures 4A–D**).

This response to homeostatic signals was confirmed by the fact that T-cells were not activated (based on CD38+ expression; **Figures 4E,F**).

Despite the absence of CMV reactivation during this acute clinical event (data not shown), we decided to evaluate *in vitro* the functionality of lymphocytes by analyzing their ability to response to pp65 antigens (which constitute the immunodominant responses described for CMV infection in elderly). Thus, taking into account the proportion of either CD4+, either CD8+ T-cells to secrete IFN-γ and/or TNF-α and/or IL-2, we found that T-cells in HF patients were as polyfunctional as elderly controls (**Figures 4G,H**), suggesting that T-cells were fully functional and able to response to antigenic stimulation if necessary.

Immune Checkpoint Analysis

Previous results suggest an immunosuppressive profile within the adaptive compartment, without defect in functional capacities of T cells. We hypothesized that T-cells regulation, mediated by immune checkpoints, could be defective. The membrane expression of immune checkpoints regulators (PD-1 and LAG-3 and 2-B4) were analyzed on memory CD4+ and CD8+ T cells (**Figure 5**). The expression of immune checkpoints inhibitors significantly increased after HF compared to control. At long term, their expression returned to controls level (**Figures 5A–D**). The maximal expression of LAG-3 was observed in post-operative timepoint within CD4+ (post-operative MFI: 240 [191–352] vs. controls MFI: 150 [133–176], $p < 0.0001$) and CD8+ (post-operative MFI: 360 [272–429] vs. controls MFI: 217 [190–245], $p < 0.0001$) T cells (**Figures 5C,D**). Conversely, the expression of 2-B4 significantly decreased after HF compared to controls, within CD4+ and CD8+ with a minimal expression at post-operative timepoint (post-operative MFI: 81 [62–107] vs. controls MFI: 190 [178–200], $p = 0.0001$ and post-operative MFI: 307 [230–345] vs. controls MFI: 423 [358–448], $p = 0.001$, respectively; **Figures 5E,F**).

These results reinforce the transitory suppressive status in adaptive compartment, occurring early after the HF and the recovery to the homeostatic status at long term.

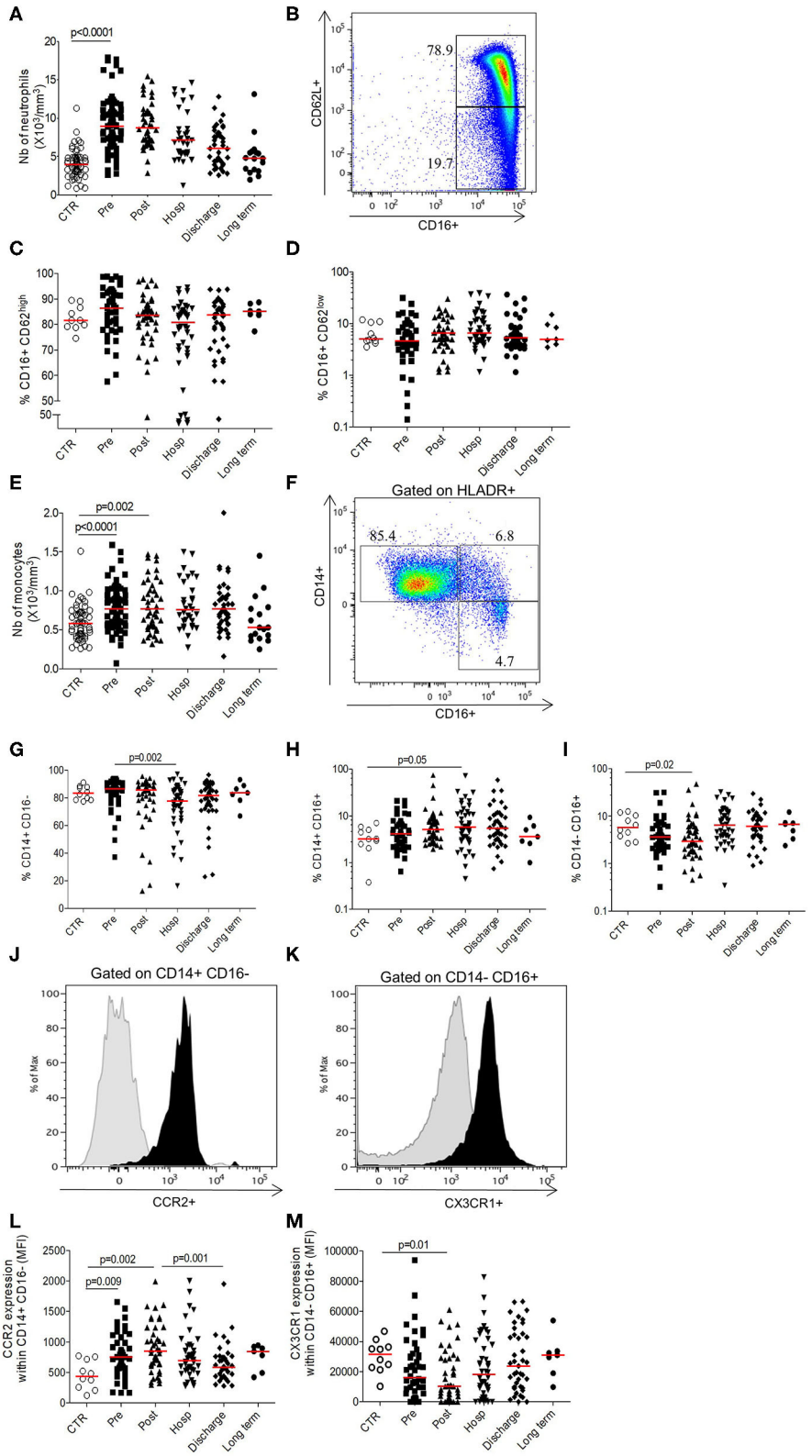

FIGURE 1 | Longitudinal analysis of innate phenotype. **(A)** Number of neutrophils (10^3 cells/mm^3); **(B)** Gating strategy depicting neutrophils staining based on size/structure and CD62L/CD16 expression; **(C)** Percentage of inflammatory neutrophils expressing $CD16^+CD62L^{high}$; **(D)** Percentage of suppressive

(Continued)

FIGURE 1 | neutrophils expressing $CD16^{+}CD62L^{low}$; **(E)** Number of monocytes (10^3cells/mm^3); **(F)** Gating strategy depicting monocytes staining based on size/structure criteria, HLADR, CD14, and CD16 expression; **(G)** Percentage of conventional $CD14^{+}CD16^{-}$ monocytes; **(H)** Percentage of intermediate $CD14^{+}$ $CD16^{+}$ monocytes; **(I)** Percentage of non-conventional $CD14^{-}CD16^{+}$ monocytes; **(J)** Representative histogram of CCR2 expression within conventional monocytes (black; control isotype in gray overlay); **(K)** Representative histogram of CX3CR1 expression within non-conventional monocytes (black; control isotype in gray overlay); **(L)** CCR2 expression within conventional monocytes (expressed in mean fluorescence intensity); **(M)** CX3CR1 expression within non-conventional monocytes (expressed in mean fluorescence intensity). Data are plotted for age-matched control individuals (CTR) or for hip fracture patients at different times of follow-up (pre-surgery: PRE; post-surgery: POST; during hospitalization: HOSP; at hospital discharge: DISCHARGE and between 6 and 12 months post-fracture: LONG TERM). Each dot represents an individual. The lanes indicate the medians. Statistical significance is determined by the nonparametric Mann–Whitney test: $p < 0.05$ was considered significant.

DISCUSSION

The objective of this study was to pinpoint immune modifications occurring after HF which represents an acute assault that can accelerate abruptly the progressive health decline associated with aging.

In fact, HF constitutes an important geriatric problem with high level of mortality and loss of functional autonomy. Despite improvement of clinical care through specific orthopedic geriatric unit creation (20), part of mechanisms implicated in the bad prognosis of HF keep unknown and could be a key to enhance medical care.

Longitudinal analysis of immune modifications is a strength in our study. Indeed, the four early time points permitted to highlight that immune changes appear in 1st hours following HF. Most of the time, these alterations falling into place at hospital discharge or at long term. Another strength of this work is the size of the cohort and the population characteristics. Patients included are typical of geriatric population with age over 80 years old, multiple comorbidities, polymedication, and frailty.

Our main results are: an increased number of innate compartments (neutrophils, monocytes) with a higher proportion of inflammatory monocytes in the first days following HF; a transitory decreased number of NK cells, T, and B lymphocytes. Despite these differential mobilizations, cells keep their ability to respond to environmental stimuli or homeostatic signals.

Moreover, there is a transient alteration in the regulation of T-cells activation with an increase expression of immune checkpoint inhibitors and a decrease expression of immune checkpoint activators, as previously described in trauma by Laudanski et al. (21) and in sepsis by Zhang et al. (22).

Overall, there is shift to a pro-inflammatory phenotype in innate compartment and to an anti-inflammatory phenotype in the adaptive compartment in the first days after HF. All these modifications are transitory forming a regulation loop before return at homeostatic status at long term.

HF represents an intense acute stress in old patients. It induces systemic reaction and notably immune responses. Another model of acute stress in geriatric population is sepsis. For several years, immune modifications by sepsis are described, associating an intense pro-inflammatory phase called "cytokine storm" and a suppressive phase with some similarities with our results (22).

During sepsis, phenotypic changes in monocytes are quite similar to what we observed in HF where the increased proportion of CD16+ monocytes is associated with an decreased expression of CX3CR1 (23–25). In our study, we observed a decreased expression of CX3CR1 and an increased expression of CCR2 indicating an intense turnover and recruitment of monocytes from bone marrow, potentially enabling migration to the fracture site.

In the 1st days of sepsis, there is a major leukocytosis similar to what we observed in this study of HF. However, in sepsis, neutrophils acquire a pro-inflammatory profile associating a decrease in the expression of CD16 and L-selectin (26). We did not observe differences in the expression of CD16 and L-selectin in our cohort. One hypothesis could be the bacterial origin of sepsis that mobilized intensively neutrophils as first line of defense against bacteria.

Concerning the adaptive compartment, early stage of sepsis is associated with global lymphopenia (27). CD4+, CD8+, B cells, and NK cells drastically decrease. Furthermore, inhibitory immune checkpoint (PD-1 and LAG-3) expression within T cells increases leading to T cells impairment and inhibition of innate cell function (28). We observed similar results in our study reinforcing the hypothesis of immunosuppression within adaptive compartment. Thus, negative signaling could contribute to T-cell anergy in trauma patients, as suggested by Bandyopadhyay et al. (29). Similarly, PD-L1 blockade has been shown to improve immune dysfunction of spleen dendritic cells and T-cells in multiple organs dysfunction syndromes (30).

If immune scar observed in HF is similar to the one observed in sepsis, it could be interesting to consider these two common complications in elderly populations as unique models to propose strategies to restore immunity after prolonged stress-induced immune suppression. Most recently, the concept of metabolic dysfunction has emerged as a factor underlying impaired function of the innate and adaptive immune systems of severely injured patients (31).

A comprehensive assessment of immune mechanisms implicated in the patients prognosis after HF appears important and could pave the way to news immune therapeutics approach.

In this regard, a recent study elegantly showed that hip fracture and surgical trauma cause significant increases in PD-1 expression in aged mice compared to sham controls. Antibody

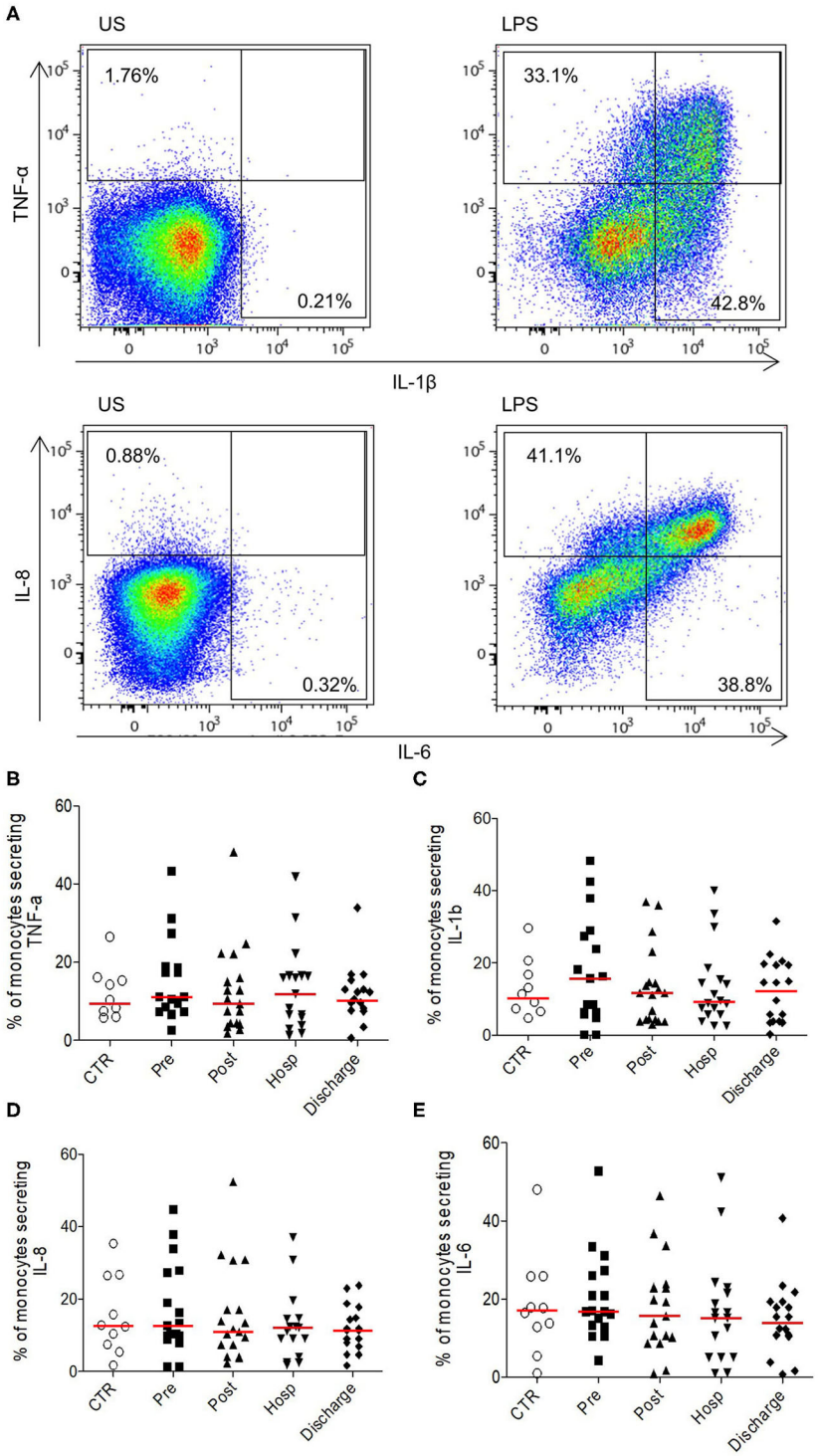

FIGURE 2 | Ability of monocytes from HFP to produce pro-inflammatory cytokines upon stimulation. **(A)** Representative flow cytometry profile of cytokines secretion (TNF-α and IL-1β upper panel; IL-8 and IL-6 bottom panel) in unstimulated monocytes (US, left panel) or in LPS conditions (right panel). Monocytes were identified according to size/structure, HLADR, CD14, and CD16 expression; **(B)** Percentages of monocytes secreting **(B)** TNF-α, **(C)** IL-1 β, **(D)** IL-8, and **(E)** IL-6. Data are plotted for age-matched control individuals (CTR) or for hip fracture patients at different times of follow-up (pre-surgery: PRE; post-surgery: POST; during hospitalization: HOSP; at hospital discharge: DISCHARGE and between 6 and 12 months post-fracture: LONG TERM). Each dot represents an individual. The lanes indicate the medians. Statistical significance is determined by the nonparametric Mann–Whitney test: $p < 0.05$ was considered significant.

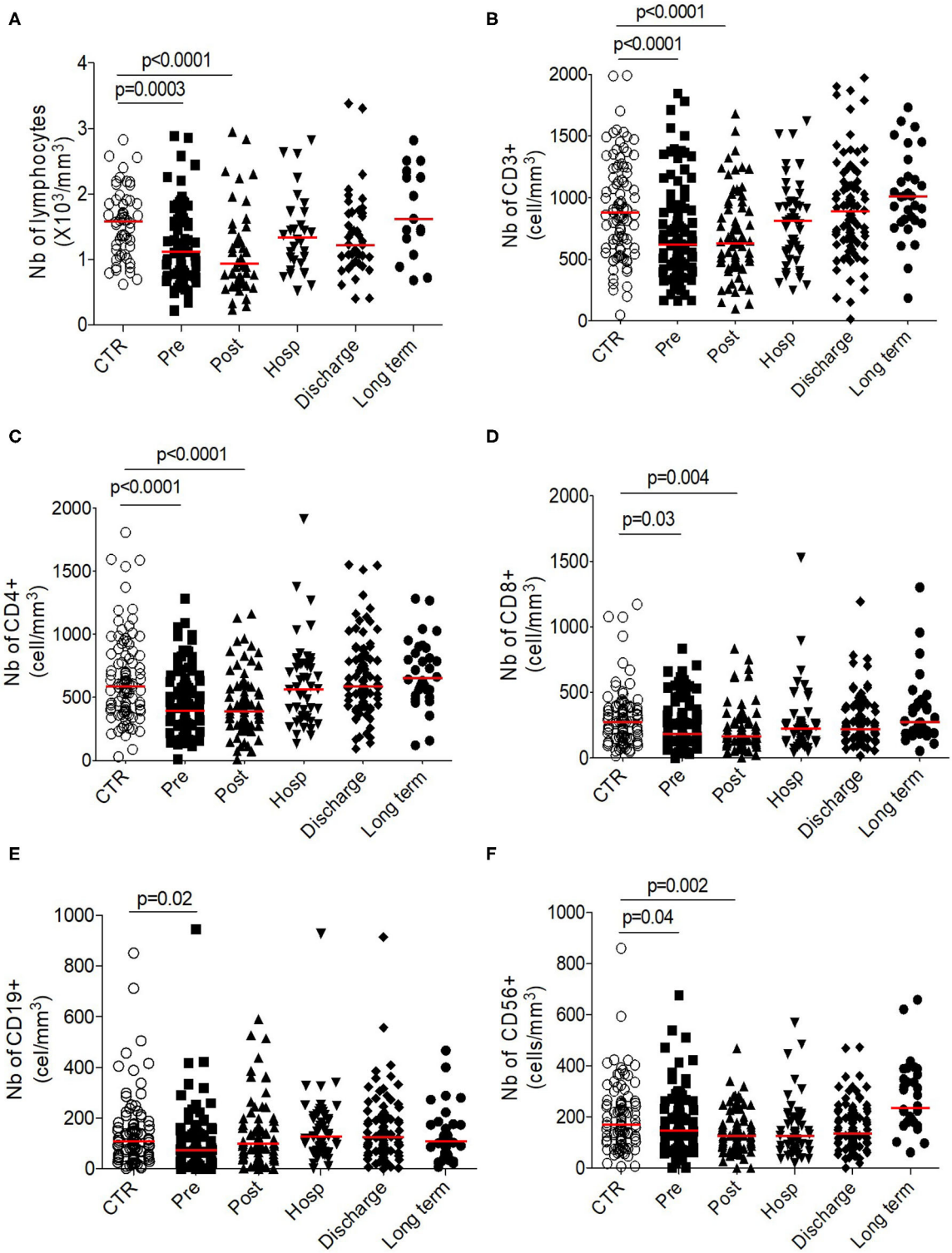

FIGURE 3 | Longitudinal analysis of adaptive phenotype. Number (10^3 cells/mm^3) of **(A)** total lymphocytes, **(B)** T-cells, **(C)** CD4+ T-cells, **(D)** CD8+ T-cells, **(E)** CD19+ B cells, and of **(F)** CD56+ NK cells. Data are plotted for age-matched control individuals (CTR) or for hip fracture patients at different times of follow-up (pre-surgery: PRE; post-surgery: POST; during hospitalization: HOSP; at hospital discharge: DISCHARGE and between 6 and 12 months post-fracture: LONG TERM). Each dot represents an individual. The lanes indicate the medians. Statistical significance is determined by the nonparametric Mann–Whitney test: $p < 0.05$ was considered significant.

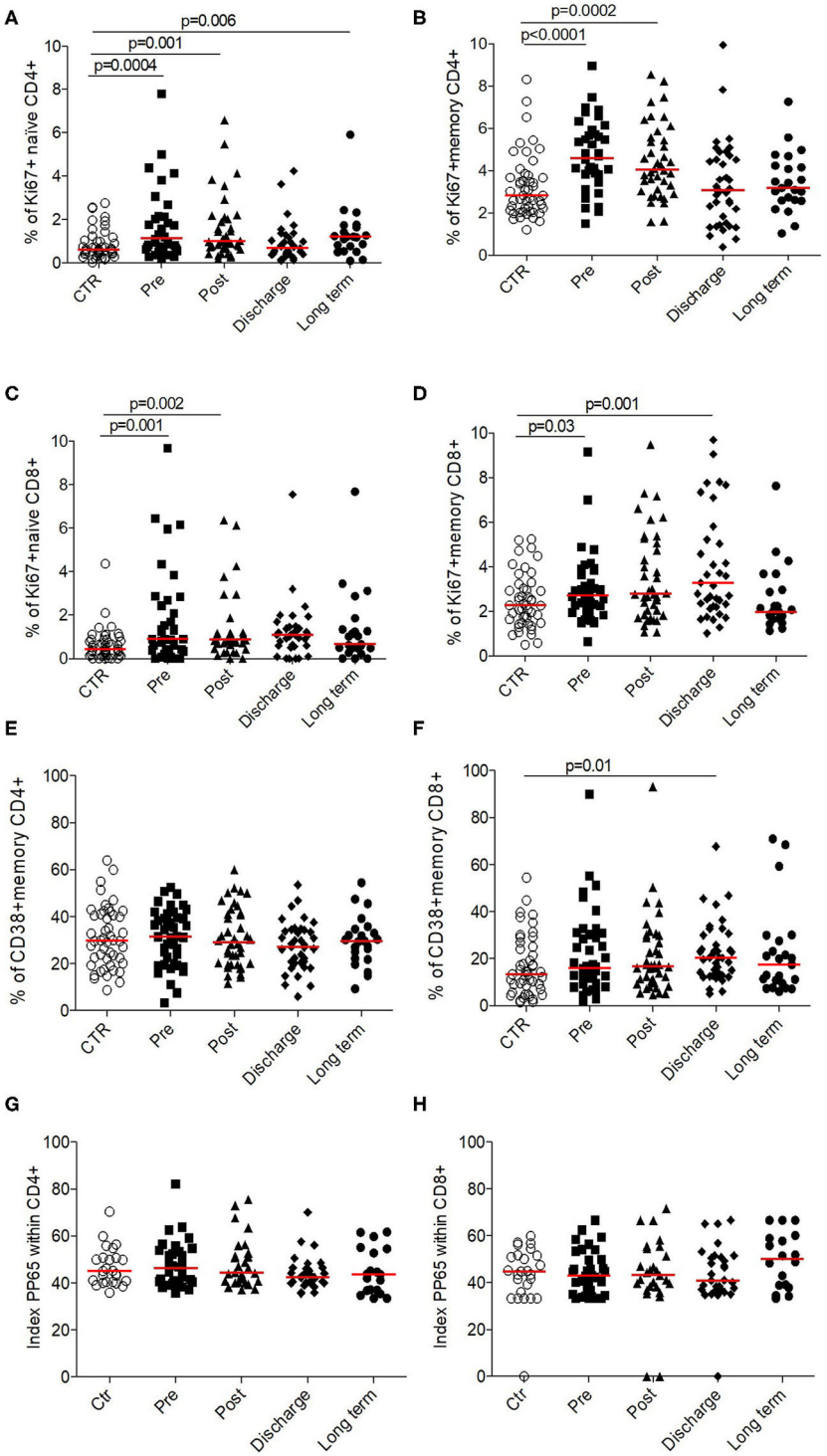

FIGURE 4 | Functionality of $CD4^+$ and $CD8^+$ T cells in HF patients (longitudinal analysis) compared to control. Proliferation was evaluated by the % of cells expressed $Ki67^+$ for **(A)** CCR7+ CD45RA+ naïve $CD4^+$, **(B)** memory $CD4^+$, **(C)** CCR7+ CD45RA+ naïve $CD8^+$, and **(D)** memory $CD8^+$. Activation was evaluated by the % of cells expressed CD38+ within **(E)** memory $CD4^+$ and **(F)** $CD8^+$. Priming capacity was evaluated by the polyfunctionality index within **(G)** $CD4^+$ and **(H)** $CD8^+$. Data are plotted for age-matched control individuals (CTR) or for hip fracture patients at different times of follow-up (pre-surgery: PRE; post-surgery: POST; during hospitalization: HOSP; at hospital discharge: DISCHARGE and between 6 and 12 months post-fracture: LONG TERM). Each dot represents an individual. The lanes indicate the medians. Statistical significance is determined by the nonparametric Mann–Whitney test: $p < 0.05$ was considered significant.

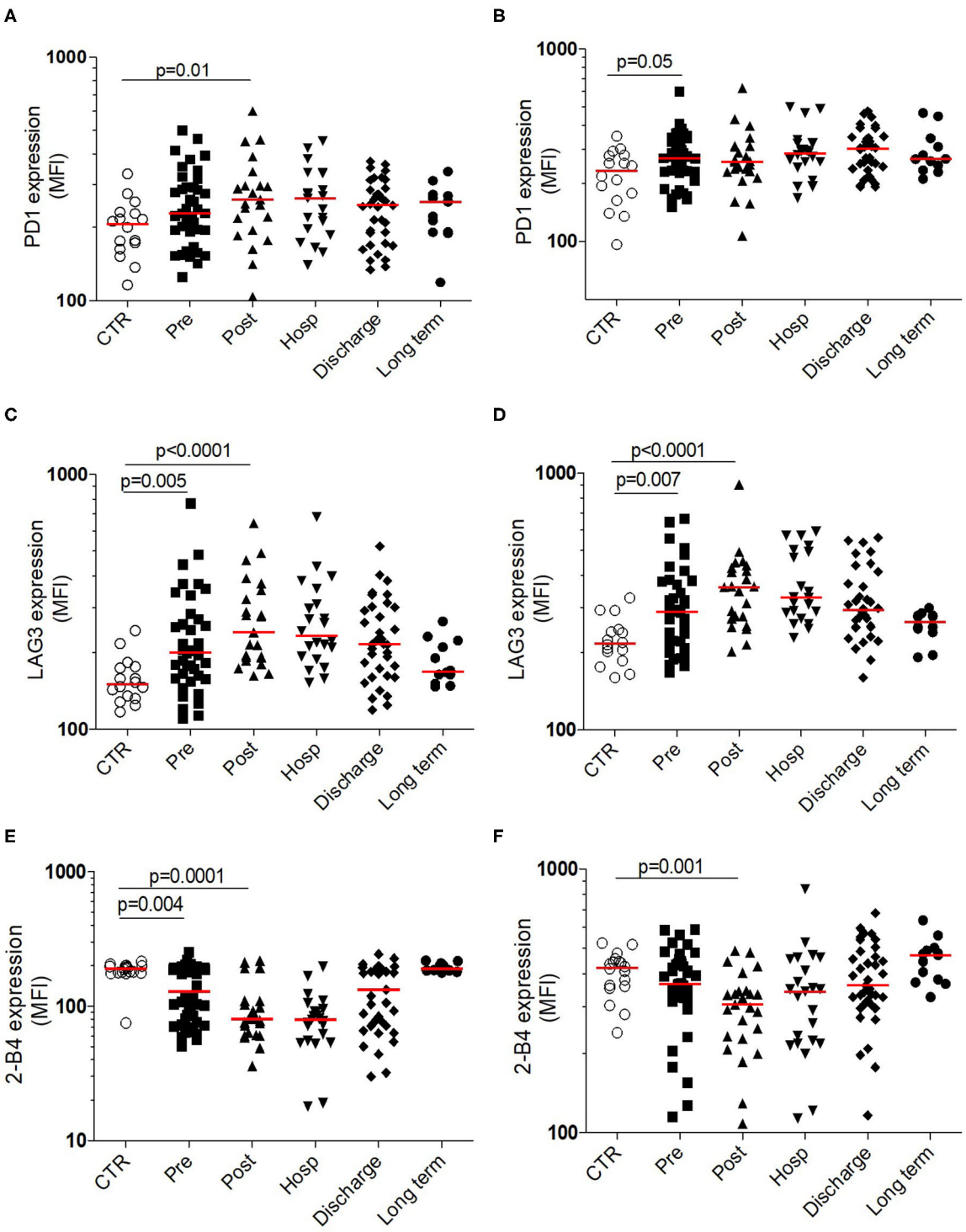

FIGURE 5 | Longitudinal analysis of immune checkpoint expression within T cells. Surface membrane expression within memory CD4+ **(Left)** and memory CD8+ T cells **(Right)** for PD-1 (**A,B** upper panels) LAG-3 (**C,D** middle panels) and 2-B4 expression (**E,F** bottom panels). All results are expressed in mean fluorescence intensity. Data are plotted for age-matched control individuals (CTR) or for hip fracture patients at different times of follow-up (pre-surgery: PRE; post-surgery: POST; during hospitalization: HOSP; at hospital discharge: DISCHARGE and between 6 and 12 months post-fracture: LONG TERM). Each dot represents an individual. The lanes indicate the medians. Statistical significance is determined by the nonparametric Mann–Whitney test: $p < 0.05$ was considered significant.

blockade of PD-1 partially reverses T cell apoptosis, decreases the systemic inflammatory response and susceptibility to bacterial lung infection, and reduces mortality (32).

During sepsis, many approaches have been deployed to target immune checkpoints reviewed in (28) and represent therefore a nice model to understand underlying mechanisms improving clinical patients outcome. Targeting immune checkpoints which could potentially reverse innate and adaptive system hypo-responsiveness during the 1st days following hip fracture could benefit elderly patients in preventing and treating immune tolerance. However, such a therapy needs to be evaluated in this particular population, where advanced age of the individuals may play a role in their capacity to respond to treatment.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité de Protection des personnes (CPP) Pitié Salpêtrière, Paris, France. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HV performed experiments, analyzed the data, and wrote the paper. CB, SF, and TF performed experiments and analyzed the data. HL, FK, and JB recruited patients and analyzed clinical data. JB designed clinical study, recruited patients, and analyzed clinical data. DS designed research, performed experiments, analyzed the data, and wrote the paper. All authors contributed to the article and approved the submitted version.

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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.2020.571759/full#supplementary-material

Supplementary Figure 1 | Longitudinal analysis of chemokines expression within monocytes subtype. CCR2 expression within **(A)** intermediate and **(B)** non-conventional monocytes (expressed in mean fluorescence intensity). CX3CR1 expression within intermediate **(C)** and conventional **(D)** monocytes (result expressed in mean fluorescence intensity). Data are plotted for age-matched control individuals (CTR) or for hip fracture patients at different times of follow-up (pre-surgery: PRE; post-surgery: POST; during hospitalization: HOSP; at hospital discharge: DISCHARGE and between 6 and 12 months post-fracture: LONG TERM). Each dot represents an individual. The lanes indicate the medians. Statistical significance is determined by the nonparametric Mann–Whitney test: $p < 0.05$ was considered significant.

Supplementary Figure 2 | Longitudinal analysis of adaptive phenotype. **(A)** % of $CD4^+$ T cells, **(B)** % of $CD8^+$ T cells, **(C)** % of $CD19^+$, and **(D)** % of $CD56^+$. Data are plotted for age-matched control individuals (CTR) or for hip fracture patients at different times of follow-up (pre-surgery: PRE; post-surgery: POST; during hospitalization: HOSP; at hospital discharge: DISCHARGE and between 6 and 12 months post-fracture: LONG TERM). Each dot represents an individual. The lanes indicate the medians. Statistical significance is determined by the nonparametric Mann–Whitney test: $p < 0.05$ was considered significant.

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REVIEW
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Immunesenescence: A Predisposing Risk Factor for the Development of COVID-19?

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Bearing a strong resemblance to the phenotypic and functional remodeling of the immune system that occurs during aging (termed immunesenescence), the immune response to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of Coronavirus disease 2019 (COVID-19), is characterized by an expansion of inflammatory monocytes, functional exhaustion of lymphocytes, dysregulated myeloid responses and the presence of highly activated senescent T cells. Alongside advanced age, male gender and pre-existing co-morbidities [e.g., obesity and type 2 diabetes (T2D)] are emerging as significant risk factors for COVID-19. Interestingly, immunesenescence is more profound in males when compared to females, whilst accelerated aging of the immune system, termed premature immunesenescence, has been described in obese subjects and T2D patients. Thus, as three distinct demographic groups with an increased susceptibility to COVID-19 share a common immune profile, *could immunesenescence be a generic contributory factor in the development of severe COVID-19?* Here, by focussing on three key aspects of an immune response, namely pathogen recognition, elimination and resolution, we address this question by discussing how immunesenescence may weaken or exacerbate the immune response to SARS-CoV-2. We also highlight how aspects of immunesenescence could render potential COVID-19 treatments less effective in older adults and draw attention to certain therapeutic options, which by reversing or circumventing certain features of immunesenescence may prove to be beneficial for the treatment of groups at high risk of severe COVID-19.

Keywords: aging, COVID-19, immunesenescence, immune dysfunction, inflammaging, SARS-Cov_2

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel highly-infectious betacoronavirus originally found in Wuhan, China in December 2019 (1). Transmitted by direct contact with infected individuals, contaminated surfaces or via respiratory droplets, SARS-CoV-2 is the causative agent of Coronavirus disease 2019 (COVID-19), which as of June 2020 had infected over 7 million people resulting in over 400,000 deaths (2). Whilst for the majority of individuals COVID-19 is a self-resolving mild to moderate respiratory tract infection, ∼20% of infected patients develop severe respiratory complications (e.g., dyspnea and pneumonia), which,

in extreme cases (∼5%), progress to acute respiratory distress syndrome (ARDS), respiratory failure, organ damage, and death (3–6).

Epidemiological analyses of COVID-19 outbreaks have revealed the disease to be highly prevalent amongst older adults, with one study of 1,591 patients reporting 87% of cases were in adults aged 51 years and over (7, 8). Furthermore, older adults are more prone to developing severe COVID-19 and its associated poor outcomes (4, 6, 9–15). For example, 91 and 81% of COVID-19 related deaths have occurred in people aged 65 years and over in the UK and USA respectively, with the majority of deaths occurring in those aged 85 years and over (16, 17). Moreover, the recovery times of older adults who survive COVID-19 are more protracted, involving more serious clinical manifestations that often require hospitalization and prolonged therapy (10, 14, 18).

The scientific community has moved rapidly to gain an understanding of the immune response to SARS-CoV-2 and how it influences patient outcome. Summarized recently by Vabret et al. (19) the current literature details a hyper-inflammatory state in severe COVID-19 patients that is characterized by a sustained raised level of pro-inflammatory cytokines such as interleukin (IL)-6, expansion of inflammatory monocytes and T cells, dysregulated myeloid responses, functional exhaustion of lymphocytes and impaired innate immune function. This immunological profile bears a strong resemblance to the remodeling of the immune system that occurs during physiological aging. Termed immunesenescence, immune aging is associated with marked alterations in the composition, phenotype and functional responsiveness of the innate and adaptive arms of the immune system that compromises the older adults ability to combat infections allowing for pathogen dissemination in a vicious cycle that leads to further inflammation and ultimately tissue damage. Furthermore, aging is accompanied by a state of chronic low-grade systemic inflammation, termed *inflammaging*, meaning older patients start with a higher inflammation status prior to infection. Immunesenescence is viewed as a major contributory factor in the increased susceptibility of older adults to infection (20, 21) as well as their poor vaccination responses (22). In addition to older adults, males (3, 4, 6, 13, 23, 24) as well as patients with pre-existing co-morbidities such as diabetes (4, 13–15, 25) and obesity (11, 24, 26–29) are at an increased risk of severe COVID-19.

Immunologically, immunesenescence and *inflammaging* appear to be more profound in older males when compared to females (30, 31), whilst an accelerated aging phenotype, termed premature immunesenescence has been described in obese subjects and patients with type 2 diabetes (T2D) (32–34). Although 85–90% of T2D patients are overweight or obese, not all adults who are obese develop T2D and most studies suggest the prevalence is below 50% (35). For this reason, we have considered three distinct demographic groups with an increased susceptibility to COVID-19 that appear to share a common immune profile, posing the question *could immunesenescence be a generic contributory factor in the development of severe COVID-19?* Here, by focussing on three key aspects of an immune response, namely pathogen recognition, elimination and resolution, we will address this question by discussing how immunesenescence may weaken or exacerbate the immune response to SARS-CoV-2. We also highlight how aspects of immunesenescence could render potential COVID-19 treatments less effective in older adults and draw attention to certain therapeutic options, which by reversing or circumventing certain features of immunesenescence may prove to be beneficial for the treatment of groups at high risk of severe COVID-19.

PATHOGEN RECOGNITION

Pathogen Recognition Receptor Expression and the Early Anti-viral Response

Comprised of four different families, namely the toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), nucleotide-binding oligomerization domain-like receptors (NLRs) and the C-type lectin receptors (CLRs), pathogen recognition receptors (PRRs) are evolutionary conserved germline-encoded receptors responsible for the early detection of invading pathogens. Located at the cell surface, in endosomes and in the cytosol, PRRs are expressed predominantly by cells of the innate immune system, in particular monocytes and dendritic cells (DCs). As a single-stranded RNA virus, detection and initiation of the immune response against SARS-CoV-2 will be mediated by the RNA-sensing endosomal PRRs TLR 3, 7 and 8, and the cytoplasmic-residing RLRs and NLRs.

Ligation of PRRs activates interferon regulatory factors (IRFs), a family of transcription factors that drive the production of type I (α/β) and type III (γ) interferons (IFNs) (36). By inhibiting viral replication, enhancing innate immune responses and modulating T cell expansion and memory formation (37), IFNs provide strong anti-viral effects. SARS-CoV-2 appears particularly sensitive to IFNs, with *in vitro* culture studies revealing viral replication in kidney epithelial cells and primary human intestinal epithelial cells is potently inhibited by type I and type III IFNs, respectively (38, 39). In one of the few studies to have investigated the IFN response to SARS-CoV-2 in patients (40, 41), Hadjadj et al. identified a distinct type I IFN signature in severe COVID-19 patients (40). Compared to individuals with mild to moderate disease, critically ill patients presented with marked downregulation of IFN-stimulated genes in whole blood leukocytes, significantly lower plasma levels of IFN-α2 and reduced IFN activity in serum (40).

Studies that have examined the effect of age on the expression of RNA-sensing PRRs have reported significantly reduced expression of TLRs 3, 7, and 8 in myeloid DCs (mDCs) or plasmacytoid DCs (pDCs) isolated from older adults (42, 43). Accompanying these changes in PRR expression is an age-related impairment in the generation of type I and III IFNs (44). pDCs or monocytes from older adults secrete significantly lower amounts of IFN α, β, or γ in response to specific ligation of TLRs 7/8 and RIG-I, with the reduction in IFN α and β synthesis post-RIG-I activation attributed to impaired activation of IRFs (43, 45–47). Furthermore, and of particular importance in the context of SARS-CoV-2, age-related impairments in type I IFN production

have been described for monocytes and pDCs challenged with influenza A virus and West Nile virus (WNV) (42, 47–50), two RNA viruses that also cause significant morbidity and mortality in older adults (51–53). As prompt and efficient type I IFN responses are critical for preventing poor outcome following coronavirus infections (54, 55), an age-related impairment in IFN production may result in more robust virus replication and higher viral loads. On this note, it has been suggested that COVID-19 patients with type I IFN deficiency, a criterion we propose older adults would fulfill, may benefit from IFN α or β supplementation (40). In an open-label, randomized, phase 2 trial in COVID-19 patients, Hung and colleagues demonstrated that, when compared to anti-viral drug treatment alone, a combined therapy of anti-viral drugs and IFN-β significantly shortened the duration of viral shedding, time to symptom resolution and length of hospital stay in patients with mild to moderate disease (56). Whilst this therapeutic approach is worthy of consideration for geriatric COVID-19 patients, it should be noted that *in vitro* studies with monocytes from older adults have demonstrated reduced up-regulation of IFN-stimulated genes following influenza A virus challenge (47). Thus, increasing type I IFN levels in older adults via IFN supplementation may be offset by an age-related impairment in IFN responsiveness.

Ligation of PRRs also triggers the secretion of pro-inflammatory cytokines via the activation of nuclear factor kappa B (NF-κB) and mitogen activated protein kinase (MAPK) signaling pathways. Compared to those with mild-to-moderate disease, patients with severe COVID-19 infection present with significantly elevated circulating concentrations of a range of pro-inflammatory cytokines such as IL-6 and tumor necrosis factor-alpha (TNF-α) (57–61). Although not observed in all studies (43, 62), the majority of groups that have investigated cytokine production triggered by RNA-sensing PRRs have found this function is maintained with age (42, 43, 46, 47, 62). For example, in response to stimulation with TLR3, TLR 7/8 and RIG-I specific ligands, as well as influenza A virus, mDCs or monocytes isolated from young and older adults generate comparable levels of TNF-α, IL-6 and/or IL-12 (42, 43, 46, 47, 62). In the context of COVID-19, these data imply that the pro-inflammatory cytokine response to SARS-CoV-2 elicited by monocytes and mDCs would be similar across different age groups. However, this may not be the case for patients with pre-existing co-morbidities. For instance, compared to normal-weight controls, monocytes isolated from obese subjects generate significantly greater amounts of TNF-α and CCL5 following stimulation with viral ssRNA (63), whilst in monocytes from T2D patients, basal expression of components of the TLR signaling pathway such as the adaptor proteins MyD88 and TRIF as well as the p65 subunit of NF-κB are significantly increased (64). Thus, we propose that this remodeling of innate immune cells in obese and T2D patients would lead to a more robust pro-inflammatory response to SARS-CoV-2 when compared to that of healthy age-matched controls, culminating in greater systemic inflammation and more severe disease.

Generated via the activation of the NLRP3 inflammasome, a multi-subunit complex comprising of the NLR protein NLRP3, the adaptor protein ASC and caspase-1, IL-1β promotes anti-microbial resistance via the modulation of innate and adaptive immune responses (65). However, if dysregulated, production of this pro-inflammatory cytokine can promote lung injury and severe pulmonary fibrosis (66, 67). Coinciding with elevated plasma levels of IL-1β (3), single cell transcriptomic analysis of peripheral blood mononuclear cells (PBMCs) has shown a greater abundance of classical $CD14^{++}$ $CD16^{-}$ $IL1\beta^{+}$ monocytes in COVID-19 patients when compared to healthy controls (HCs) (68), whilst analysis of RNA extracted from whole blood found increased IL-1β gene expression preceded a decline in respiratory function (69). In terms of patient groups at high risk of severe COVID-19, significantly increased NLRP3 expression and ssRNA-induced IL-1β generation has been reported for monocytes and monocyte-derived macrophages isolated from T2D patients and obese subjects, respectively (63, 70, 71), suggesting potential exaggeration of inflammasome-mediated immune responses to SARS-CoV-2 in these cohorts. Conversely, aging appears to be associated with impaired activation of the inflammasome. Investigated primarily in animal models, significantly reduced inducible expression of NLRP3, ASC and/or caspase-1 has been described in lung homogenates, macrophages and/or DCs from aged mice, with these changes in expression resulting in decreased synthesis of IL-1β upon stimulation (72–74). Highlighting the importance of the inflammasome in host protection, models of influenza infection and secondary *Streptococcus pneumoniae* infection have shown the age-associated decrease in NLRP3 inflammasome expression and activity results in impaired cell infiltration to sites of infection, increased pathogenic load in the lung and higher rates of morbidity and mortality (72, 74). In terms of human aging and its impact on the inflammasome, no change (47) or a significant reduction (45) in IL-1β production by monocytes challenged with influenza A virus or TLR 7/8 ligands, respectively has been reported. Given the importance of the inflammasome in host defense against viral infections (75, 76), we suggest that the older COVID-19 patient with no pre-existing co-morbidities would elicit an impaired inflammasome-mediated immune response to SARS-CoV-2 that would increase their susceptibility to severe disease.

PATHOGEN ELIMINATION

Neutrophils

Currently, few studies have reported upon the neutrophil response to SARS-CoV-2. These studies have shown neutrophilia (3, 4), an elevated neutrophil-to-lymphocyte ratio (60, 61, 77–79) and neutrophil infiltration in the lungs (80, 81) to be features of severe COVID-19 and poor patient outcomes. In the only laboratory-based study, Zuo et al., using cell-free DNA (cfDNA), myeloperoxidase-DNA complexes and citrullinated histone H3 as surrogate markers of *in vivo* neutrophil extracellular trap (NET) formation, reported elevated levels of all three markers in serum samples obtained from hospitalized COVID-19 patients when compared to HCs (82). Significantly higher cfDNA and myeloperoxidase-DNA complexes were recorded in those who required mechanical ventilation, suggesting a potential relationship between enhanced NET formation and disease severity (82). Previously linked to the pathogenesis of acute lung

injury and the onset of ARDS in critically-ill patients (83–86), the authors suggested robust NET formation may propagate the inflammatory storm that appears to precede the onset of severe COVID-19 (3, 82, 87).

In the context of immunesenescence, both murine and human-based studies have reported a significant age-related reduction in NET formation (88–90). Thus, in contrast to younger adults and those with inflammatory co-morbidities (91–93), we would speculate that in older adults with no pre-existing health conditions, any elevation in circulating NET components post SARS-CoV-2 infection would not be a direct consequence of enhanced NET formation. Rather, we suggest that reduced clearance may be responsible. Once dismantled by the endonuclease deoxyribonuclease (DNase)-1, NETs are engulfed by macrophages and degraded in lysosomes, a process facilitated by the opsonisation of NET fragments by the complement protein C1q (94). Whilst no study to our knowledge has investigated the effect of age on DNase-1 activity, there are reports that aging is associated with reduced endocytic and phagocytic activity of macrophages (95–97) as well as reduced lysosomal activity (98). When viewed alongside data from critically-ill patients, in whom DNase activity and uptake of NETs by alveolar macrophages (AM) is significantly reduced (99, 100), then older adults with severe COVID-19 are a group that would be predicted to present with a high systemic NET load, a scenario, which in a cohort of patients with severe influenza A infection was associated with the development of multiple organ dysfunction syndrome (101).

NET production, whether assessed by a measurement of circulating markers (e.g., MPO-DNA complexes) or *ex vivo* generation, is significantly increased in obese subjects and individuals with T2D (91–93), patient groups that are not only at high risk of developing severe COVID-19 (102, 103) but who experienced poor outcomes in the 2009 H1N1 influenza A virus pandemic (104). Whilst multiple factors will underlie the susceptibility of obese and T2D subjects to severe COVID-19, it is intriguing to speculate that remodeling of the innate immune response, in this case a heightened sensitivity for NET generation, could be one such factor, particularly given the cytotoxic and pro-thrombotic nature of NETs (105, 106).

NETs may represent a potential therapeutic target for the prevention of poor outcomes such as ARDS in COVID-19. In a recent article, Barnes et al. discussed the therapeutic options that are available to manipulate NET formation and how some of these approaches are already being tested in clinical trials in COVID-19 patients (80). Improvements in clinical indices were reported in a cohort of severe COVID-19 patients that were co-treated with anti-viral agents and dipyridamole, an adenosine-receptor agonist that inhibits NET formation *in vitro* (107, 108). However, whether the observed benefits were related to the modulation of NET production was not addressed (108). Nevertheless, the success that enhancing NET degradation has had in terms of improving clinical markers in patients with virus-associated bronchiolitis (109, 110) and reducing both lung injury and mortality rates in murine models of pneumonia (84), should encourage researchers and clinicians to pursue NETs as therapeutic strategies. This is particularly pertinent to older adults, where administration of therapeutic doses of DNase would completely eradicate NETs (94), thereby bypassing the need for macrophage clearance, which is a process that is likely to be impaired with age.

Associated with lymphocytic and neutrophilic infiltrate, post-mortem histological examination of lung tissue has shown severe COVID-19 results in extensive diffuse alveolar damage (81). In response to a panel of inflammatory mediators, which included IL-8, C5a, leukotriene B4 and sputum, we have shown aging is associated with impaired migratory accuracy of neutrophils (111). This defect, which was detected in individuals aged ≥60 years, was accompanied by enhanced degranulation and neutrophil proteinase activity, leading us to propose that aging is associated with an increase in neutrophil-mediated bystander tissue damage (111). Interestingly, a similar situation may be observed in T2D patients, whose neutrophils also exhibit impaired migration *in vitro* (112, 113). Furthermore, compared to HCs, circulating levels of the protease inhibitor alpha-1 antitrypsin are significantly lower in T2D subjects (114). Thus, in the context of SARS-CoV-2, we suggest that the meandering neutrophils of both older adults and T2D patients would, via excessive proteinase release, promote more widespread tissue damage and increased systemic inflammation.

Monocytes and Macrophages

Accompanied by an emergence into circulation of large atypical vacuolated monocytes (115), SARS-CoV-2 infection is associated with alterations in the composition of the peripheral monocyte pool. For example, whereas frequencies of $CD14^{++}$ $CD16^{-}$ classical monocytes have been reported to be significantly reduced in COVID-19 patients when compared to HCs (115), the proportions of intermediate ($CD14^{++}16^{+}$) and non-classical ($CD14^{+}16^{++}$) monocytes are significantly increased (115, 116), with analysis also revealing the percentage of intermediate $CD14^{++}16^{+}$ monocytes to be significantly higher in patients requiring intensive care unit (ICU) treatment when compared to those with milder disease (116).

Moreover, single cell analysis of PBMCs has reported the presence of a monocyte subset unique to severe COVID-19 patients that is enriched in genes encoding a range of cytokine storm related cytokines such as IL-1β, IL-6, and TNF-α (117). Phenotypically, mirroring the immunological changes that occur during sepsis, monocytes from COVID-19 patients exhibit significantly reduced surface expression of the antigen presenting molecule HLA-DR (118). *Ex vivo* examination of intracellular cytokine levels has revealed an increased frequency of $GM\text{-}CSF^{+}$ and $IL\text{-}6^{+}$ monocytes in both ICU and non-ICU COVID-19 patients, with the percentage of $IL\text{-}6^{+}$ monocytes correlating with disease severity (116). Similarly, a greater proportion of $CD14^{++}$ $CD16^{-}$ $IL1\beta^{+}$ monocytes were detected in COVID-19 patients by RNA sequencing, which found expression in $CD14^{++}$ monocytes of pro and anti-inflammatory genes were up and down-regulated, respectively when compared to HCs (68). Whilst more studies are required, emerging data implies a role for IL-6 in driving the SARS-CoV-2-mediated remodeling of the monocyte pool (117, 118), with one group demonstrating a significant reduction in the expression of genes

involved in "leukocyte chemotaxis" and the "acute inflammatory response" in monocytes obtained from COVID-19 patients following treatment with the IL-6 receptor monoclonal antibody Tocilizumab (117).

Physiological aging and obesity are associated with remodeling of the circulating monocyte pool, with older adults and obese subjects exhibiting elevated frequencies of intermediate and non-classical monocytes when compared to younger adults and lean subjects, respectively (119–126). Interestingly, Ong et al. have recently assigned a senescent-like pro-inflammatory phenotype to both non-classical and intermediate monocytes (123). Associated with high expression of the phosphorylated p65 subunit of NK-κβ, both monocyte subsets secreted, in the absence of *ex vivo* stimulation, an array of pro-inflammatory cytokines and chemokines, which included TNF-α, IL-6, and CCL4 (123). Importantly, this basal increase in monocyte activity was associated with significantly elevated plasma levels of IL-6 and TNF-α (123). Thus, in the absence of infection, obese and older adults exhibit a state of heightened peripheral inflammation upon which the abovementioned SARS-CoV-2-mediated changes in monocyte biology would be super-imposed. When combined with the maintained (42, 43, 46, 47, 62) or increased (63) generation of pro-inflammatory cytokines by RNA-stimulated monocytes of older adults and obese subjects respectively, we speculate that this high level of basal inflammation would predispose these groups to hyper-inflammation that would hasten the onset of severe COVID-19.

Single cell RNA sequencing (scRNA-seq) of bronchoalveolar lavage fluid (BALF) has revealed the composition of macrophages within the lungs of COVID-19 patients differs based on disease severity. Categorizing macrophages as monocyte-derived, pro-fibrotic or alveolar, Liao et al. found BALF obtained from patients with severe disease was dominated by monocyte-derived and pro-fibrotic macrophages, with the former subset expressing a strong pro-inflammatory gene signature (127). Offering potential insights into the secondary complications that may develop in severe COVID-19 patients as a consequence of this remodeling of lung-resident macrophages, two studies have implicated monocyte-derived AMs in the development of post-injury lung fibrosis and viral-induced pneumonia (128, 129). In the context of immunesenescence, it has been proposed that as a consequence of life-long exposure to environmental challenges, monocyte recruitment to the lung increases with age, such that over time, monocyte-derived macrophages become the predominant subset within the lungs (130). If correct, then a more robust pulmonary inflammatory response to SARS-CoV-2 in older adults may increase their susceptibility to developing severe COVID-19.

Natural Killer Cells

Natural killer (NK) cells are innate immune cells that play a major role in the early recognition and elimination of virally-infected cells. In a murine model of severe SARS-CoV-1 pulmonary infection, Glass et al. demonstrated viral clearance in the absence of NK cells (131), a finding that suggests these innate lymphocytes are not required for host protection against coronaviruses. However, the significant number of studies that have demonstrated marked alterations in the composition and function of the circulating NK cell pool of COVID-19 patients (19) makes a discussion of the NK cell response to SARS-CoV-2, particularly in the context of immunesenescence, necessary.

COVID-19 patients with mild-to-moderate disease present with significantly reduced circulating numbers of total NK cells, driven by a reduction in both $CD56^{DIM}16^{+}$ and $CD56^{BRIGHT}$ NK cell subsets (40, 41, 79, 118, 132–134). Accompanying these numerical changes are significant alterations in NK cell phenotype, with scRNA-seq and flow cytometric analyses revealing the peripheral NK pool of COVID-19 patients is dominated by immature, highly activated and functionally compromised cells (40, 41, 134). Focussing on the latter, increased frequencies of NK cells expressing the inhibitory receptors TIM3 and NKG2A have been detected in patients with mild/moderate and severe COVID-19 (40, 134) with the increase in NKG2A expression potentially reflecting the stimulation of NK cells by pro-inflammatory cytokines (135). Upon recognition of its ligand HLA-E, signaling through NKG2A inhibits NK cell cytotoxicity (NKCC) (136, 137). Thus, one would predict that NK cells isolated from COVID-19 patients would exhibit reduced functional responses. Indeed, albeit to a non-viral stimulus, Zheng and colleagues found the frequencies of $CD107a^{+}$, $IFN\gamma^{+}$, $TNF\alpha^{+}$, and $IL\text{-}2^{+}$ NK cells in PBMC samples acquired from COVID-19 patients were significantly lower following PMA and ionomycin challenge when compared to HCs (134). As blood samples were acquired at the time of hospital admission, these results imply an immediate breakdown of NK-mediated anti-viral immunity (134). Interestingly, when patients were reanalysed following anti-viral therapy, a marked reduction in the percentage of $NKG2A^{+}$ NK cells was noted, leading to the suggestion that downregulation of NKG2A may correlate with disease control (134).

A prominent feature of NK cell immunesenescence is reduced NKCC, a defect we have previously attributed to impaired polarization of the pore forming protein perforin to the immunological synapse (138). Accompanying this decline in lytic activity is an age-related reduction in cytokine and chemokine production (139–141). NK cell function is regulated by the balance of signals transmitted through surface expressed activatory and inhibitory receptors (142). As discussed by others (19), it is currently unknown as to which ligands for activatory receptors are expressed on the surface of SARS CoV-2 infected cells. Possible candidates are stress-inducible ligands, which are recognized by the activatory receptors NKG2D, NKp30, and NKp46. Whilst age has no effect upon the expression of NKG2D (138, 143), a number of studies have described an age-associated decline in the frequency of $NKp30^{+}$ and $NKp46^{+}$ NK cells (138, 144, 145). Thus, in the older adult with severe COVID-19, superimposed on a baseline reduction in NKCC and activatory receptor expression would be a SARS-CoV-2 driven induction of functional exhaustion via the up-regulation of NKG2A (134). Moreover, with *in vitro* studies having shown that exposure to IL-6 and TNF-α, two cytokines whose circulating levels are elevated in COVID-19 patients (3, 77, 146) impairs NKCC and reduces perforin, NKp30 and NKp46 expression (147–149), then the SARS-CoV-2-induced cytokine storm would exacerbate the

abovementioned functional and phenotypical features of NK cell immunesenescence, which would be predicted to further reduce NK cell anti-viral activity.

Recent studies in the field of cancer immunotherapy have shown that manipulation of NKG2A signaling can restore NKCC and promote anti-tumor immunity (136, 150, 151). Based on its success, Yaqinuddin and colleagues have proposed mirroring this therapeutic approach for the treatment of COVID-19 patients, where administration of the humanized anti-NKG2A antibody Monalizumab would rejuvenate the anti-viral immune response of COVID-19 patients by counteracting the NKG2A-driven inhibition of NKCC (152). However, for older adults with severe COVID-19, any therapeutic value of this approach may be offset by the age-related impairments in perforin polarization, NKCC and the reduced expression of NK cell activating receptors.

T Cell Responses

Lymphopenia is a common hematological observation in patients infected with SARS-CoV-2. $CD3^+$, $CD4^+$, and $CD8^+$ T cell counts are significantly lower in patients with severe COVID-19 when compared to those with mild disease (57, 60, 133, 134, 153), with numbers increasing significantly in subjects who respond clinically to anti-viral treatment (133). Elevated circulating concentrations of pro-inflammatory cytokines (133, 154), induction of apoptosis (40) and pulmonary infiltration (5, 127) are some of the mechanisms that have been proposed to underlie SARS-CoV-2-induced lymphopenia. Indicative of *in vivo* activation, increased proportions of $CD4^+$ and $CD8^+$ T cells expressing CD69, CD38, CD44, or HLA-DR have been reported in COVID-19 patients (5, 116, 155–158) as has the presence of pathogenic GM-CSF^+/IL-6^+ and GM-CSF^+/IFN^+ $CD4^+$ T cells, with those experiencing severe disease presenting with significantly increased frequencies when compared to those with mild COVID-19 (116). Pointing toward a state of functional exhaustion or senescence, markedly higher percentages of $CD4^+$ or $CD8^+$ T cells expressing a variety of molecules such as NKG2A, PD-1, TIGIT, TIM-3 and CD57 have been detected in SARS-CoV-2-infected patients (116, 134, 154, 159), with their presence coinciding with significantly reduced intracellular cytokine generation upon *ex vivo* stimulation (134, 158).

Characterized by the gradual replacement of functional epithelial cells with fat and fibrous tissue (160), thymic involution is a defining feature of T cell immunesenescence, which results in a decline in the production of naïve T lymphocytes (161). This reduction in thymic output is offset by the homeostatic proliferation of pre-existing naïve and memory T cells, a scenario that results in a contraction in the diversity of the circulating T cell receptor (TCR) repertoire of older adults (162). As well as aging, obesity is associated with reduced thymic function. Yang and co-workers found the generation of naïve T cells was significantly lower in obese younger adults when compared to age-matched lean controls (163). As a broad TCR repertoire is crucial for the detection of novel pathogens, the reduced diversity within the T cell pool of older adults and obese subjects may contribute to their increased susceptibility to SARS-CoV-2 infection and put them at risk of eliciting a blunted immune response to any future COVID-19 vaccine.

Owing to impaired metabolism, shortened telomeres and aberrant intracellular signaling (33, 164, 165), reduced proliferation, cytokine production, cytotoxicity and migration are examples of some of the functional impairments that have been reported for T cells isolated from older adults and those with inflammatory co-morbidities (33, 166, 167). The peripheral T cell pools of these adults are enriched with functionally exhausted ($TIGIT^+$, $PD\text{-}1^+$), highly activated ($TIGIT^+$ $HLA\text{-}DR^+$ $CD38^+$), senescent ($CD28^-57^+$, $CCR7^-45RA^+$) and terminally differentiated ($CD27^-28^-$) $CD4^+$ or $CD8^+$ T cells (33, 168–170). The most profound changes are witnessed within the $CD8^+$ T cell subset, with the accumulation of $CD8^+28^-$ T cells of particular significance (171). Saurwein-Teissl et al. found an expansion of $CD8^+28^-$ T cells was associated with reduced antibody responses in older adults following influenza vaccination (172). The efficiency of T cell responses are also hampered by age-associated alterations in the expression of co-stimulatory molecules on the surface of antigen presenting cells. Relevant to SARS-CoV-2, monocytes isolated from older adults have been shown to exhibit reduced expression of CD80 and CD86 following ligation of the RNA-sensing PRRs TLR7/8 (173). In subsequent vaccination studies, it was shown that expression of these co-stimulatory molecules was positively associated with antibody responses (173).

Based on scRNA-seq data that has shown the presence of highly expanded and functionally-competent $CD8^+$ T cells in the BALF of mild COVID-19 patients, it has been suggested that a robust adaptive immune response is critical to controlling SARS-CoV-2 infection (127). If correct, then combined with the aforementioned remodeled T cell pool of older adults and individuals with inflammatory co-morbidities, the SARS-CoV-2 driven induction of lymphocyte exhaustion (116, 134, 154, 159) would hamper both the initiation and maintenance of such a response. Furthermore, due to the reduced vaccine efficacy that occurs as a consequence of both innate and adaptive immune dysfunction, alternative therapeutic strategies such as administration of the immunomodulatory drugs metformin and pioglitazone, have been proposed to protect these high risk groups against severe COVID-19 (174).

B Cells

Marked alterations have been described in the composition of the circulating B cell pool of SARS-CoV-2 infected patients. Relative to HC's, significantly reduced frequencies of naïve IgM^+CD27^-, memory $CD21^+27^+$ and $CD5^+$ B cells have been reported (175, 176), and are accompanied by a concurrent elevation in the proportion of $CD38^+27^+$ plasmablasts (175–177). When analyzed by disease severity, significant alterations in plasmablast and memory $CD21^+27^+$ B cell frequencies were observed only in patients with severe COVID-19 disease, with the proportions of both subsets returning to levels comparable to those of HCs upon recovery (175, 176). Demonstrating a rapid and robust B cell response to SARS-CoV-2 infection, elevated circulating levels of virus specific IgM, IgG, and IgA antibodies have been detected, with this seroconversion evident within 7–14 days post-symptom onset (19, 68, 175, 176, 178). Interestingly, in a small pilot study of five critically-ill COVID-19 patients, transfusion of convalescent

plasma containing neutralizing SARS-CoV-2 specific antibodies was shown to improve clinical status (179). In terms of the longevity of the antibody response, SARS-CoV-2 specific IgG antibodies have been detected in serum samples acquired from COVID-19 patients 7 weeks post-infection (180). However, due to the infancy of the current pandemic, it is currently unknown as to whether this initial antibody response and generation of memory B cells will protect against re-infection. That said, data from previous coronavirus outbreaks, in which a progressive decline in both SARS-CoV-1 specific IgG memory B cells and IgG antibodies were reported (181, 182), suggests that SARS-CoV-2 antibody responses will wane over time.

Attributed to a range of factors such as changes in the bone marrow microenvironment and skewing of haematopoietic stem cell differentiation toward the myeloid lineage, murine-based studies have shown aging is associated with a reduction in mature B cell production (183). In line with this observation, human aging is accompanied by a reduction in the size of the peripheral B cell pool, with both the frequency and absolute numbers of $CD19^+$ B cells significantly lower in older adults (183–185). However, whether human aging is associated with changes in the composition of the peripheral B cell pool is unclear. For example, whilst some groups have reported an age-related increase in the percentage or number of circulating $CD27^+$ memory B cells (185), others have demonstrated an age-associated decline in this subset (183, 184). Similarly, the frequency of IgM memory B cells have been reported to be either decreased (186) or unchanged with age (184).

Results of human and animal-based studies have revealed that aging is associated with reduced B cell proliferation and differentiation into plasma cells, which secrete antibodies that are weaker and of lower affinity when compared to those produced by plasma cells of younger subjects (186–189). Critical steps in a humoral immune response are class switch recombination (CSR) and somatic hypermutation (SHM). Taking place in germinal centers, these two processes are responsible for the generation of isotype-switched high-affinity antibodies. Essential for both CSR and SHM is activation-induced cytidine deaminase (AID), a DNA-editing enzyme, whose expression is regulated by the transcription factor E47. Culminating in defective class switching, the expression of both AID and E47 has been shown to be significantly lower in B cells from aged mice and humans (184, 190, 191). Alongside these intrinsic defects, B-cell extrinsic factors also contribute to the age-related impairment in humoral immunity. For example, attributed to reduced surface expression of Fc receptors, follicular dendritic cells of aged mice exhibit reduced antigen trapping and presentation (192), whilst the age-related decline in CD40L expression on the surface of activated $CD4^+$ T cells would reduce the delivery of co-stimulatory signals to antigen-expressing B cells (193).

B cell immunesenescence is considered a major underlying factor in the reduced efficacy of vaccination in older adults. Characterized by decreased antibody concentrations, delayed peak antibody titres and lower seroprotection (194–196), the humoral response to a range of vaccinations such as influenza (197) and Hepatitis A (196) is significantly reduced in older adults. Furthermore, accompanying this impairment in initial antibody responses is an age-associated decline in antibody persistence, with one study reporting non-protective antibody titres to be present in older adults 6–10 years following vaccination with tetanus toxoid (198). In the context of COVID-19, these studies highlight the need for research groups involved in designing a SARS-CoV-2 vaccine to consider the impact that age will have on its efficacy, and whether one vaccine will confer protection amongst all groups of society. With this in mind, it may be that a vaccination strategy specific for older adults is required. This could involve the co-administration of an adjuvant or delivery of a booster vaccine, two strategies that have previously proven successful in augmenting antibody titres and conferring seroprotection in aged rhesus monkeys and humans (199, 200).

Inflammaging

Physiological aging is accompanied by a sub-clinical chronic low-grade state of systemic inflammation, *inflammaging*. This phenomenon is characterized by elevated serum levels of acute phase proteins (e.g., C-reactive protein) and pro-inflammatory cytokines (e.g., TNF-α, IL-6, and IL-8) (201). Previous papers that have discussed COVID-19 in the context of aging and immunesenescence have speculated that *inflammaging* would predispose the older adult to severe infection by fuelling an exaggerated pro-inflammatory response to SARS-CoV-2 (202, 203). However, based on emerging data that suggests excessive pro-inflammatory responses in older adults negatively regulates their immune responses (204, 205), we propose the following alternative hypothesis: *inflammaging predisposes older adults to severe COVID-19 by suppressing the immune response to SARS-CoV-2*. Whilst *in vitro and in vivo* studies have demonstrated that exposure to pro-inflammatory cytokines can modulate the phenotype and/or function of innate and adaptive immune cells (147–149, 206), it is the work of Akbar et al. that have specifically linked hyper-inflammation to impaired antigen specific immune responses during aging. Using a human experimental system that investigates antigen-specific immunity *in vivo*, the group has consistently demonstrated an age-related impairment in the delayed type hypersensitivity (DTH) response to varicella zoster virus (VSV) antigen (204, 205, 207). Attributed to aberrant activation of P38 MAPK signaling, the decreased VZV antigen responsiveness of older adults is associated with an accumulation of $CCR2^+$ monocytes that inhibit T cell proliferation via the production of prostaglandin E2 (PGE_2) (204, 205).

In terms of COVID-19, it is interesting that the aging lung is characterized by a state of heightened basal inflammation, with levels of IL-6, amongst other cytokines, significantly higher in the BALF of healthy older adults when compared to their younger counterparts (208–210). It has been suggested that a life-long accumulation of senescent cells may be responsible for this age-associated increase in pulmonary inflammation (210). Whilst data from murine models support this assumption (211), it is currently unknown in humans as to whether aging is associated with an increased senescent cell burden in the lungs. However, it is interesting that metatranscriptomic sequencing of BALF from COVID-19 patients aged 40-61 years detected an up-regulation of CCL2 (212), a chemokine produced in large

amounts by senescent cells (213, 214). Moreover, CCL2 is the chemoattractant for $CD14^{++}$ $CCR2^{+}$ classical and intermediate monocytes, which in the abovementioned VZV models were more abundant at sites of antigenic challenge in older adults and negatively regulated the adaptive immune response (204). Thus, in response to SARS-CoV-2, the pulmonary immune response of older adults may share features reminiscent of the impaired cutaneous immune response described in DTH models, in that, via the CCL2-mediated recruitment of PGE_2-secreting monocytes, a hyper-inflammatory response would impede T cell function.

Residing in a state of permanent cell cycle arrest, yet remaining metabolically active, senescent cells are a rich source of pro-inflammatory cytokines, chemokines, growth factors and proteases (215). Due to this inflammatory profile, termed the senescent associated secretory phenotype (SASP), and their presence in various tissues of older adults and T2D patients (216–218), senescent cell accumulation is considered to be one factor underlying the heightened systemic inflammatory status of these individuals. Recently, it was demonstrated that certain viruses such as influenza virus exhibit enhanced replication efficiency in senescent cells (219). In terms of coronavirus, entry of SARS-CoV-1 into host cells has been shown to be dependent upon surface expression of vimentin, a filament protein that interacts directly with the spike protein of SARS-CoV-1 (220). Since vimentin was recently found to be expressed on the surface of senescent lung fibroblasts (221), and the fact that SARS-CoV-1 and SARS-CoV-2 utilize the same mechanism of attachment to host cells, a number of groups have proposed increased SARS-CoV-2 replication would occur in individuals with a high senescent cell burden (222–224). Thus, an increased presence of senescent cells may predispose to the development of severe COVID-19 via two mechanisms: (1) reduced immune cell clearance by contributing to the aforementioned inflammation-induced suppression of innate and adaptive immunity (204, 205, 225) and (3) increasing viral load by acting as a site of enhanced SARS-CoV-2 replication. Interestingly, a number of clinical trials assessing the therapeutic benefit of drugs that directly eliminate senescent cells or suppress their SASP are already underway in patients with COVID-19 (223, 226). Results of such studies will help researchers address whether a high senescent cell burden is indeed a risk factor for the development of severe COVID-19.

Belonging to one of two distinct subsets, namely monocytic or granulocytic, myeloid-derived suppressor cells (MDSC's) are a heterogeneous collection of immature cells. Via a range of mechanisms, which include the generation of ROS and nitric oxide, arginine metabolism, induction of T regulatory cells and the production of anti-inflammatory cytokines, MDSC's are potent immune suppressors, inhibiting the proliferation and activation of innate (NK cells, DC's and macrophages) and adaptive (T and B cells) immune cells (227, 228). Whilst the presence of MDSC's during acute inflammatory responses is seen as beneficial (due to their involvement in the resolution of inflammation), in the setting of chronic inflammation, where MDSC's persist, their suppressive activity is considered detrimental to the host (229). Thus, the elevated frequency of MDSC's reported in older adults, obese subjects and T2D patients (230–232) has been proposed as a potential mechanistic explanation for the increased susceptibility to infection and poor vaccination responses elicited by these individuals (233, 234). Given that such inflammatory mediators as PGE_2, IL-6, TNF-α, and GM-CSF promote the expansion and activation of MDSC's (227), the hyperactive immune response and cytokine storm described in SARS-CoV-2-infected patients has resulted in a handful of studies investigating whether MDSC's may contribute to the pathogenesis of COVID-19.

Relative to HC's, significantly elevated frequencies of MDSC's (235) and granulocytic-MDSC's (G-MDSCs) (236) have been detected in peripheral blood samples obtained from mild and severe COVID-19 patients. Suggestive of driving reduced anti-viral immune responses, significant negative associations were reported between MDSC frequency and the percentage of $perforin^{+}$ $CD3^{+}$T cells and $perforin^{+}$ NK cells (235), whilst in *ex vivo* cultures, depletion of G-MDSC's from PBMC samples of severe COVID-19 patients restored the proliferative capacity and cytokine production of T cells (236). In terms of disease severity, MDSC frequency has been reported to be significantly higher in patients with severe COVID-19 when compared to subjects with mild disease (236), whilst single cell transcriptomics has revealed the presence of immature $CD14^{+}MPO^{+}Ki67^{+}HLA\text{-}DR^{lo}$ suppressive monocytes and immature $ARG1^{+}CD101^{+}S100A8/A9^{+}$ neutrophils only in patients with severe disease (237). Furthermore, there is evidence to suggest that MDSC's persist in severe patients, with one study reporting G-MDSC's comprised >30% of total PBMC's in samples acquired from 3 severe COVID-19 patients at day 18 post-hospital admission (236). Thus, it has been hypothesized that a SARS-CoV-2-induced expansion of MDSC's may promote immune paralysis and that current therapeutic approaches targeting the cytokine storm may have the additional benefit of augmenting anti-viral immune responses by reducing MDSC proliferation and activation (235).

Age-Associated Changes in Pulmonary Immune Responses

Thus, far, our discussion of how immunesenescence may predispose to severe COVID-19 has focussed on the changes that occur in the composition, phenotype and/or function of circulating immune cells. As a respiratory tract infection, it is important to discuss the pulmonary immune response.

As the resident immune cell of the lungs, studies that have examined the effect of age on the pulmonary immune response have focussed predominantly upon the AM. Gene profiling of resting AMs has shown aging induces wide-spread transcriptional changes in aged mice (97), with up-regulation of inflammatory pathways related to oxidative burst and IL-8 supporting the notion that aging is associated with heightened basal inflammation within the lung (97). Intertwined with this pulmonary inflammaging is reduced AM function (95, 97, 238), with the work of Hinojosa et al. suggesting the elevation in basal inflammation is linked to impaired cytokine production via an up-regulation in AMs of A20, a negative regulator of NK-κβ and MAPKs (239). As both these signaling elements

function downstream of the RNA sensing PRRs TLR7/8 and RIG-1, pro-inflammatory cytokine production by AMs following SARS-CoV-2 stimulation may be reduced with age. This theory is supported by the significantly reduced production of IL-6 by AMs from aged mice following *ex vivo* stimulation with the TLR7/8 agonist R848, an impairment that was reported alongside a down-regulation of TLR8 gene expression and a reduced induction of genes related to IL-6 signaling in lung tissue from aged mice following viral infection (240). Other age-related defects reported in the pulmonary immune response include reduced NKCC (241), impaired migration of pulmonary DCs to draining lymph nodes (DLNs) (242), diminished virus-specific $CD8^+$ T cell responses (242–245) and delayed immune cell infiltration (74, 245). Results of adoptive transfer experiments point toward an immune suppressive environment rather than cell-intrinsic defects as the cause of some of the abovementioned functional impairments, with one study attributing the age-associated impairment in pulmonary DC and T cell responses to elevated levels of the immune suppressive eicosanoid PGD_2 in the lungs of aged mice (97, 242).

Insights into how aging may specifically affect the pulmonary immune response to SARS-CoV-2 are offered by the results of murine and non-human primate models of SARS-CoV-1 infection (242, 246–251). Replicating the situation in humans, disease severity and lethality in these models are higher in aged animals when compared to their younger counterparts (242, 247–249), and interestingly, the immune dysregulation that occurs in aged mice infected with SARS-CoV-1 is greater than that detected during influenza A virus infection (242). Features of the pulmonary immune response of aged animals to SARS-CoV-1 include: reduced DC migration to DLNs (242), impaired $CD8^+$ viral-specific T cell responses (242), decreased macrophage and DC activation (247), reduced T cell proliferation (247) and enhanced pro-inflammatory cytokine responses (246, 248, 249). Those studies that have reported an age-related increase in viral-induced inflammation have shown this exaggerated response is associated with significant lung damage, leading to the suggestion that a pathological immune response may contribute to the increased morbidity and mortality rates in older adults following coronavirus infection (246). Using two distinct approaches, namely antagonism of PGD_2 signaling (242) or prophylactic treatment with the TLR3 agonist poly IC (251), it is possible to enhance the pulmonary immune response of older animals to SARS-CoV-1 and increase host survival (242, 251). Demonstrating reversal of immunesenescence, these therapeutic strategies have been proposed as a potential means of improving clinical outcome in older adults at high risk of severe respiratory infections (242, 251).

RESOLUTION OF INFLAMMATION

A co-ordinated multi-step program that involves the clearance of apoptosed neutrophils by macrophages (efferocytosis) and the generation of specialized pro-resolving lipid mediators (SPMs), the resolution of inflammatory responses is an active process that protects against unwarranted tissue damage (252). Whilst we await data relating specifically to features of the resolution phase in SARS-CoV-2-infected patients, a series of murine and human-based studies have shown aging (97, 225, 253), obesity (254, 255) and T2D (254, 256) are all associated with delayed resolution of inflammatory responses.

Attributed to a p38 MAPK driven reduction in the expression of T-cell immunoglobulin mucin protein 4 (TIM-4), a receptor expressed by macrophages that recognizes phosphatidylserine on the surface of apoptosed neutrophils, De Maeyer and colleagues recently demonstrated an age-associated impairment in efferocytosis (225). In a human dermal model of acute sterile inflammation, this defect in efferocytosis resulted in the accumulation of annexin V^+ neutrophils and delayed resolution (225). Mirroring these observations, reduced clearance of apoptosed cells by macrophages has been reported in the experimental settings of obesity (97, 254, 257) and diabetes (254, 256, 258), with decreased PI3-K signaling (257) and elevated PGE_2 levels in inflammatory exudate (254) identified as potential underlying causes. In addition to defective efferocytosis, reduced concentrations of SPMs have been measured at sites of acute inflammation in murine models of aging (253) and diabetes (256). Augmenting SPM levels via exogenous administration shortened resolution time *in vivo*, with this improvement linked to increased efferocytosis and the reprogramming of monocytes to a pro-resolving phenotype (253). Based on these data, we propose that, via their delayed induction of resolution programs, groups at high risk of COVID-19 would experience prolonged inflammatory responses following SARS-CoV-2 infection. By exacerbating their pre-existing heightened pro-inflammatory status, this impairment in resolution would promote further immune dysregulation and bystander tissue damage that would result in delayed viral clearance and an extended time to recovery. On this note, coinciding with impaired efferocytosis *in vitro*, Wong et al. observed greater neutrophil retention in the lungs and higher myeloperoxidase levels in the BALF of aged mice following influenza A virus infection (97). Interestingly, adoptive transfer of AMs from young mice into aged mice significantly reduced the degree of lung damage measured 3 days post influenza A virus challenge (97).

Associated with pathogen dissemination, impaired lung function and increased mortality (259, 260), down-regulation of ALOX5 (the gene responsible for the synthesis of the SPM lipoxin) and reduced production of the SPM protectin D1 (PD1) have been reported in murine models of severe influenza infection. Based in part on the fact that in these models administration of PD1 improved survival rates and pulmonary function (260), SPM treatment has been proposed as a therapy by which to promote the resolution of lung inflammation and reduce tissue damage in COVID-19 patients (261). Importantly, treatment regimens that include exogenous application of SPMs and inhibition of P38 MAPK have been shown in human and animal models to overcome the delay in inflammatory resolution that occurs as a consequence of aging and the presence of co-morbidities (225, 253, 256). Thus, it appears that resolution of inflammation can be manipulated in groups at high risk of severe COVID-19. However, as histological examination of lung tissue obtained from a SARS-CoV-1 infected patient found

increased expression of plasminogen activator inhibitor-1 (262), a negative regulator of efferocytosis (263), there may be obstacles beyond impaired SPM generation that need to be overcome in order to successfully promote the resolution of coronavirus-induced inflammatory responses in older adults and those with inflammatory co-morbidities.

FUTURE DIRECTIONS

Immunesenescence and the Development of a COVID-19 Vaccine

A recent report by the World Health Organization provided information on the 26 candidate COVID-19 vaccines that are currently undergoing clinical testing and details of a further 139 that are in preclinical evaluation (264). The speed of COVID-19 vaccine research was highlighted by the fact that within 68 days of being declared a pandemic, results of the first animal and human based studies to test potential vaccines were published (265). Across rodents and non-human primates, the efficacy of an adenovirus-vectored vaccine encoding the spike protein of SARS-CoV-2 (266), a purified inactivated virus vaccine (267) and a series of DNA vaccines expressing different forms of the spike protein (268) have been tested, with preliminary results demonstrating the generation of robust humoral and cell-mediated responses that significantly reduce viral load and prevent the development of pneumonia (266, 267). Moderna Therapeutics recently announced the results of their phase 1 human trial of a potential COVID-19 vaccine (269). Using an mRNA vaccine that encodes for a pre-fusion stabilized form of the SARS-CoV-2 spike protein, the company reported seroconversion following a single dose in all 45 participants, with those who received two doses generating antibody levels akin to those measured in patients that have recovered from COVID-19 (269). However, it will be important to consider the impact of age and co-morbidities on the efficacy of any potential vaccine.

To date, a number of animal-based studies have investigated the effect of age on the efficacy of SARS-CoV-1 vaccines (270–272). In response to infection with homologous or heterologous viral strains, Bolles et al. found that aged mice vaccinated with an adjuvanted-double-inactivated whole SARS-CoV-1 virus were not completely protected against virus-induced mortality and exhibited both increased morbidity and pulmonary viral load when compared to young mice (270). Underlying this impairment in vaccine efficacy was a significant age-associated reduction in serum neutralizing antibody titres (270). However, in a related study, Sheahan et al. used a virus replicon particle vaccine platform that specifically targeted DCs, and showed that this strategy resulted in comparable antigen-specific IgG responses between young and aged mice and protected older mice from SARS-CoV-1-mediated clinical disease (272). Taken together, these data not only demonstrate the importance of testing any potential COVID-19 vaccine in all age groups but highlight how vaccine design will be critical for inducing protective antibody responses in aged hosts. On this note, a number of therapeutic strategies have been proposed and/or trialed in an attempt to combat the reduced efficacy of vaccinations against viral antigens in older adults (273). To date, these have included immunostimulant patches (274), the use of TLR agonists as adjuvants (275), the fusion of viral proteins with TLR agonists (276), high dose vaccination (277–279) and the use of PGD_2 antagonists (242).

It is becoming increasingly recognized that obesity is a risk factor for infectious disease and poor vaccination responses (280–283). Data from mice (284–286) and human (287, 288) studies have reported reduced influenza vaccine efficacy in obese subjects, which in murine studies was associated with increased lung pathology, higher viral titres and greater mortality rates upon secondary infection (284, 285). Studies are underway to investigate methods of counteracting the negative effects of obesity on vaccine responses. Of note, whilst the use of adjuvants and/or high dose vaccination have been shown to increase neutralizing antibody titres in obese mice, the levels generated as well as the breadth and magnitude of the antibody response was significantly lower when compared to lean controls, ultimately resulting in reduced protection upon viral challenge (289). Thus, when viewed alongside the abovementioned age-related impairment in vaccine efficacy, these results imply that a "one size fits all" policy may not be appropriate for a COVID-19 vaccine, with high risk groups requiring a tailored vaccine designed to overcome the deficits of their remodeled immune systems.

Enhancing Immune Function in Older Adults

Through pharmacological and non-pharmacological approaches, which include nutritional intervention (290, 291) and the administration of protein kinase inhibitors (204, 205, 225, 292, 293), clinical studies in older adults have shown it is possible to reverse immunesenescence.

Associated with reduced circulating frequencies of functionally exhausted PD-1 positive $CD4^+$ and $CD8^+$ T cells, Mannick et al. demonstrated a significantly enhanced serological response to influenza vaccination in older adults treated with the allosteric mammalian target of rapamycin (mTOR) inhibitor RAD001 prior to antigenic challenge (292). More recently, the same group reported that a combined therapy of RAD001 and BEZ235, a competitive mTOR inhibitor, significantly reduced the annualized rate of respiratory tract infections in adults aged $\geq$65 years (293). mRNA sequencing analysis of circulating leukocytes revealed this protective effect was accompanied by an up-regulation in genes related to anti-viral type I IFN signaling (293).

Oral administration of the potent and selective P38 MAPK inhibitor losmapimod has been shown to boost cutaneous immune responses in older adults. In a model of DTH, P38 inhibition was found to significantly increase VZV antigen specific immunity (205). Mechanistically, at the site of antigenic challenge, this improved immune response was associated with significantly reduced infiltration of PGE_2 producing $CCR2^+$ monocytes and increased T cell proliferation, whilst systemically, a significant decline in serum CRP levels was reported

(204, 205). Importantly, losmapimod treatment also augments the resolution response of older adults (225), meaning that the improved immune response that would occur following P38 inhibition would not be offset by bystander tissue damage that would arise from the delay in the resolution of inflammatory responses that accompanies physiological aging (225).

Taken together, these studies demonstrate that it is possible to enhance anti-viral immunity and resolution responses in older adults. In the context of SARS-CoV-2 infection, these treatment regimens could be applied in a prophylactic manner to prevent the spread of COVID-19 and boost immune responses to future vaccines. Having already proven successful in elderly subjects, these therapeutic strategies have an advantage over many other potential treatments whose efficacy would be hampered by the remodeling of the immune system that occurs with age.

CONCLUDING REMARKS

The similarities that exist between the immune response that precedes or accompanies the onset of severe COVID-19, and the re-modeled immune systems of older adults and those with inflammatory co-morbidities, lend support to the idea that immunesenescence may predispose to COVID-19 infection and disease severity (**Figure 1**). However, current evidence is at best circumstantial (202, 203), with the lack of cross-sectional and prospective studies examining the SARS-CoV-2-induced immune response in these high risk groups hindering our ability to address this hypothesis. That said, it appears that such studies are underway (10, 154, 294). For example, in a recent study, Liu et al. divided a cohort of 221 COVID-19 patients into two distinct age groups, and found older adults (≥60 years of age) presented with significantly elevated inflammatory indices (10). Furthermore, a study at University College London has acquired pre-infection blood and throat swab samples from people ≥70 years of age who will be assessed weekly for COVID-19 related symptoms (294, 295). Working on a hypothesis that prior exposure to coronaviruses may lead to an exaggerated immune response against SARS-CoV-2, one aim of the study is to determine pre-infection antibody titres against other coronaviruses (295). The results of this study, which also plans to search for

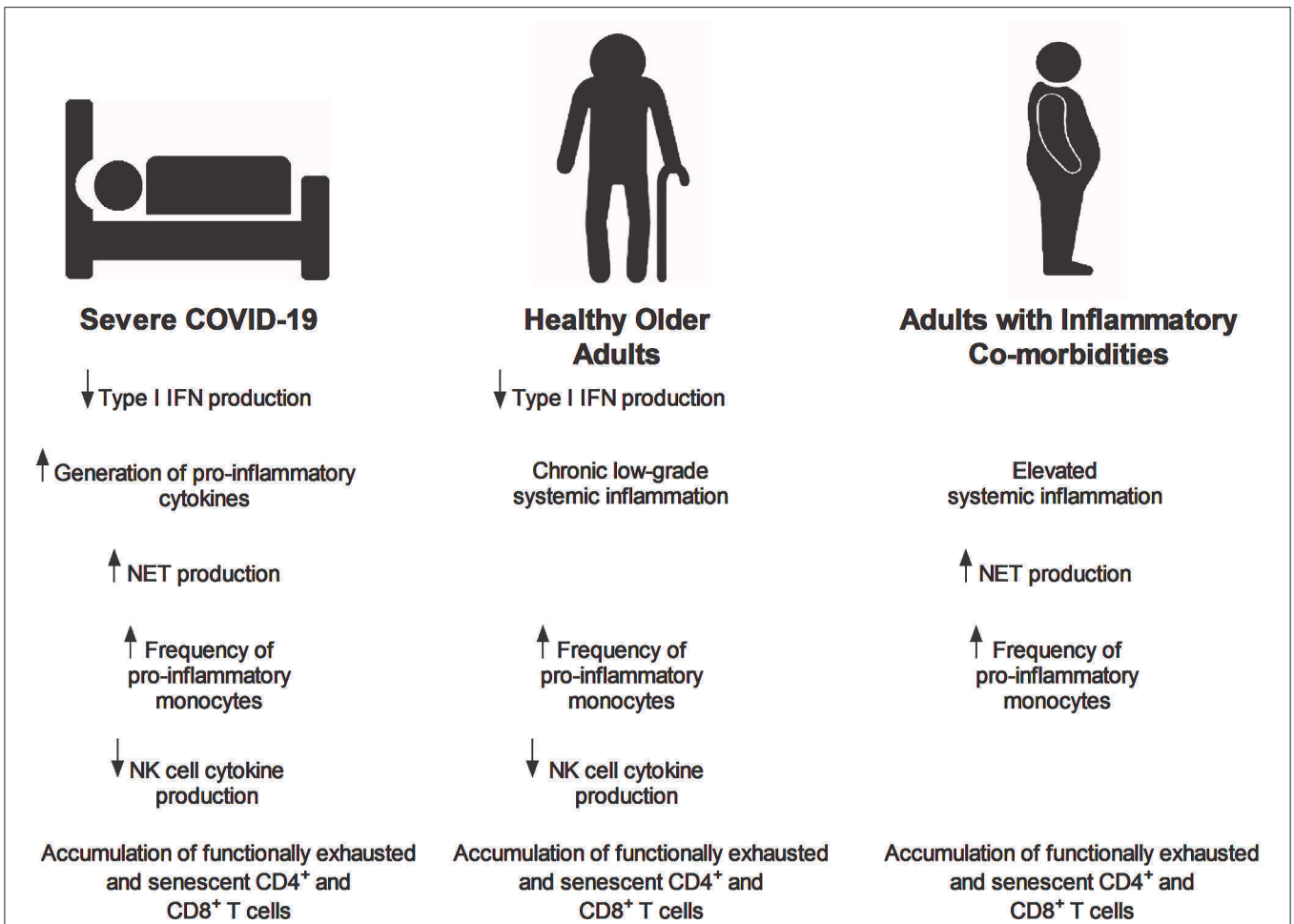

FIGURE 1 | Immunesenescence: a risk factor for severe COVID-19? Similarities between the immune profile of patients with severe COVID-19, healthy older adults and adults with inflammatory co-morbidities (obesity and type 2 diabetes). IFN, Interferon; NET, Neutrophil extracellular traps; NK, Natural killer.

biomarkers that are predictive of outcome in those subjects who develop COVID-19 (294, 295), will provide a much needed insight into how the immune system of older adults responds to SARS-Cov-2 and whether it is a contributory factor in patient outcome.

AUTHOR CONTRIBUTIONS

JH wrote the manuscript. JL critically appraised the manuscript. All authors have seen and approved the final submission.

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REVIEW
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Targeting Inflammation and Immunosenescence to Improve Vaccine Responses in the Elderly

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One of the most appreciated consequences of immunosenescence is an impaired response to vaccines with advanced age. While most studies report impaired antibody responses in older adults as a correlate of vaccine efficacy, it is now widely appreciated that this may fail to identify important changes occurring in the immune system with age that may affect vaccine efficacy. The impact of immunosenescence on vaccination goes beyond the defects on antibody responses as T cell-mediated responses are reshaped during aging and certainly affect vaccination. Likewise, age-related changes in the innate immune system may have important consequences on antigen presentation and priming of adaptive immune responses. Importantly, a low-level chronic inflammatory status known as inflammaging has been shown to inhibit immune responses to vaccination and pharmacological strategies aiming at blocking baseline inflammation can be potentially used to boost vaccine responses. Yet current strategies aiming at improving immunogenicity in the elderly have mainly focused on the use of adjuvants to promote local inflammation. More research is needed to understand the role of inflammation in vaccine responses and to reconcile these seemingly paradoxical observations. Alternative approaches to improve vaccine responses in the elderly include the use of higher vaccine doses or alternative routes of vaccination showing only limited benefits. This review will explore novel targets and potential new strategies for enhancing vaccine responses in older adults, including the use of anti-inflammatory drugs and immunomodulators.

Keywords: aging, immunosenescence and inflammaging, vaccine, T lymphocytes, anti-inflammatories

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INTRODUCTION

Human aging is associated with a general decline in physiological functions and increased susceptibility to disease. A dysregulation of the immune system, known as immunosenescence, is characteristic of aging and has been linked with negative clinical outcomes in older adults (1). One of the most appreciated consequences of immunosenescence is an impaired response to new infections and vaccination in older people (2). Four vaccines are currently recommended for individuals over 65 years of age to protect against infections that disproportionately affect older adults, including influenza, herpes zoster, pneumococcal disease and tetanus and diphtheria. However,

responses to these vaccines are often impaired in older individuals placing them at further risk of disease (3, 4). This has considerable implications for vaccination against emerging infectious diseases such as COVID-19 that have a disproportionately larger effect on older subjects (5).

While most studies report antibody responses as a correlate of vaccine efficacy, it is now widely appreciated that this may fail to identify important changes occurring in the immune system with age that may affect vaccine efficacy (6, 7). The impact of immunosenescence on vaccination goes beyond the defects on T and B cell responses and changes in innate immunity and increased systemic inflammation, also referred to as inflammaging, may have additional consequences on vaccine efficacy (8). While the mechanisms of immune aging are not yet fully understood, it is now apparent that this process is dynamic and multifaceted, with a decline in many primordial functions but also gain of new functions as well as changes in the microenvironment. Globally, age-related changes in the immune system are better described as a remodeling than a decline in immune functions (9). A better understanding of the full spectrum of changes characterizing immunesenescence is fundamental to the development of novel and improved vaccines for older adults.

HOW CAN IMMUNOSENESCENCE AND INFLAMMATION AFFECT VACCINE RESPONSES?

Changes affecting both innate and adaptive immune function with age may lead to impaired vaccine responses in older people. Immunosenescence is primarily linked to the involution of primary lymphoid organs (bone marrow and thymus), resulting in depletion of the peripheral pool of naive B and T cells (10). To maintain peripheral cell numbers, there is a clonal expansion of antigen-experienced cells resulting in extreme differentiation and altered functionality (11). Consequently the immune space becomes filled with antigen-specific memory cells leading to a contraction of the immune repertoire and impaired responses to neo-antigens (12). In parallel with this, the effects of aging on hematopoiesis result in a lineage skewing towards an increase in myeloid versus lymphoid precursor (13). Although the numbers of most circulating innate immune cells may not be significantly reduced with age, alterations in their functionality have a particular impact on antigen presentation due to decreased antigen uptake, reduced phagocyte functions and altered cytokine production (13, 14). In addition to cell-intrinsic changes, alterations in the microenvironment including a low-grade chronic inflammatory status and architectural changes occurring in lymph nodes may play previously underappreciated roles in shaping vaccine responses with age (1, 15). Excessive baseline inflammation has been recently associated with poor responses to vaccination (16) however more research is needed to reconcile this evidence with the current paradigm that adjuvants enhance immune responses to vaccines by promoting local inflammation. It is plausible that stronger local inflammatory signals are needed to overcome background inflammation or that specific inflammatory pathways should be triggered to overcome local inhibitory responses. Thus a better understanding of the role of inflammation in vaccination and of the mechanisms of action of adjuvants is needed to be able to fine tune immune responses and selectively stimulate pathways that lead to long-lasting immune protection. In this review, we will describe the most recent data on the effects of aging on immune responses to vaccination and discuss, in light of the current knowledge, how can immunesenescence and inflammaging be targeted to improve vaccine responses in older adults.

Age-Related Changes in Adaptive Immunity

Changes in the T Cell Compartment

The effects of aging are particularly evident in the T cell compartment and reduced vaccine responses in older people are, at least in part, due to defective T cell memory responses with age (17). Different mechanisms may be contributing to reduced T cell responsiveness with age (18), but the loss of proliferative capacity (19) and decreased TCR function (20–22) and TCR diversity (23) are certainly determining factors. Prior antigen exposure, in particular latent viral infections such as cytomegalovirus (CMV) and Epstein-Barr Virus (EBV) have a significant impact on immunosenescence by shaping the immune repertoire with large proportions of terminally differentiated cells with reduced proliferative capacity and features of replicative senescence (24–26). Despite this, data on the impact of CMV infection on vaccine responses are controversial, with studies showing an association between CMV-seropositivity and impaired antibody responses to vaccination in older adults (3, 27) while others have found enhanced antibody responses to influenza vaccination in CMV-seropositive compared to CMV-negative individuals (28, 29). Nevertheless, it has been shown that CMV seropositivity is a better predictor of a decline in T cell responses to influenza challenge rather than antibody responses in vaccinated older adults (30, 31). When using functional assays of $CD8^+$ T cell cytolytic activity upon *ex vivo* influenza challenge, CMV-seropositivity was associated with impaired cytolitic responses to influenza, measured by granzyme B levels in virus-challenged T cells (30, 31).

Mechanistically, we have described that highly differentiated T cells with features of senescence exhibit decreased TCR responsiveness as a results of loss of key components of the TCR signalossome (20, 22). Interestingly, these cells concomitantly express NK lineage receptors and acquire TCR-independent functionality (32). Thus, non-proliferative senescent-like T cells, in particular $CD8^+$ T cells, are reprogrammed to acquire broad, innate-like killing activity regulated by a group of stress sensing molecules known as sestrins (32). Studies in human centenarians have found an expansion of these NK-expressing T cells in old individuals compared to young (33) while others have shown that the expression of NK cell markers on $CD8^+$ T cells is particularly evident in individuals with high levels of CD57, indicative of an aged immune system (34). The biological significance of the acquisition of innate-like receptors and functions by T cells is unclear, but we believe that this may serve as a beneficial adaptation to ensure broad and rapid effector function with age, independently

of antigen-specificity, and this may represent a relatively unexplored opportunity to enhance vaccine-elicited immunity (35, 36). Despite the loss of proliferative potential, aged T cells are metabolically active and exhibit increased production of pro-inflammatory cytokines and thus may have detrimental effects on the tissue microenvironment, contributing to age-associated low-grade inflammation (37–39).

Changes in the B Cell Compartment

As with T cells, there is an age-dependent accumulation of late-stage memory B cells, while the circulating pool of naïve B cells progressively decreases, skewing the B cell repertoire and limiting the number of clones available to respond to novel antigens (40). B cells experience significant functional changes with age with reduced proliferative potential and impaired capacity for differentiation into plasma cells after antigen challenge (41). Senescent B cells have also been shown to spontaneously secrete pro-inflammatory cytokines contributing to age-related chronic inflammation and further immune dysregulation (42). Overall, these changes have been associated with poor health outcomes (43) and diminished responses to vaccination in old age (44). Several studies have shown that older adults have lower antibody responses following vaccination compared to younger adults and have been reviewed elsewhere (45). The quality of these antibody responses is also compromised with reduced diversity in the antibody repertoire (46, 47). This is particularly well described for influenza vaccination (48, 49), although responses to pneumococcal vaccines are equally compromised (50). Intrinsic defects of B cells, such as reduced somatic hypermutation and isotype switch as well as reduced numbers of plasma cells contribute to reduced antibody responses after vaccination and this correlates with decreased vaccine efficacy (41).

Changes in Innate Immunity With Age

Alterations in the phenotype and function of innate immune cells with age are increasingly well recognized (13, 14) and particularly relevant for vaccine-induced immune responses. Reduced chemotaxis, alterations in signaling pathways following antigen recognition and aberrant cytokine production have been described in neutrophils (51, 52), monocytes/macrophages (53, 54) and dendritic cells (DCs) (55, 56) derived from older persons further affecting their capacity to process and present antigen to T cells. Toll-like receptor (TLR) signaling has a crucial role in vaccination by linking innate and adaptive immune responses (57). Although the surface expression of TLRs does not show a consistent change with age, altered cytokine production and impaired downstream TLR signaling have been described in older adults (58). Interestingly, an age-dependent decrease in TLR function in human DCs has been linked with poor antibody responses to influenza immunization, providing evidence for the impact of an aging innate immune system in vaccine responses (59). Moreover, intracellular cytokine production in the absence of TLR ligand stimulation was elevated in cells from older compared with young individuals (59), suggesting a dysregulation of cytokine production that may contribute to age-related inflammation. Changes affecting the local microenvironment at the site of injection may have a significant effect on vaccine responses. Neutrophils and tissue-resident macrophages contribute to a pro-inflammatory environment at the site of vaccine injection that is important for recruiting other immune cells and for the priming of adaptive immune responses (60). However, as it will be discussed in more detail there is a growing appreciation that excessive local inflammation may be detrimental to vaccine responses (16).

The effects of age on the phenotype and function of NK cells have been described elsewhere (13, 61) and may as well affect the efficacy of vaccination in older people. As discussed later, NK cells have a previously unrecognized role in vaccination, contributing for protection during the early phases post-vaccination by mechanisms that involve the generation of innate immune memory (62). Thus, the effects of aging on cytotoxicity and cytokine secretion mediated by NK cells may have wider implications for immune responses to vaccination in older adults (63).

Age-related changes in innate T cells are less well described however a decreased frequency and change in phenotype of peripheral γδ T cells (64) and mucosal-associated invariant T (MAIT) cells (65) have been reported in older adults compared to young. Recently it has been described that MAIT cells in older adults have an increased baseline inflammatory profile that was associated with reduced *Escherichia coli*–specific responses in aged MAIT cells compared with their young adult counterparts (66).

Inflammaging

Aging is associated with a chronic and systemic sterile inflammatory state termed inflammaging (67). This is supported by the findings of higher levels of tumor necrosis factor (TNF), IL-6 and other pro-inflammatory cytokines in the serum of older individuals compared to young (68, 69). A variety of stimuli may sustain inflammaging, not only chronic antigen stimulation by pathogens, but also activation of the inflammasome by endogenous cell debris and misplaced self-molecules and microbial translocation due to increased gut permeability (70). Although the innate immune system, in particular the monocyte-macrophage network are thought to be at the center of inflammaging (70, 71), accumulating evidence indicates that senescent cells in general, including senescent T and B cells have an important contribution with their senescent-associated secretory phenotype (SASP) (72). Regardless of the origin, this low-grade systemic inflammation is predictive of frailty and earlier mortality (73) and is an established risk factor for many age-related diseases including heart disease, age-related macular degeneration, type II diabetes, osteoporosis and cancer (74, 75).

There is accumulating evidence that increased chronic background levels of inflammation might be detrimental for vaccine responses (76–81). Nakaya et al. investigated gene signatures predictive of influenza vaccine responses in young and old adults and found that pre-vaccination signatures associated with T and B-cell function were positively correlated with antibody responses at day 28 after vaccination, while

monocyte- and inflammation-related genes were negatively correlated with antibody responses (76). Similarly, studies on HBV vaccination in the elderly revealed that a more pronounced inflammatory gene expression profile at baseline predicted a poorer response to vaccination (77, 78). Our group has shown that older individuals exhibit reduced cutaneous immunity to varicella zoster virus recall antigen challenge associated with increased baseline local inflammation (79). Subsequently we demonstrated that infiltrating monocytes play a crucial role in the inhibition of cutaneous immunity, by a mechanism driven by increased cyclooxygenase 2 (COX2) expression and production of prostaglandin E2 (PGE2), ultimately leading to reduced proliferation of skin resident-memory T cells and reduced responses to antigenic stimulation (82). Overall, these findings support the concept that elevated baseline inflammation may have a significant role in the age-related hypo-responsiveness to vaccination and thus reducing background inflammation might be a promising strategy to enhance vaccine responses (83). This may be a particularly important consideration for older subjects who develop severe inflammation after SARS-Cov-2 where reducing inflammation may boost vaccine efficacy (84).

CURRENT STRATEGIES TO IMPROVE VACCINE EFFECTIVENESS

Current recommendations for vaccination in older adults include vaccines against influenza, herpes zoster, pneumococcal disease and a booster against tetanus and diphtheria. Despite being able to mitigate the severity of the disease to some degree, these vaccines often fail to induce protective immunity in the elderly. Several approaches are currently in place to improve vaccine effectiveness in this population [discussed in detail elsewhere (4)] and largely focus on the use of adjuvants, higher antigen doses and alternative routes of immunization.

Influenza Vaccines

Adjuvanted influenza vaccines are now the first choice for those over 65 years in countries such as Austria and the United Kingdom (UK) to overcome the low effectiveness of standard vaccines in the elderly (85). Data from the 2018/19 influenza season in the UK, the first season after the introduction of adjuvanted vaccines for persons above 65 years, demonstrated better protection from pneumonia-associated hospitalizations and laboratory-confirmed influenza cases with adjuvanted compared to non-adjuvanted vaccines (86). Studies have demonstrated that the addition of MF59® to influenza vaccine enhanced antibody production with increased seroconversion and seroprotection rates (87), improved antibody binding affinity and a more diverse antibody epitope repertoire (88) and induced broader serological protection against drifted strains (89) providing support for the use of adjuvants in influenza vaccination of older populations. Despite this, a study comparing cell-mediated immune responses to vaccination in adults ≥ 65 years old randomized to receive one of 4 seasonal influenza vaccines—standard subunit, MF59 adjuvanted subunit and split-virus vaccines given intramuscularly or intradermally —found no benefit of the MF-59 adjuvanted formulation over non-adjuvanted formulations delivered by intramuscular and intradermal routes (90).

Alternatively, the use of high-dose influenza vaccines in individuals over 65 years has also been shown to induce higher antibody titers and seroprotection rates compared to standard-dose vaccine (91), leading to their approval for clinical use in person aged 65 and older (92). Meta-analysis of randomized controlled trials (RCTs) showed that high-dose vaccines (split-virus and subunit recombinant hemagglutinin formulations) were more effective than standard-dose vaccines in preventing influenza-like illness, influenza hospitalization and all-cause mortality in adults ≥65 years old (93). When looking at T cell-mediated immune responses, high-dose influenza vaccines had little impact on the development of functional T cell memory in older adults compared to standard-dose vaccines (31).

Another approach to improve influenza vaccine immunogenicity in older people is the use of alternative routes of vaccination. Most vaccines are delivered by intramuscular or subcutaneous injection, bypassing the mucosal immune compartment. Intranasal and intradermal routes for influenza vaccination have been developed with the aim of enhancing immunogenicity, particularly cell-mediated and mucosal immunity. Although studies suggest that intradermal influenza vaccination may enhance immunogenicity compared to standard intramuscular vaccines in persons over 65 years of age (94), pooled analysis of RCT found no significant differences in seroprotection and seroconversion rates in older adults with intradermal vaccine compared to intramuscular (95) and intradermal influenza vaccines are no longer recommended. T cell responses were also not different between intramuscular versus intradermal injection in a randomized study comparing influenza vaccines in adults ≥ 65 years old (90).

It should be noted that when comparing different types of influenza vaccines, the formulation may differ. Current licensed inactivated influenza vaccines are manufactured using either split-virus or subunit formulations. They are all designed and licensed based on hemagglutinin antibody responses but while they may induce similar antibody responses, the differences become more evident when measuring cellular immune responses to vaccination (96). Split-virus vaccine lack some of the purification steps of subunit vaccines and therefore may contain a larger amount of internal viral proteins such as matrix protein (M1) and nucleoprotein (97) that are important to elicit T cell responses (98). Co et al. showed that the presence of influenza internal proteins, M1 and NP, contained in standard-dose split-virus vaccines but not in subunit vaccines, were necessary for stimulating $CD8^+$ T cell responses measured by IFN-gamma production and by cytotoxicity assays *in vitro* (96). Importantly, a study evaluating the clinical effectiveness of split-virion versus subunit trivalent influenza vaccines in older adults using a case-positive, control test–negative study design, demonstrated a vaccine effectiveness of 77.8% (95% confidence interval [CI], 58.5%–90.3%) for the split-virion compared with 44.2% (95% CI, −11.8% to 70.9%) for the subunit vaccine (99).

Unfortunately, there are not many studies performing head-to-head comparisons between the different available influenza vaccine options for older adults comparing both humoral and T cell responses. A randomized clinical trial comparing immunogenicity of currently available vaccine options for older adults—standard-dose quadrivalent vaccine, MF59-adjuvanted trivalent vaccine, high-dose trivalent vaccine, or recombinant-hemagglutinin quadrivalent vaccine – is currently under way and it will be important for identifying improved vaccination strategies for influenza in older adults (100).

Herpes Zoster Vaccines

Herpes zoster results from the reactivation of latent varicella-zoster virus (VZV) infection. Although the reactivation of VZV can occur throughout life, the risk increases substantially with age and in conditions associated with a decline in T cell immunity. A live-attenuated VZV vaccine (Zostavax®) is approved for older adults to boost VZV-specific cell-mediated immunity (CMI). Evidence that the vaccine is partially effective in older patients comes from the Shingles Prevention Study that demonstrated a reduction in the incidence of herpes zoster and post-herpetic neuralgia by 51% and 67%, respectively (101). However, the efficacy of the vaccine was age-dependent, dropping from 64% in the age group 60–69 years to 41% in the age group 70–79 years. In addition to this, data on long-term follow-up indicates that vaccine-induced immune responses decline over time. Revaccination can have a booster effect although current evidence is not sufficient to support revaccination of older people (102).

A new adjuvanted recombinant zoster vaccine (Shingrix®) has been recently approved to prevent herpes zoster in older adults. It consists of recombinant VZV glycoprotein E and a liposome-based AS01B adjuvant system. This system consists of two adjuvants, 3-O-desacyl-40-monophosphoryl lipid A (MPL) and QS-21 formulated in a liposomal delivery system (AS01B) (103). MPL is a TLR agonist, activating the innate immune system at the site of the injection and enhancing antigen-presentation (104). Whist the molecular mechanisms underlying the adjuvant effect of QS-21 are not yet fully understood, it has been demonstrated that it induces strong and persistent Th2 humoral and Th1 cell-mediated immune responses (105). It is thought that the use of liposomal formulations facilitates the escape of the antigen into the cytosol enhancing antigen-presentation through MHC-I pathway leading to cross-presentation to $CD8^+$ T cells and an early IFN-gamma response that promotes vaccine immunogenicity (106). Interestingly, the AS01B adjuvant system seems to require the synergistic action of the three components together for optimal adjuvant effect (107). The efficacy of the adjuvanted recombinant vaccine has been demonstrated in two randomized placebo-controlled Phase III clinical trials, where the administration of two doses resulted in 97.2% protection against HZ in persons over 50 years of age (108) and 89.8% in adults over 70 years of age (109). While long-term follow-up is still ongoing, robust antibody and $CD4^+$ T cell responses were found for at least 3 years after the vaccination, although $CD8^+$ T cell correlates of protection were not identified (110). A meta-analysis comparing the two vaccines in adults over 50 years of age confirmed the superiority of the adjuvant recombinant subunit vaccine compared to the live attenuated vaccine for the prevention of herpes zoster infection despite a greater risk of adverse events at injection sites (111). An additional advantage of the recombinant zoster vaccine over the live-attenuated vaccine is its suitability to use in immunocompromised patients, including HIV-infected patients (112) and in transplant recipients (113).

Pneumococcal Vaccines

The currently available 23-valent polysaccharide vaccine (PPV-23) has been used for many years in older adults and is still the first choice in many countries. However this vaccine does not generate adequate immunological memory, as purified polysaccharides do not induce persistent antigen-specific memory B cells (114). Furthermore, responses to PPV-23 were impaired in older adults compared to young individuals (115). A 13-valent conjugate vaccine (PCV-13) has been introduced and is now the first line choice for older adults in several countries as it has improved immunogenicity compared to the polysaccharide vaccine (116). Conjugation of polysaccharide antigens enables the uptake and antigen presentation in the context of MHC-II to $CD4^+$ T helper cells resulting in the generation of memory B cells specific for the polysaccharides (114). A large randomized placebo-controlled trial demonstrated that the conjugate vaccine is effective in persons over 65 years of age, reducing the number of hospitalizations due to community-acquired pneumonia caused by vaccine-type strains by 45.6% and the number of cases of invasive pneumococcal disease by 75% (117). It is still debatable which pneumonoccal vaccine is more suitable to the elderly and this is largely reflected in the heterogeneity of the recommendations for pneumococcal vaccines from country to country. PCV-13 induces stronger and long-lasting memory responses compared to PPV-23, however PPV- 23 covers more serotypes. This is particularly relevant in the context of the serotype replacement that is seen as a consequence of routine childhood vaccination with PCV-13 leading to the reduction in the incidence of pneumococcal disease caused by vaccine serotypes while other serotypes become more prevalent (118).

Tetanus and Diphtheria Vaccines

Antibody responses to tetanus and diphtheria vaccines are also suboptimal in old age. In addition to reduced antibody concentrations in the elderly, protection is short-lasting and a second booster after 5 years did not lead to additional long lasting immunity in older people (119).

Overall, immune responses to currently recommended vaccines are suboptimal in older people. Despite the important successes achieved with strategies currently in place to improve vaccine responses in the elderly, most available vaccines still fail to elicit long-lasting immune responses and insufficiently trigger cell-mediated and mucosal immunity. Therefore, novel approaches should be explored to enhance immunogenicity and efficacy of vaccines in this population.

NOVEL STRATEGIES FOR ENHANCING VACCINE RESPONSES

Implementing New Correlates of Vaccine Efficacy

Although real estimates of vaccine efficacy can only be established in randomized, placebo-controlled trials against laboratory-confirmed cases, the standard of practice is to use surrogate markers of vaccine-induced protection against disease (120–122). Hemaglutinin inhibition (HI) assays detecting antibody responses to vaccine strains are the most widely used correlates of protection induced by vaccines. Nevertheless, studies in older adults have found a poor correlation between antibody responses to influenza vaccine and protection against laboratory-confirmed cases of influenza (7, 123). The limitations associated with over-reliance on HI assays to ascertain vaccine responses have been reviewed elsewhere (124), however there is growing appreciation that the use of HI antibody titers as a sole measure of vaccine efficacy may fail to detect important changes in cellular immunity that occur with age (6, 7). It has been shown that older adults exhibit lower T cell responses to influenza compared to young controls (125) and that preexisting $CD4^+$ T cells against conserved internal influenza proteins are important for limiting virus replication and disease severity (126). Additionally, Sridhar et al. showed that, in the absence of crossreactive neutralizing antibodies, $CD8^+$ T cells specific to conserved viral epitopes correlated with crossprotection against symptomatic influenza (127). However, T cell correlates of protection based on the frequency of IFN-gamma-producing $CD4^+$T (126) and $CD8^+$ T cells (128) have only been established in young adults and have not yet been validated in older adults. On the other hand, other studies have demonstrated that *ex vivo* T cell parameters (e.g., interferon (IFN)-gamma and IL-10 ratio, granzyme B levels) measuring cellular immune responses to influenza challenge performed better than antibody titers as correlates of vaccine efficacy in older adults (7, 129). Correlates of protection based on functional assays of CD8+ T cell cytolytic activity are important to better predict vaccine efficacy and should ideally be incorporated into the evaluation of protective immunity in the elderly (7). Nevertheless, there is still limited data on functional T cell responses to vaccines, particularly in older adults, such as $CD8^+$ T cell-mediated *ex vivo* virus inhibition assays as described in HIV vaccine development (130). Although recent data indicates that innate immune cells may be important contributors for developing effective cytolytic-mediated immunity to infection this requires a functional readout of the response to vaccination.

Novel correlates of vaccine effectiveness are needed and an evolving area of interest is the contribution of neutralizing and cross-reactive antibodies induced by vaccination to enhanced protection against disease (131). The use of functional assays such as antibody-dependent cell mediated cytotoxicity (ADCC) and serum neutralization assays to detect cross-reactive antibodies that may not necessarily be detected in HI assays has been suggested as alternative correlates of protection however they are difficult to standardize across laboratories. Likewise, the incorporation of methods to assess antibody binding affinity, specificity, and epitope diversity of polyclonal antibodies would be important for a more comprehensive assessment of the quality of immunization-induced antibody responses and for developing more effective vaccines (132). Sequencing B and T cell receptors to analyze repertoire clonality and diversity could represent a valuable tool to predict vaccine efficacy by identifying vaccine-induced clones that will respond better and for longer to a given immunogen (133, 134). Although difficult to implement as routine measure of vaccine efficacy, assessment of repertoire clonality and diversity would be important to direct the development of next-generation vaccines that provide long-lasting immunity against infection.

Searching for Novel Adjuvants to Stimulate the Immune System

Adjuvants act as enhancers of vaccine-induced immunogenicity at multiple dimensions: inducing local proinflammatory cytokine production, recruiting and activating innate immune cells, stimulating antigen presentation and ultimately boosting humoral and cellular immune responses (135). For many years, aluminium salts have been the only adjuvant in use in human vaccines. In recent years, high-throughput screening approaches have led to the discovery of many novel adjuvants. However, to date only two adjuvants (MF59 and AS01B) are currently licensed for persons older than 65 years, while the majority failed to translate to effective therapeutics mostly due to their side-effects (136). As our understanding of the mechanisms that boost immunogenicity rapidly increases, new adjuvants are being developed with focus on generating multifaceted immune responses. Recent research efforts have also focused on developing new ways to deliver old adjuvants in order to improve their function while reducing side-effects (137). The requirements for effective novel adjuvants are to boost innate and adaptive immune responses to vaccines and induce long-term protective memory as well as to counterbalance the low-grade inflammatory state that might hamper vaccine responses (136, 138). The incorporation of pathogens associated molecular patterns (PAMPs) in vaccine formulations that act as ligands for pattern recognition receptors (PRRs) on innate immune cells is a strategy already in place for enhancing vaccine-specific responses. PRR activation leads to inflammatory cytokine and type I IFNs production, facilitating antigen cross-presentation and activation of cytotoxic T cells (135). Due to their ability to induce strong cell-mediated responses, TLR ligands are attractive sources for developing new adjuvants (57, 139, 140). Some TLR agonists are already in clinical stage as vaccine adjuvants. Monophosphoryl lipid A is among the first of a new generation of TLR agonists to be already approved and in clinical use worldwide as an adjuvant in several vaccine formulations including a vaccine against hepatitis B virus (FENDrix) and human papilloma virus (Cervarix) (141). Another TLR4 agonist, glucopyranosyl lipid adjuvant (GLA) formulated in a squalene-in-water emulsion (SE), has been

shown in a first-in-human trial to improve magnitude and quality of humoral and T-helper 1 type cellular responses elicited by the ID93 tuberculosis vaccine (142). The stimulatory effect of GLA-SE is well preserved in older adults (143) and *in vitro* studies in the context of vaccination with a split-virus influenza vaccine in older adults confirmed the activation of DCs to induce a Th1 response, increasing the interferon-γ to IL-10 ratio and the cytolytic (granzyme B) response to influenza virus challenge, both of which have been shown to correlate with protection against influenza in older adults (144). However, the response to TLR agonists was impaired in aged compared to young mice (145) and the age-related defects in TLR function and cytokine production might limit the utility of TLR ligands in older adults (58, 59). Although more research is needed, the use of combinations of TLR agonists has been proven effective in experimental models and might be a possible strategy for more effective vaccination in the older population (140).

Triggering Innate Immune Memory

Effective vaccination strategies should aim at inducing protective adaptive immunity but also incorporate novel means of triggering innate immune memory to induce life-long protection against infection (146). Recent findings suggest that NK cells may play important roles in vaccination, through the modulation of adaptive immune responses and generation of innate immune memory (62, 63). NK cells can be activated following immunization through cytokines produced in response to adjuvants (147) or by direct stimulation of receptors, including TLRs (148). Thus, vaccine adjuvants can be optimized to promote activation and recruitment of NK cells to target tissues where they can positively or negatively regulate antigen presenting cells and downstream T cell responses (149). Additionally NK cells may contribute to enhanced vaccine responses through the generation of long-lived 'memory' NK cells capable of mediating rapid effector functions following re-exposure to antigen, reminiscent of T-cell memory responses (62, 150, 151). The concept of innate immune memory is relatively new and a better understanding of how memory NK cells are generated and can mediate specific recognition of antigen is important to define strategies promoting the development of these cells during vaccination.

Targeting T Cells to Induce Broad Protective Immunity

An ongoing challenge in vaccination is the development of vaccines that are able to induce broad protective immunity. This is particularly relevant for influenza where next-generation vaccines inducing T cell immunity may potentially overcome the limitations of current available vaccines that rely on antibodies to provide narrow subtype-specific protection and are prone to antigenic mismatch with circulating strains. The concept of "universal" vaccines is based on the possibility of inducing heterosubtypic immunity, whereby T cells can target diverse influenza strains by recognizing highly conserved peptides (127, 152). Studies conducted during the 2009 H1N1 pandemic provided key insights into the role of cross-reactive T-cells in mediating heterosubtypic protection in humans. We conducted influenza studies to map T cell responses before and during infection in adults with no detectable antibodies to pandemic H1N1 and found that preexisting $CD4^+$ T cells targeting highly conserved protein epitopes exhibited cytotoxic activity across strains and were important to limit viral replication and disease severity (126). By mapping the type of epitopes that were able to generate heterotypic responses across strains, the results of this work and others (153) can aid the development of broadly protective T cell vaccines (154). This may be particularly important in the context of pandemics where there is no preexisting immunity. Interestingly, a recent study done in COVID-19 convalescent patients detected circulating SARS-CoV-2-reactive $CD4^+$ T cells in 40%–60% of unexposed individuals, supporting the importance of cross-reactive heterotypic T cell responses for clinical protection and limiting disease severity (155).

Exploring New Pathways for the Development of Broadly Protective Vaccines

Innate T-cells (MAIT cells, γδ cells, and NKT cells) are attractive vaccine targets as they can link both innate and adaptive immunity by mediating TCR-dependent and independent (innate-like) functions (156). A common feature of innate T cells is their capacity to respond rapidly to danger signals and pro-inflammatory cytokines (such as IL-12, -15, -18 and Type I IFNs) in a TCR-independent mechanism and participate in the early stages of defense against certain infections. MAIT cells are abundant in human lungs where they have been shown to contribute to protection against influenza infections (157) and mucosal tissues, such as the intestinal mucosa, making them attractive targets for mucosal vaccine design. Recent studies have shown that MAIT cell frequencies can be rapidly 'boosted' through mucosal administration of synthetic MAIT cell ligands with TLR agonists (157, 158) and this could be particularly beneficial for the elderly who have impaired MAIT cell immunity.

Bystander activation by cytokines is a feature shared by a subset of conventional T cells, particularly $CD8^+$ T cells. We have recently shown that as T cells differentiate toward senescence they become less responsive to TCR conventional signaling while acquiring innate-like functions (32). The reprogramming of highly differentiated $CD8^+$ T cells from TCR to NKR functional activity provides them broad protective functions that can be beneficial in the context of aging (35) and might be also relevant for vaccination.

Another area of potential interest is the use of monoclonal antibodies that selectively block inhibitory receptors to boost T cell function. In light of the unprecedented results obtained with the use of checkpoint inhibitors (e.g., PD-1, CTLA-4) in cancer, new avenues of research are open for the use of these immunomodulators in other settings, including vaccination (159, 160). Interestingly, improved vaccine responsiveness has been linked to reduced frequencies of $CD4^+$ and $CD8^+$ T

lymphocytes expressing PD-1. For instance, immunological responses to the live-attenuated zoster vaccine in individuals over 50 years of age were correlated with pre-vaccination levels of regulatory T cells and PD1-expressing T cells, regardless of the age of the vaccine (161). *Ex vivo* blocking experiments corroborated a role of PD1 and CTLA4 as modulators of decreased VZV responses (161). A study on the responses to a trivalent inactivated influenza vaccine in lung cancer patients receiving PD-1 blockade therapy compared to age-matched healthy controls showed comparable serological protection but an increased rates of immune-related adverse events (IRAEs) (162) although a subsequent study found no increase in incidence or severity of IRAEs in patients on immune checkpoint inhibitors who received the flu vaccine (163). While more research is needed on the safety and efficacy of such combinations of immune checkpoint inhibitors with vaccines, this combinatorial approach has been tested and proved efficient in preclinical and clinical trials using therapeutic cancer vaccines with anti-PD1 (164, 165) or anti-CTL4 (166) monoclonal antibodies. As the expression of inhibitory receptors on T cells has been shown to increase with age and differentiation (37, 167) the selective blockade of inhibitory receptors known to regulate T cell activity could be explored as means of boosting cellular responses in the elderly prior to or during vaccination.

Blocking Baseline Inflammation to Boost Vaccine Responses

Responses to vaccination vary widely across individuals and are generally poorer in particular groups including not only the elderly but also individuals with autoimmune diseases, HIV infection (168) and cancer (169). A common feature among these groups is the presence of a chronic inflammatory background that has been associated with adverse health outcomes (170). Furthermore there is a growing appreciation that pre-existing inflammation may be a determinant of vaccine responsiveness and thus modulating baseline inflammation prior to vaccination has become an attractive area of research to boost vaccine responses (16, 83, 171). Using high-throughput technology researchers have identified baseline transcriptional signatures that predict protective immune responses to vaccines (76, 78–81). Most of the signatures identified so far are indicative of broad immune activation and excessive inflammation. For example, a study comparing responses to the yellow fever vaccine in an African cohort compared with a Swiss cohort found that an activated immune profile of NK cells, monocytes and differentiated T and B cell subsets was associated with reduced responses to vaccination (81). Our group has previously shown that older individuals have decrease ability to mount recall responses to VZV antigen challenge in the skin (172) and this was subsequently associated with increased baseline local inflammation (79). Ingenuity pathway analysis indicated that this inflammation was driven by the activation of p38 MAP kinase pathway in the skin of old individuals compared with young. Short-term systemic treatment with an oral p38 MAPK inhibitor (Losmapimod) significantly increased the cutaneous VZV response in older subjects (79), supporting the concept that anti-inflammatory interventions may be promising strategies for boosting immunity during aging. Furthermore, oral administration of an mTOR inhibitor (Rapamycin) prior to influenza vaccination of older adults resulted in increased antibody titers against all three strains of a trivalent influenza vaccine by more than 20% in individuals aged above 65 years (173). Other immunomodulator agents such as metformin, imiquimod (174) and anti-inflammatory drugs inhibiting COX2 expression (175) (e.g., aspirin and NSAIDS) that are currently approved for clinical use in other settings may represent attractive approaches to promote more effective vaccine responses by transiently alleviating chronic inflammation prior to vaccination. Finally, it is likely that targeting other sources of inflammaging by changing the composition of the microbiome (176) or selectively removing senescent cells using senolytic drugs (177) may represent further opportunities for enhancing vaccine immunity in the setting of chronic inflammation.

REFLECTIONS ON COVID-19 VACCINATION STRATEGIES FOR THE ELDERLY

The discussion about the impact of aging on immunity and vaccination is particularly relevant at the moment as the COVID-19 pandemic placed again the spotlight on the vulnerability of older adults to emerging infectious diseases. Epidemiological data reveals that individuals over 60 years of age are disproportionately affected by SARS-CoV-2 infection experiencing the most severe forms of disease and the highest hospitalization rates (178–180). Age is a strong predictor of death among patients hospitalized with COVID-19 (181, 182) and a review of epidemiological data from different countries revealed an exponential increase in case fatality rates with age, regardless of the geographic region (183). Despite being the most affected risk group, older adults are the least likely to respond to a new vaccine. This represents a major challenge for vaccine development and thus it is critical to understand how immunosenescence and inflammaging impact on vaccine responses to ensure that vaccination remains effective in this age group (184). To meet this need, leading vaccine developers Oxford University/AstraZeneca (ClinicalTrails.gov number: NCT04516746), NIAID/Moderna Therapeutics (NCT04405076) and BioNTech/Pfizer (NCT04368728) are currently recruiting adults over 55 years of age to evaluate efficacy, safety and immunogenicity of their vaccine candidates in older individuals. However, due to intricacies of clinical trial design with strict inclusion/exclusion criteria most COVID-19 vaccine studies may fail to include a sufficient number of older individuals, in particular those in their 70s and 80s. As of 3 of September 2020, the COVID-19 vaccine development landscape includes 33 vaccine candidates in clinical trials, of which 6 candidates are currently in phase III clinical trials (185).

Despite the promising preliminary reports of their phase I/II trials (186, 187), current vaccine front-runners have not yet published results on the vaccine safety and immunogenicity in elderly. Relaxing the eligibility criteria and ensuring an adequate representation of the groups most affected by COVID-19 disease - such as elderly people, those with comorbidities and people from black, Asian and minority ethnic groups – is of key importance for successful vaccination strategies for COVID-19.

Trials in older adults are also important to understand why immune responses to COVID-19 infection and vaccination may vary from person to person. A recent study performed deep immune profiling of 125 COVID-19 patients and identified immune profiles associated with poor clinical outcomes (97). Severe COVID-19 disease was associated with an immunotype characterized by the paucity of circulating follicular helper cells and the presence of highly activated $CD4^+$ and $CD8^+$ T cells, with increased frequencies of highly differentiated $CD8^+$ T cell "EMRAs" and exhausted $PD1^+$ $CD8^+$ T cells, providing evidence for the association between an immunosenescent phenotype and disease severity. Other studies have shown that severe COVID-19 disease correlated with elevated serum concentrations of inflammatory cytokines including interleukin-6 (IL-6), granulocyte colony-stimulating factor (G-CSF), IP-10, MCP1, macrophage inflammatory protein 1α (MIP1α) and tumor necrosis factor (TNF) (188–191). Among these, IL-6 has received particular attention (189) providing support for several clinical trials on IL-6 receptor antagonists as potential treatments for severe COVID-19 disease (192). Accumulating evidence suggests that the pathophysiological hallmark of COVID-19 disease is severe inflammation with descriptions of a cytokine storm syndrome (193, 194) induced by a dysregulated monocyte/macrophage response (195, 196). As previously discussed, the presence of low-grade sterile inflammation characterized by high baseline serum concentrations of pro-inflammatory cytokines including IL-6 is a hallmark of aging (70) and is predictive of early mortality (73). Thus, it can be speculated that inflammaging is one of the mechanisms underlying increased morbidity and mortality due to SARS-CoV-2 infection in older adults (196). As pre-existing inflammation may also be detrimental to vaccine responses it has been proposed that reducing inflammation with short-term course of mTOR or p38 MAPK inhibitors and possibly other anti-inflammatory agents (e.g., steroidal drugs such as dexamethasone) may be used as a strategy for improving COVID-19 vaccine responses in older people (84).

CONCLUDING REMARKS AND UNSOLVED QUESTIONS

Despite the important successes achieved with current vaccines, most available vaccines still fail to elicit long-lasting immunity in older adults. Current vaccine strategies must evolve to be able to enhance cell-mediated and mucosal immunity in addition to inducing long-lasting antibody responses. However, to date most clinical trials leading to vaccine approval in older adults rely entirely on antibody responses as correlates of protection and thus novel correlates of vaccine effectiveness are needed that fully reflect the changes occurring with age in the immune system. The use of system vaccinology approaches can aid researchers in identifying signatures that predict protective immune responses and this information can be used for optimization of current vaccination strategies. Responses to vaccination vary widely across individuals and baseline immune profiles matter to determine the outcome of vaccination. Recent data suggests that excessive baseline inflammation is deleterious and may hamper immune responses and thus novel approaches aimed at reducing inflammation may offer novel opportunities to improve vaccine responses in older individuals. Yet the prevailing view is that adjuvants improve vaccine responses by promoting local inflammation. Thus more research is needed to understand the role of inflammation in vaccine responses and to reconcile these seemingly paradoxical observations. It could be speculated that the effects of systemic versus local inflammation are distinct and that the beneficial effects of anti-inflammatory drugs on vaccine response result from the systemic reduction of the low-level chronic inflammation. Additionally, chronic immune activation may be associated with desensitization or tolerance to new antigenic stimulation resulting in poor immune responses. Thus stronger adjuvants may be needed to overcome this tolerogenic state and alleviate the consequences of chronic inflammation. There is a need to develop newer and more specific adjuvants, able to fine tune immune responses and selectively stimulate pathways that lead to long-lasting immune protection. As our understating of immunosenescence and inflammaging increases new individualized approaches could point towards the development of more effective vaccines for older individuals.

AUTHOR CONTRIBUTIONS

BP has done the literature search and writing. AA and X-NX contributed for the writing and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Immunosenescence and Inflammaging: Risk Factors of Severe COVID-19 in Older People

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Old individuals are more susceptible to various infections due to immunological changes that occur during the aging process. These changes named collectively as "immunosenescence" include decreases in both the innate and adaptive immune responses in addition to the exacerbated production of inflammatory cytokines. This scenario of immunological dysfunction and its relationship with disease development in older people has been widely studied, especially in infections that can be fatal, such as influenza and, more recently, COVID-19. In the current scenario of SARS-CoV-2 infection, many mechanisms of disease pathogenesis in old individuals have been proposed. To better understand the dynamics of COVID-19 in this group, aspects related to immunological senescence must be well elucidated. In this article, we discuss the main mechanisms involved in immunosenescence and their possible correlations with the susceptibility of individuals of advanced age to SARS-CoV-2 infection and the more severe conditions of the disease.

Keywords: COVID-19, coronavirus, aging, immunosenescence, inflammaging, SARS-CoV-2

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INTRODUCTION

Human history is marked by major epidemics, and viral respiratory infections have been major villains in this scenario. The 20th century was certainly marked by the devastating outbreak of the Spanish flu, caused by an influenza A virus of the H1N1 subtype (1). Currently, in the 21st century, coronaviruses appear to show their potential, with three epidemics in the past two decades.

Coronavirus epidemics include severe acute respiratory syndrome (SARS) coronavirus (CoV) (SARS-CoV-1), which occurred between 2002 and 2003, and Middle East respiratory syndrome (MERS)-CoV, which occurred in 2012 (2). Since December 2019, SARS-CoV-2, a new type of coronavirus, has caused respiratory infections ranging from mild to severe clinical conditions and death, and the disease caused by it has been called coronavirus disease 2019 (COVID-19) (3).

The current SARS-CoV-2 outbreak originated in the city of Wuhan in China (4), rapidly spread worldwide and was declared a pandemic by the World Health Organization. By September 20, 2020, COVID-19 had already infected more than 30 million people and caused over 950,000 deaths (5). In this global pandemic scenario, the United States and Brazil are the countries with the highest

number of cases and deaths from COVID-19 (5). In Brazil, approximately 51% of SARS cases due to COVID-19 occur in old individuals (over 60 years of age), accounting for 73% of deaths (6).

In fact, it was observed that older people have a higher severity of the disease and are considered the main risk group for COVID-19 (4). This observation has been reinforced by SARS-CoV-2 infection in experimental models, where infected aged Syrian hamsters developed alveolar and perivascular edema (7). A greater severity in individuals of advanced age has also been reported in SARS-CoV-1 (8) and MERS (9). Interestingly, in addition to advanced age, male gender appears to be another risk factor for COVID-19 (10). Additionally, other conditions such as obesity, hypertension, and metabolic diseases are also risk factors for COVID-19 (11).

The following additional four coronaviruses circulate globally among the population: alpha (229E and NL63) and beta (OC43 and HKU1) CoVs. These coronaviruses generally cause mild infections of the upper respiratory tract similar to the common cold (12). However, there are reports of more severe respiratory diseases caused by OC43 and 229E, mainly in older individuals and individuals with chronic immune deficits (13).

Thus far, it is known that cytokine storm in the lungs may be among the immunological components involved in the pathogenesis of COVID-19 in the aged population. Although it has been suggested that alveolar macrophages from older individual have an anti-inflammatory profile, they can develop higher and uncontrolled responses of cellular activation and cytokine production after a pathogen insult and a lower ability to control tissue damage due to infection leaving the lung in a compromised state (14–16). In fact, at baseline state, the lungs of old individuals show increase in levels of complement and surfactant proteins and pro-inflammatory cytokines (15, 16). Interestingly, half of fatal cases of COVID-19 experience a cytokine storm, of which 82% are over the age of 60 (17).

Notably, a series of immunological changes occur with age, causing older individuals to develop immunosenescence (18). These factors can contribute to as pulmonary as systemic exacerbated inflammatory response in older individuals and seem to play a role in increasing susceptibility to respiratory infections.

In fact, a better knowledge of these mechanisms can contribute to the understanding of the infection dynamics in this scenario. Thus, here, we review the main factors related to the senescence of the innate and adaptive immune responses that can be responsible for both the severity and pathogenesis of COVID-19.

BRIEF BACKGROUND OF CORONAVIRUS INFECTION

Coronaviruses have a positive single-stranded RNA genome of approximately 30 kb that forms the viral nucleocapsid with the nucleocapsid (N) protein. This structure is surrounded by an envelope formed of a lipid bilayer in which the spike (S) proteins, membrane (M) protein, and envelope (E) protein are inserted (19).

The coronavirus subfamily consists of four genera, i.e., α, β, γ, and δ coronaviruses, and the α and β genera are responsible for human infections (20, 21). Among the coronaviruses that infect humans, seven are known to cause diseases with flu-like symptoms, but SARS-CoV-1, MERS-CoV, and, more recently, SARS-CoV-2 have gained greater notoriety for their high transmission capacity and severe infections (22–24).

The transmission of coronaviruses occurs mainly through respiratory droplets and close contact between people. Once in the body, the viruses enter target cells when protein S binds to specific input receptors. SARS-CoV-2 S protein binds angiotensin-converting enzyme 2 (ACE2), which is present on the surface of several human cells (25). In addition, several studies have been suggesting that the MERS-CoV receptor, dipeptidyl peptidase 4 (DPP4), can also be used by SARS-CoV-2 during infection (26, 27). The interaction between SARS-CoV-2 and ACE2 recruits the transmembrane protease serine 2 (TMPRSS2), which promotes S protein priming and facilitates viral entry in the host cell (28). Other cellular proteins, such as the protease furin, can also promote SARS-CoV-2 S protein cleavage indicating their potential involvement in viral entrance (29). Once inside the cell, the envelope fuses with the endosomal membrane and releases the viral genome into the cytoplasm where replication and assembly of new viral particles occurs (30).

Coronavirus infection can affect the airways, causing cough, headache, and fever. In more severe cases, the infection can cause tissue damage, especially to the lung tissue, due to the high degree of inflammation generated to fight the virus, leading to the development of pneumonia and dyspnea, which can progress to death (31). Among patients with COVID-19, the highest incidence of severe cases occurs in individuals affected by comorbidities such as lung diseases, diabetes, and hypertension (32–34). Age also appears to be a risk factor for the disease, as worse outcomes and higher mortality rates are observed in older patients (35–37).

IMMUNOSENESCENCE: INNATE IMMUNITY AND SUSCEPTIBILITY TO COVID-19

Can Inflammaging Enhance Immunopathogenesis in Old Individuals?

The aging process can be understood as a progressive and natural decrease in the biological functions of an organism (18). Despite its enormous plasticity and capacity for renewal, the immune system is also affected during the aging process. Since a functional immune response is essential for maintaining homeostasis and health, the immune aging process, called immunosenescence (**Figure 1**), contributes to the increased susceptibility to infections, cancers and autoimmune diseases (38–40).

A very striking feature of the immunosenescence process is a low-grade proinflammatory state, with an increase in serum

inflammatory mediators, such as IL-6, IL-1RA, TNF-α, IL-1, and C-reactive protein (CRP) (41, 42). This low-grade inflammatory state named "inflammaging" is associated with the diminished ability to mount efficient immune responses during the aging process (42) (**Figure 1**).

Inflammaging is caused by a set of hormonal, metabolic and immune factors that constantly provide stimuli that are recognized by innate receptors, favoring an inflammatory environment (43). In addition, senescent cells commonly experience changes in their intracellular homeostasis, including telomeric perturbations and oxidative stress, leading to the activation of signaling pathways such as nuclear factor κB (NF-κB) and increased secretion of cytokines, chemokines, growth factors and lipids (44, 45). This condition in which senescent cells change their secretory phenotype is called the senescence-associated secretory phenotype (SASP) and is a potential contributor to inflammaging (46). The exacerbated inflammatory process associated with age may also be due to a failure to resolve inflammation since many regulatory factors are also deficient in older individuals (47–49).

The inflammatory stimuli that support the phenomenon of inflammaging can be triggered by several factors, including chronic infections and microbiota changes, which are going to be more detailed further in this text. However, sterile components naturally produced during cell cycle can also contribute to this phenomenon. Cellular debris resulting from the cell death process that occurs daily due to chemical and physical stresses as well as the accumulation of metabolic products and cellular catabolic products, such as lipofuscins and beta-amyloid proteins play a crucial role in inflammaging (50, 51). Under the physiological conditions of cell proliferation, such components are usually diluted between dividing cells. However, as the cell proliferation rate reaches its lowest levels due to aging, these molecules accumulate and can be recognized by pattern recognition receptors (PRRs) (52, 53).

In addition, infectious processes during aging can further accentuate the inflammatory condition by releasing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (54). During cytomegalovirus (CMV) infection, which infects 40–100% of the population worldwide (55), inflammatory mediators such as prostaglandin E2, IL-6 and TNF-α are released, highlighting the important contribution of this pathogen to inflammaging (56–58). However, a 10-year longitudinal study compared the impact of CMV infection on the serum levels of inflammatory cytokines in 249 individuals and showed that cytokine production in CMV-seropositive and CMV-seronegative individuals is similar (59).

Studies focusing on the current SARS-CoV-2 pandemic have already investigated the association between the pathogenesis of the disease and the inflammatory process. Regardless of the age group, patients affected by COVID-19 have higher plasma concentrations of inflammatory cytokines, such as TNF-α and

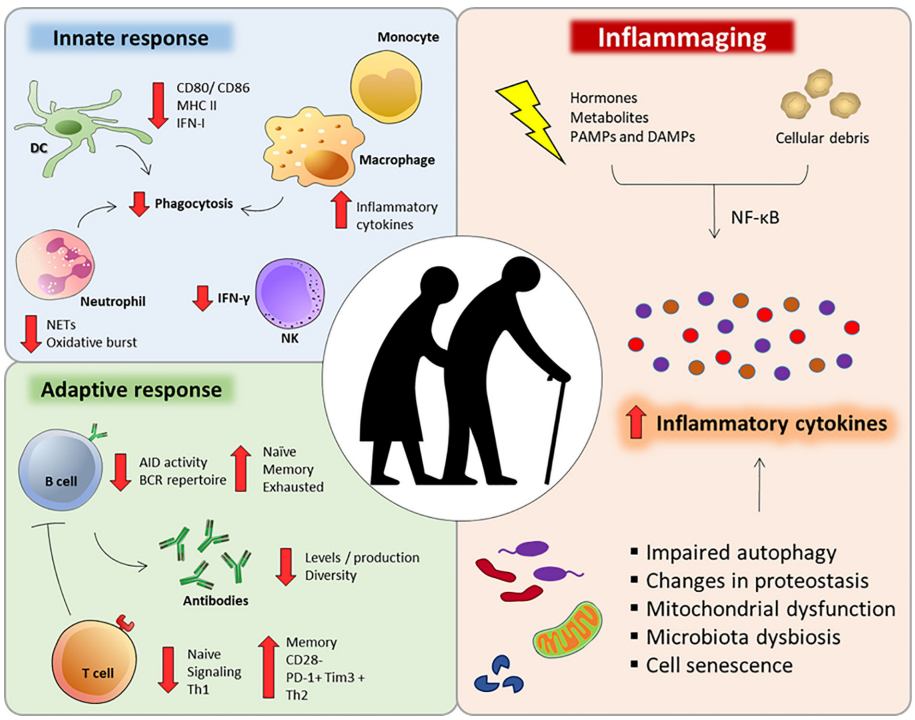

FIGURE 1 | Major immunological alterations observed during immunosenescense. Aging interferes in a number of innate and adaptive immune cells aspects that can impair or compromise their function and response. Additionally, several factors can dysregulate intracellular homeostasis during aging, intensifying the secretion of inflammatory cytokines and chemokines (inflammaging).

IL-6, and the chemokines and molecules that activate cells, such as CXCL10, CCL2, CCL3, G-CSF, IL-2, IL-7, and IL-10 (24, 60). There is also a circulating increase in others well-known inflammatory markers, such as CRP, ferritin, D-dimer, and serum amyloid A (SAA) (61–64).

Additionally, *in vitro* SARS-CoV-1 studies have found that the viral cytopathic effect induces apoptosis in Vero E6 and HEK293 cells (65, 66) and that MERS-CoV promotes apoptosis in lung and kidney cells *via* Smad7 and FGF2 (67). A similar effect has also been observed in the HCoV virus (229E) in monocytic cells (68). These findings suggest that target cell apoptosis is a factor contributing to the tissue damage caused during *in vivo* infection. Potential DAMPs released during apoptosis can contribute to the local and systemic inflammatory response by activating PRRs, further aggravating the infection. Additionally, *in silico* studies have indicated that a strong protein-protein interaction exists between the viral S protein and TLR4, a PPR, suggesting that SARS-CoV-2 directly activates proinflammatory pathways (69).

One of the main intracellular pathways resulting from the activation of PRRs is NF-κB, which is the main pathway responsible for inducing the inflammatory response and the appearance of the SASP phenotype (70). DAMPs can also signal *via* the NLRP3 receptor, leading to the activation of the inflammasome pathway and secretion of the inflammatory cytokines IL-1β and IL-18 (71). Interestingly, serum IL-18 levels increase with age, indicating that the pathway strongly contributes to inflammaging (72). Higher levels of IL-18 were also observed in the serum of COVID-19 patients and were associated with disease severity and clinical outcome (73). Moreover, monocytes infected *in vitro* with SARS-CoV-2 presented the formation of NLRP3 puncta, and the same could be observed in mononuclear cells isolated from COVID-19 patients, indicating activation of the inflammasome pathway (73). In fact, NLRP3 inhibitors have already been proposed as potential drugs for the treatment of COVID-19 (74).

In addition, the autophagy pathway seems to be directly related to the development and progression of the inflammaging process. This pathway consists of specialized protein machinery that promotes the recycling of cellular content, generating nutrients and energy for maintaining homeostasis (75). Therefore, autophagy contributes to the elimination of the debris and products of cellular metabolism, preventing its recognition by PRRs and the consequent inflammation (76). However, it has been shown that there is a reduction in the activity of the autophagy pathway during aging (77). Additionally, deficiencies in other pathways that regulate proteostasis during aging, such as reduced proteasome activity, contribute to the accumulation of misfolded protein aggregates that can activate inflammatory pathways (78).

Preliminary studies in DAF2 mutant invertebrates, a longevity study model, indicate that silencing autophagy pathway genes reduces life expectancy in these organisms (79). Additionally, in a clinical trial, Mannick et al. (2018) demonstrated that enhancing the autophagy pathway using mTOR inhibitors reduces the incidence of infections in older individuals and promotes the expression of antiviral genes and a better response to vaccination against the influenza virus, corroborating the importance of the autophagy pathway in the immune response and fighting infections in individuals of advanced age (80).

Another consequence of reduced autophagy during aging is a lower rate of mitophagy, which leads to the accumulation of dysfunctional and damaged mitochondria, changes in the respiratory chain and the generation of reactive oxygen species (ROS) (81, 82). Oxidative phosphorylation products, such as ATP and ROS, induce an inflammatory response by activating the inflammasome pathway (83, 84). In an experimental model, it has been verified that the influenza virus induces the production of mitochondrial ROS, contributing to inflammation, higher viral titers and increased neutrophil infiltration in the airways and lungs (85). It has also been found that oxidative stress generated by H5N1 infection induces the formation of oxidized phospholipids, which activate the TLR4-TRIF pathway in pulmonary macrophages, inducing the inflammatory response (86). In fact, in the context of COVID-19, it was recently shown that mitoquinol and N-acetyl cysteine, two antioxidant drugs, prevented SARS-CoV-2 infection in human primary monocytes (87).

Additionally, mitochondrial lesions generated by stress lead to the release of DAMPs, such as mitochondrial DNA (mtDNA) rich in CpG motifs and bacterial DNA, and, therefore, can activate the inflammatory response *via* TLRs, NLRs and cGAS (88, 89). In this context, a positive correlation was found between the increase in mtDNA and proinflammatory cytokines such as TNF-α, IL-6 and CCL5 during aging (90).

Furthermore, it has been speculated that mitochondrial dysfunctions could be involved in the older population's greater susceptibility to viral infections since the functioning of MAVS, a protein that assists the RIG-IRF-IFN cascade located in the mitochondrial membrane, depends on the integrity of the mitochondria and oxidative phosphorylation (91, 92). SARS-CoV-1 infection induces mitochondrial fission and MAVS degradation, suppressing the host's antiviral response (93).

The intestinal microbiota can also play an important role in modulating the proinflammatory response during aging (94, 95). Over time, the composition and diversity of the microbiome changes, leading to dysbiosis in the host and a predominance of Th1-type responses (95, 96). Simultaneously, there is an increase in intestinal permeability with aging, favoring bacterial translocation and inflammaging (95). It has been observed that centenary individuals have a greater prevalence of opportunistic bacteria with proinflammatory characteristics in the intestinal microbiota and a reduced number of bacteria with anti-inflammatory properties. These data are strongly correlated with the serum levels of inflammatory cytokines such as IL-6 and IL-8, suggesting that the microbiota also contributes to the maintenance of inflammaging (97).

Some studies suggest that an exacerbated immune response is mainly responsible for the worsening of SARS-CoV-1 and MERS-CoV infections by contributing more to tissue damage than the actual infection, regardless of the age group (98).

Regarding COVID-19, these effects do not seem to be any different. Excessive immune activation and production of proinflammatory cytokines are commonly observed in patients with COVID-19 (99). This exacerbated immune response involving high levels of cytokine release is known as cytokine storm syndrome. Although inflammatory responses are crucial for pathogen clearance, uncontrolled immune responses can be destructive by leading to systemic inflammation, vascular hyperpermeability, multiple organ failure and eventually death (100). In viral infections that reach the lungs, cytokine storm syndrome contributes to apoptosis in epithelial and endothelial cells, leading to fluid leakage in the lungs, the accumulation of leukocytes and tissue fibrosis (101), which, in turn, cause ARDS (102).

Considering the abovementioned aspects, it is possible that the inflammaging process favors the greater severity of COVID-19 in the aged population (**Figure 2B**). Although experimental reports are still scarce in the literature, several researchers have proposed that inflammaging could contribute to the more severe outcomes of COVID-19 in older patients (10, 103). In fact, Guaraldi and colleagues demonstrated that treatment with tocilizumab, a monoclonal anti-IL-6 receptor antibody, could attenuate COVID-19 severity in patients older than 60 years (104).

Other Aspects of Innate Immunity That May Favor SARS-CoV-2

The cells of the innate immune system can be quantitatively and qualitatively affected by the aging process. In the case of monocytes, there is a prevalence of nonclassical and intermediate subtypes associated with a lower phagocytic capacity (43). Monocytes from older individuals also secrete less IFN-α, IFN-γ, IL-1β, CCL20 and CCL8 when stimulated with adjuvants of the innate immune response (105), although some studies have suggested that these cells have a greater capacity to secrete proinflammatory cytokines under baseline conditions or after stimulation in older individuals (106–109). Recently, Zheng and colleagues reported an increase in the monocyte population in aged healthy adults, especially classical CD14 monocytes (110). Monocytes from aged individuals have higher expression of inflammatory genes, such as IL1B, TNF and CXCL8, and increased activation of the NF-κB, Toll-like receptor, inflammasome, and MAPK signaling pathways (110).

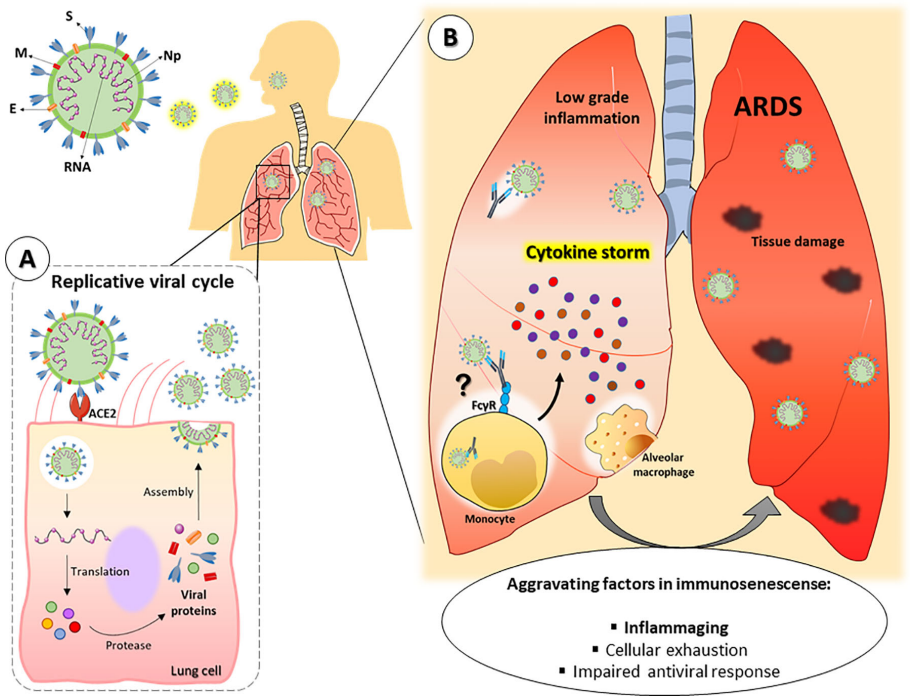

FIGURE 2 | Hypothetical framework of SARS-CoV-2 pulmonary infection in old individuals. SARS-CoV-2 consists of single RNA strand and the following proteins: Spike (S), membrane (M), envelope (E) and nucleocapsid (Np). **(A)** After entering the organism, the virus infects lung cells by binding to the receptor angiotensin-converting enzyme 2 (ACE-2) and establishes its replicative cycle releasing new viral particles. **(B)** Older people have a constitutive low-grade proinflammatory state that, along with other peculiarities of the immunosenescence, can favor the cytokine storm syndrome, leading to a faster progression to ARDS and severe manifestations of COVID-19. In addition, tissue resident or lung-infiltrating immune cells (e.g., neutrophils, monocytes and alveolar macrophages) can contribute to disease severity either by dysfunctional responses associated to immunosenescence or by facilitating viral internalization through ADE. ARDS = Acute respiratory distress syndrome. ADE = Antibody-dependent enhancement.

In the case of infection by SARS-CoV-2, there is a greater production of IL-6 and GM-CSF in the peripheral blood by $CD14^+$ $CD16^+$ monocytes (61). Additionally, *in vitro* infection of human monocytes with SARS-CoV-2 leads to the production of several inflammatory cytokines, such as IL-6, IL-1β, TNF-α, and IFN-I (87). Moreover, COVID-19 patients of advanced age have more monocytes than younger patients (110). These cells with an inflammatory profile can migrate into the lungs, contributing to the exacerbated inflammatory response and consequent tissue damage characteristic of the pathogenesis of the disease. In fact, because of their inflammatory properties, monocytes have been suggested to be among the main contributors to the disparate severity of COVID-19 in older patients (111).

The changes occurring in immunosenescence also affect antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages (112). In both cell populations, antigen presentation is compromised in the old population, possibly due to the lower expression of CD80, CD86 and MHC-II after exposure to a stimulus (113, 114) and a lower production of superoxide anion by macrophages after treatment with IFN-γ (115). *In vitro* studies investigating DCs derived from peripheral blood monocytes show that infection with MERS-CoV induces the expression of MHC-II and CD86 and promotes the production of IFN-γ, CXCL10, IL-12, and CCL5 (116). However, whether DCs from the older people respond similarly to infection by MERS or other coronaviruses is unclear.

In addition, SARS-CoV-1 is capable of infecting monocyte-derived DCs, rendering these cells producers of inflammatory cytokines, such as TNF-α and IL-6, and chemokines, such as CCL2, CCL3, CCL5 and CXCL10 (117). However, it was not possible to identify the production of antiviral cytokines, such as IFN-α, IFN-β, IFN-γ and IL-12p40, which may indicate a possible viral escape mechanism mediated by blocking these pathways. In fact, Hu et al. showed that the SARS-CoV-1 N protein interacts with TRIM25, preventing the generation of IFN-I *via* RIG-I (118). In COVID-19 patients, an imbalanced production of IFN-I has also been reported, and it seems to correlate with disease severity (119, 120). Moreover, SARS-CoV-2 infection elicits reduced expression of IFN-I and interferon-stimulated genes (121). In addition, pretreatment with IFN-α or IFN-β reduced SARS-CoV-2 titers in *in vitro* infection studies (122, 123). In fact, IFN-I administration has shown promising results in COVID-19 patient clinical trials (124). In a phase 2 study, the triple combination of lopinavir–ritonavir, ribavirin and interferon beta-1b was efficient in reducing symptoms, shortening the duration of infection and hospital stay in patients with mild to moderate COVID-19 (125).

Interestingly, in old individuals, the population of plasmacytoid DCs (pDCs), which is among the main mechanisms of fighting viral infections, is reduced and has less capacity for IFN-α secretion when stimulated with influenza virus due to the deficient expression of TLR7 and TLR9 (126–128). Complementarily, aged human monocytes have imbalanced IFN-I and IFN-III production in response to influenza infection due to defective induction of IFN transcription (129). Taken together, these findings suggest that a reduced IFN-I response in advanced age can contribute to poor clinical outcomes of COVID-19.

Another peculiarity that is possibly associated with the greater susceptibility of old individuals to viral infections is the reduced ability of DCs to perform cross-presentation due to dysfunction in mitochondrial activity and changes in the membrane potential of this organelle (130). These data indicate that the greater susceptibility of the advanced age population to infections may be associated with a lower functional capacity of phagocytes to eliminate pathogens and promote adequate activation of the adaptive immune response.

Aging also contributes to changes in alveolar macrophages (131, 132). During aging, there is a reduction in this cell population in the lungs, which is associated with the downregulation of pathways related to the cell cycle and upregulation of pathways associated with the inflammatory response (14). In fact, alveolar macrophages in animals with an advanced age are in a greater state of cellular activation, secrete more proinflammatory cytokines in response to a *Mycobacterium tuberculosis* stimulus and are refractory to an IFN-γ stimulus (16). Additionally, studies using murine models of influenza infection indicate that alveolar macrophages have a lower ability to control tissue damage due to infection (14). In addition, there is lower expression of the CD204 receptor, suggesting a reduced phagocytosis capacity of cellular debris that could contribute to increased tissue damage (14). In an experimental model of infection by coronavirus hepatitis virus type 1 (MHV-1), the depletion of alveolar macrophages contributes to the reduction in mortality and morbidity caused by the infection (133). In fact, SARS-CoV-2 infection in transgenic mice bearing human ACE2 leads to macrophage infiltration into the alveolar interstitium and alveolar cavities (134), and macrophage activation syndrome is associated with severe respiratory failure in COVID-19 patients (135), suggesting that this cell population plays a crucial role in the pathogenesis of the disease.

Neutrophils in old individuals are also affected by the immunosenescence process. During infections in older people, neutropenia may occur due to the lower proliferative capacity of neutrophil progenitor cells when stimulated by G-CSF (136). However, in SARS-CoV-1 infection, an increase in circulating neutrophils and an association between the infiltrates of this cell type in the lung and the severity of the injury have been observed (137). A similar scenario is observed in SARS-CoV-2 infection, where high neutrophil to lymphocyte ratio in peripheral blood have been reported in severely ill patients (138). Besides, lung infiltration of neutrophils was observed in autopsied COVID-19 patients, revealing capillary extravasation and neutrophilic mucositis (139). These findings indicate that neutrophils not only contribute to systemic inflammation in COVID-19 but also play a crucial role in local tissue damage.

Other characteristics of senescent neutrophils include lower microbicidal activity and a deficiency in the phagocytosis of opsonized bacteria, possibly due to a reduction in CD16 and the oxidative burst mediated by Fc-type receptors (140, 141). Some studies even suggest a deficiency in chemotaxis and release of ROS and neutrophil extracellular traps (NETs) in neutrophils in

old individuals (140, 142, 143). However, there is a higher release of NETs in COVID-19 patients, and plasma from infected subjects induces NET release in neutrophils from healthy donors, indicating the participation of these cells in the immunopathogenesis of the disease (144).

The natural killer (NK) cell response is also compromised in old individuals. There is a prevalence of NK CD56dim cells, a cell population with high cytotoxic capacity and production of IFN-γ, and a decrease in NK CD56bright cells, which have a high capacity for cytokine and chemokine production (145, 146). NK cells produced in older people also produce less IFN-γ in the absence of stimulation, which helps to explain the greater susceptibility to viral infections during this stage of life (147). In an animal model of influenza infection, a decrease in NK cells in the lungs, with less capacity for IFN-γ production and degranulation, was observed (148, 149). Similarly, clinical observations of patients with COVID-19 revealed a significant reduction in this cell population during SARS-CoV-2 infection (99). In addition, Zheng et al. reported impaired NK function in severe COVID-19 patients, expressing higher levels of the NKG2A receptor, a cellular exhaustion marker, indicating impaired antiviral immunity (150). However, single-cell analysis of lung bronchoalveolar immune cells revealed a significant increase of NK cells in patients with COVID-19 when compared to healthy controls (151). In addition, in a senescent mice model of SARS-CoV-1 infection, NK cells have been shown to migrate to the lungs (152), indicating a possible contribution of these cells in coronaviruses infection pathogenesis.

Taken together, these findings lead us to propose that innate immune cell dysfunction linked to immunosenescence could be involved in the greater COVID pathogenesis in old individuals either by promoting a less efficient response for fighting the infection and/or favoring an exacerbated inflammatory response.

IMMUNOSENESCENCE: ADAPTIVE IMMUNITY AND SUSCEPTIBILITY TO COVID-19

Can Exhausted T Cells Compromise the Cellular Response Against SARS-CoV-2?

Changes due to aging are also present in the adaptive immune response and are associated with the functional impairment of T and B lymphocytes (153). The sum of these changes renders old people vulnerable to new emerging infectious diseases, as recently observed with SARS-CoV-2. The most prominent factor involves a decrease in the number of naïve cells because of thymic involution (154), an increase in memory/exhausted T cells and a reduction in B cell progenitors in the bone marrow (155). Consequently, these changes reflect the cumulative effect of previous and persistent infections in older individuals (156).

Initial studies involving patients with COVID-19 in China have observed decrease in peripheral lymphocytes was observed (24, 32). This lymphopenia was more prominent in the cases with more severe disease, and 42% of these patients were aged ≥65 years (32). SARS-CoV-1 patients also have been reported to have reduced circulating $CD4^+$ and $CD8^+$ T cells (157, 158). Indeed, in more severe cases of COVID-19, there is a reduction in $CD8^+$ T cells (159), which could prevent an adequate cytotoxic response to fight the virus. Taken together, this profile has been proposed as a biomarker for diagnosis (160).

However, recent data from Arunachalam et al. evidences an increase in effector CD8+ T cells population in infected patients in comparison to health donors in an American and Chinese cohort (161). This could reflect the fact that COVID-19 has distinct effects in different population. In addition, the enhancement of effector T cells has been associated with recovery of SARS-CoV-2 infection (162, 163).

Whether the reduction in the number of T lymphocytes in old individuals could be a condition that predisposes such patients to more severe pathogenesis by COVID-19 remains unknown. However, analysis of immune cell sequencing showed that SARS-CoV-2 enhances T cell polarization from naïve to effector cells and that aging promotes the expression of SARS-CoV-2 susceptibility genes, mainly in T cells (110). In addition to lymphopenia, other age-related comorbidities are predictive of severe/critical cases and a high fatality rate during COVID-19 (37).

Individuals of advanced age have an increase in memory T cells with oligoclonal expansion and a decrease in the T cell receptor (TCR) repertoire (164, 165). These senescent T cells are mainly characterized not only by a low proliferative potential after activation but also by a shortening of telomeres and low telomerase activity, high production of ROS and constitutive activation of p38 MAP kinase, which once activated, blocks signaling *via* TCRs (166). Therefore, the inhibition of p38 MAPK could restore the proliferation and activation of telomerase in senescent T cells.

Phenotypically, senescent T lymphocytes can be identified by the expression of surface markers ($CD28^-$, $CD27^-$, $CD57^+$ and $CD45RA^+$) (167–169). In old individuals, the decrease of CD28 has been linked to persistent antigenic stimulation, and with each cycle of proliferation, its expression on the cell surface decreases (170). In addition, telomere shortening occurs, characterizing the process of replicative senescence in T lymphocytes (171).

$CD28^+CD27^+$ undifferentiated T cells have long telomeres, while highly differentiated or senescent $CD28^-CD27^-$ cells have shortened telomeres (172). This phenotype ($CD28^-$) is also observed in persistent human immunodeficiency virus (HIV), CMV infections and chronic inflammatory diseases such as rheumatoid arthritis (171). Under these conditions, a persistent antigenic stimulus occurs that leads to replicative senescence.

Several studies indicate that senescent T lymphocytes express the exhaustion molecules $PD\text{-}1^+$ and $Tim3^+$, a phenotype also observed in infections by lymphocytic choriomeningitis virus (LCMV), HIV and HCV (173). Exhausted cells have a low functional capacity, which could prevent the adequate cellular response to the virus, favoring viral escape and intensifying the pathogenesis of COVID-19 in old individuals. We base this hypothesis on studies showing that in SARS-CoV-2 infection,

$CD4^+$ and $CD8^+$ T lymphocytes also have PD-1^+ and $Tim3^+$ expression, prominently in $CD8^+$ T cells (159, 174).

In addition, changes in cytokine expression/secretion contribute to the development of immunological senescence. For example, IL-2 is decreased in old individuals, directly impacting the activation and proliferation of T cells (175, 176), which can lead to changes in the intensity and duration of the immune response and contribute to the immunosenescence process. In addition, senescent T cells also secrete high levels of the proinflammatory cytokines IFN-γ and TNF-α (166).

Regarding $CD4^+$ helper T cells, the older people have a lower proportion of IFN-γ/IL-4 produced by memory $CD4^+$ T cells, with increased Th2 cytokines and decreased Th1 cytokines, which may be a mechanism compensating for the increase in the proinflammatory state characteristic of the aging process (177). Interestingly, patients with severe COVID-19 (mean age of 61 years) also have decreased T-cell IFN-γ production (178). In addition, it has been shown that there is a lower frequency of memory $CD4^+$ T cells producing IL-17 (179).

The functional impairment of the $CD4^+$ T cell response contributes to the increase in pathology during influenza infection in old individuals (180). The same seems to be true for COVID-19 infections, since patients affected by the most severe form of the disease (mean age of 50 years) develop pathogenic Th1 lymphocytes that coexpress IFN-γ and GM-CSF and are associated with a hyperinflammatory response in the pathogenesis of the disease (61).

$CD4^+$ T cells may also contribute to the production of cytokines in the cytokine storm, which is a main mechanism associated with the pathogenesis of COVID-19 in old individuals (181). In patients with severe COVID-19, $CD4^+$ T cells express high levels of OX40 (159), a molecule involved in the production of cytokines by T cells (182).

However, an adequate balance between pro- and anti-inflammatory immune responses is essential for preserving health in old individuals. In fact, in severe cases of COVID-19, the evolution to acute respiratory distress syndrome (ARDS) and respiratory failure is a rapid process, which can occur before adaptive response establishment, emphasizing that excessive innate immunity (such as inflammaging) and inadequate regulatory responses may favor the evolution of the infection.

Regulatory T cells (Tregs) are potentially capable of suppressing the immune response and guaranteeing homeostasis (183). The number of naïve circulating Treg cells decreases while the number of memory Treg cells increases with age (184). Although both are suppressive, these different subtypes act at distinct sites in the body, according their expression of chemokine receptors. In addition increase in memory Treg cells is associated with a poor humoral response to influenza vaccination in older individuals (184). In mice, an increase in Treg cells at the expense of helper T cells has also been observed with age (185). Interestingly, patients with more severe COVID-19 present with fewer Treg cells than patients with less severe COVID-19 (186).

Immunosenescence studies are essential for understanding the greater susceptibility of older people to severe respiratory failure induced by viral infections. The presence of exhausted lymphocytes with a low functional capacity compromises the efficient antiviral cellular response, and changes in regulation favor the inflammatory status. These aspects appear to contribute to the severity of COVID-19 due to the cytokine storm.

Can Previous Antibodies in Old Individuals Aggravate the Pulmonary Condition of COVID-19?

Another important aspect of immunosenescence associated with the adaptive immune response concerns changes in B cells and the consequent failure of the humoral response. Memory B cells have a limited B cell receptor (BCR) repertoire, leading to a decrease in the humoral response to new antigens, with less efficient antibodies and less avidity (187).

A decrease in the ability to produce high-affinity antibodies in old individuals may result from defects in T cell signaling for the adequate activation of B cells, such as inadequate support mediated by T follicular helper cells (T_{FH}) (188). Thus, many vaccines are ineffective in old individuals, rendeing them highly vulnerable to newly emerging pathogens, such as SARS and rapidly evolving viruses, such as influenza (189).

In an experimental model of influenza A infection, compared to young mice, aged mice showed a lower frequency of T_{FH} cells and germinal center B cells, with reduced IgG titers but not IgM titers, but the IgM levels do not seem to depend on age (190). Thus, during the aging process, there may be some intrinsic impairment in B cells that compromises their functionality (191).

COVID-19 cohort studies show that seroconversion is observed on approximately the 10th day after symptom onset by increased IgM and IgG antibodies against the viral proteins N and S (60, 192). An age-dependent increase in the viral load (mean age of 66 years) was observed, but there was no correlation between age and the antibody levels. Interestingly, COVID-19 patients with associated comorbidities show lower levels of specific antibodies than COVID-19 patients without associated comorbidities (192).

A subset of B cells called age-associated B cells (ABCs) **identified in mice** has been closely related to the process of immunological senescence and minimally responds BCR and CD40 binding (193–195). ABCs have the potential to inhibit the growth of B cell precursors through the effects of TNF-α, inducing pro-B cell apoptosis (196, 197).

The transcription factor E47 is involved in the regulation of most B cell functions and is negatively regulated in splenic B cells in aged mice, promoting a reduction in the activation of activation-induced cytidine deaminase (AID) and class-switch recombination (198). In older humans, B cells have an age-dependent lower expression of E47 and AID, an associated decline in the number of memory B cells that have undergone class switching (IgG^+ or IgA^+) and an increase in naïve cells ($IgG^-/IgA^-/CD27^-$) (199).

CD27 expression is related to somatically mutated B cell subsets (200, 201) and accordingly, CD27- and CD27+ B cells represent naïve and memory B cells, respectively. In fact, others studies also found higher number of naïve ($CD27^-$) than memory ($CD27^+$) B cells in individuals of advanced age (202–204).

Old people also have a reduction in the number of circulating B cells (205). In contrast, it has been observed that a double-negative **(DN)** B cell subtype (IgD^-CD27^-), which is the counterpart of ABCs in humans, is increased in the peripheral blood of older individuals (206). These **DN** B cells, also called late memory or exhausted cells, are associated with the failure to respond to the influenza vaccine in old individuals. **DN** B cells show SASP, with greater expression of proinflammatory cytokines (TNF, IL-6, and IL-8) and microRNAs associated with inflammation (miR 155/16/93) and are dependent on metabolic signaling *via* MAPK (207). These cells were increased in cases of chronic inflammation, such as HIV infection (208), and in systemic lupus erythematosus (209). For COVID-19, DN B cells are also significantly increased in severe patients (163, 210, 211) but is still unclear if this conditions is dependent on age.

In addition, recent studies have shown that seronegative healthy donors have SARS-CoV-2-specific CD4+ T cells, albeit at lower frequencies, which is indicative of cross-reactivity due to infection between circulating "common cold" coronaviruses (212–214). However, it is unknown whether the older individual could have previous cross-reactive antibodies to the new coronavirus.

In this context, a humoral immune response mechanism widely proposed to be associated with the severity of COVID-19 is related to the possible presence of a phenomenon called antibody-dependent enhancement (ADE) (215–217). ADE occurs when non-neutralizing antibodies generated in a previous viral infection bind Fcγ receptors (FcγR) present in host cells and promote viral internalization. This phenomenon has already been observed in dengue, yellow fever and HIV infection (218). In fact, ADE has also been demonstrated in other coronavirus infections, such as SARS-CoV-1 and MERS (215, 219). In COVID-19, ADE in phagocytes such as alveolar macrophages and lung-infiltrating monocytes could favor SARS-CoV-2 replication in the lung tissue (**Figure 2B**). In addition, the activation of these phagocytes through FcγR could contribute to the cytokine storm in these patients (220). Considering the decrease in the quality of antibody production in older individuals, it is reasonable to think that ADE could be involved in COVID-19 pathogenesis in advanced age patients.

As previously mentioned, different coronaviruses circulate among the population. Therefore, it is plausible that older people have been more exposed to these circulating viruses throughout their lives, thus generating a greater repertoire of antibodies, which could favor a more severe ADE-dependent COVID (**Figure 2B**). This hypothesis is reinforced since children show less susceptibility to SARS-CoV-2 infection (181) considering that their immune system is still developing and that they have had less time to be exposed to antigens. This hypothesis is also reinforced by the fact that some studies show rapid seroconversion to IgG in some patients with SARS-CoV-2 (221).

ADE can also occur when antibody concentrations decrease as a result of waning immunity, as observed by diluted antibodies for SARS-CoV-1 (219). Thus, high levels of antibodies can neutralize the virus, while subneutralizing concentrations could increase infection (222).

It is worth mentioning that highly neutralizing antibodies, such as those proposed to be generated by some SARS-CoV-2 candidate vaccines (223, 224) or those present in convalescent plasma used as treatment for some COVID-19 patients (225, 226), should not trigger ADE.

The IgG-mediated humoral response could also contribute to more severe pulmonary pathology. Compared to patients who recovered within the first 15 days after the onset of symptoms, the patients who died of SARS-CoV-1 had higher levels and faster development of neutralizing anti-S antibodies (227). In addition, in a nonhuman primate model, the previous presence of anti-S IgG antibodies resulted in more severe acute lung injury, with an increase in inflammatory cytokines (CCL2 and IL-8) and recruitment of monocytes/macrophages in the lung (228). These antibodies appear to promote activation *via* FcγR in these cells since their blockade reduced the inflammatory condition. The role of the virus-specific antibody response in lung injury in the pathogenesis of COVID-19 is still unknown.

The presence of immune complexes (ICs) worsens lung injury in viral infections by H1N1 influenza (229) and respiratory syncytial virus (230). Another severe lung disease has also been associated with IC deposition, which promotes not only FcγR-dependent cell activation but also complement system activation and consequent tissue damage (231). It is known that the aging process predisposes individuals to autoimmunity (232); however, whether the accumulation of ICs in old individuals is related to the severity of COVID-19 is unknown.

ICs have a high molecular weight, can be deposited in vessels and tissues, and can activate the complement system, thereby aggravating inflammation (233). In fact, the SARS-CoV-2 N protein has been shown to promote the activation of the complement system lectin pathway and aggravate lung injury in an animal model (234). In addition, these complement pathways were overactivated in the lungs of COVID-19 patients.

To date, no studies have proven that this senescent proinflammatory profile is dependent on B and T cells or other innate cell types and may in fact contribute to a more severe lung pathology in coronavirus-infected patients by increasing the inflammatory response and tissue injury.

CONCLUSIONS AND FUTURE PERSPECTIVES

Considering the clinical findings obtained thus far concerning SARS-CoV-2 infection and reports of diseases of a similar etiology, it is evident that the immunosenescence process, particularly the increased production of inflammatory cytokines resulting from inflammaging, plays a role in determining the prognosis of COVID-19 in old individuals. From an immunological perspective, the peculiarities of the immune system of older individuals may contribute to both the deficiency of effector mechanisms essential to fighting viral pathogens and the exacerbated inflammatory response, which can accelerate and intensify lung tissue damage. However, despite the strong evidence presented here, tests that accurately demonstrate the association between immunosenescence and the severity of

COVID-19 are essential for assisting the search for treatments and the development of vaccines for this most affected age group.

AUTHOR CONTRIBUTIONS

AP and FT contributed equally to this work. All authors contributed to the article and approved the submitted version.

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GLOSSARY

ABC	Age-associated B cell
ACE2	Angiotensin-converting enzyme 2
ADE	Antibody-dependent enhancement
AID	Activation-induced cytidine deaminase
APC	Antigen-presenting cell
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
BCR	B cell receptor
CCL	CC chemokine ligand
CD	Cluster of differentiation
cGAS	Cyclic GMP-AMP synthase
CMV	Cytomegalovirus
CoV	Coronavirus
COVID-19	Coronavirus disease 2019
CpG	Cytosine-phosphate-Guanine
CRP	C-reactive protein
CXCL	C-X-C motif chemokine ligand
DAF2	Dauer formation-2
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DN	double-negative B cell
DNA	Deoxyribonucleic acid
DPP4	dipeptidyl peptidase 4
E47	E47 transcription factor
FGF2	Fibroblast growth factor 2
Fc	Fragment crystallizable
FcγR	Fc gamma receptors
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
H1N1	Haemagglutinin-1 neuraminidase-1
H5N1	Haemagglutinin-5 neuraminidase-1
HCoV	Human coronavirus
HIV	Human immunodeficiency virus
IC	Immune complex
IFN-I	Interferon type I
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
LCMV	Lymphocytic choriomeningitis virus
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral-signaling protein
MERS	Middle East respiratory syndrome
MHC	Major histocompatibility complex
MHV-1	Murine hepatitis virus type 1
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa B
NK	Natural killer
NKG2A	CD94/NK group 2 member A
NLR	NOD-like receptor
NLRP3	NLR family pyrin domain containing 3
OX40	Tumor necrosis factor receptor superfamily, member 4 (TNFRSF4)
PAMP	Pathogen-associated molecular pattern
PD-1	Programmed cell death 1
pDC	Plasmacytoid DC
PRR	Pattern recognition receptor
RIG	Retinoic acid-inducible gene
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAA	Serum Amyloid A
SARS	Severe acute respiratory syndrome
SARS-CoV-1	Severe acute respiratory syndrome coronavirus 1
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SASP	Senescence-associated secretory phenotype
Smad7	Mothers against decapentaplegic homolog 7
STING	Stimulator of interferon genes
TCR	T cell receptor
TFH	T follicular helper cell

Th	T helper cell
Tim3	T-cell immunoglobulin and mucin-domain containing-3
TLR	Toll like receptor
TMPRSS2	Transmembrane Protease Serine 2
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cell
TRIF	TIR-domain-containing adapter-inducing interferon beta
TRIM	Tripartite motif

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Inflammaging in Endemic Areas for Infectious Diseases

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Immunosenescence is marked by a systemic process named inflammaging along with a series of defects in the immunological activity that results in poor responses to infectious agents and to vaccination. Inflammaging, a state of low-grade chronic inflammation, usually leads to chronic inflammatory diseases and frailty in the elderly. However, some elderly escape from frailty and reach advanced age free of the consequences of inflammaging. This process has been called immunological remodeling, and it is the hallmark of healthy aging as described in the studies of centenarians in Italy. The biological markers of healthy aging are still a matter of debate, and the studies on the topic have focused on inflammatory *versus* remodeling processes and molecules. The sub-clinical inflammatory status associated with aging might be a deleterious event for populations living in countries where chronic infectious diseases are not prevalent. Nevertheless, in other parts of the world where they are, two possibilities may occur. Inflammatory responses may have a protective effect against these infectious agents. At the same time, the long-term consequences of protective immune responses during chronic infections may result in accelerated immunosenescence in these individuals. Therefore, the biological markers of healthy aging can vary according to environmental, cultural, and geographical settings that reflect worldwide, and in a non-biased, non-westernized perspective, the changes that we experience regarding our contacts with microorganisms and the outcomes of such contacts.

Keywords: aging, inflammation, chronic infection, genetics, microbiota, dietary components, inflammaging

INTRODUCTION

Inflammaging, which has been described as a state of low-grade chronic inflammation associated with dysfunctional immunity, is the hallmark of immunosenescence. The consequences of both processes may lead to increased susceptibility to infection and poor responses to vaccination as well as to chronic inflammatory and degenerative diseases in the elderly. However, a critical observation coming from the studies of centenarians in Italy was that some aged individuals reach this advanced age without chronic diseases or frailty. These studies showed that unlike frail individuals, healthy European centenarians have immune-modulatory mechanisms that compensate for the inflammaging and prevent the development of chronic inflammatory diseases (1). On the other hand, many individuals living in endemic areas of infectious diseases in developing countries manage to stay clear of infection throughout life due to remodeling mechanisms of innate immunity that could be classified as inflammatory. Nevertheless, chronic exposure to infectious agents and the protective mechanisms needed to cope with it can also function as aging acceleration stimuli.

The concept of healthy aging was proposed in Europe to describe individuals who reach advanced age free of inflammatory consequences of immunosenescence (1, 2). However, this concept might be incomplete because it does not take into account environmental and geographical differences that would interfere with the effects of inflammaging. Understanding the immunological and biological consequences of living in areas where contact with infectious agents is continuous and of high intensity may provide valuable elements to broaden the concept of healthy aging.

SENIEUR PROTOCOL, CRITICISMS, AND CRITERIA TO RECOGNIZE AGING AS HEALTHY

The description of inflammaging as a major event in immunosenescence has fostered a growing interest among researchers and physicians in its effects on age-related diseases and on healthy aging, as its counterpart (3–6). Not only the number of elderlies is increasing around the world, but today they expect to live much longer (7). In the case of Brazil, recent data shows that the country has experienced an unprecedent demographic process of aging of its population when compared to more developed countries although it is not clear which is the real size of elderly population (specially centenarians) due to incorrect recording of age over the years and the quality of data (8). In this scenario, a first challenge we still have to face is to define what healthy aging is. A misinterpretation of any age-associated condition as age-determined can alter study results and its usefulness.

The first results on the immunological alterations brought about by aging were conflicting due to bias in patient selection, which was one of the reasons for the creation of the SENIEUR protocol (9). This protocol was proposed in 1984 by Ligthart and coworkers to better distinguish immunosenescence from age-associated diseases, and it consists of a set of criteria in which clinical information as well as laboratory data are evaluated. Any overt disorder that might influence the immune system should be excluded, including Crohn's disease, collagen-vascular diseases, tumors, and infections. This generated a uniformity in patient selection for immunological studies and was a huge achievement. In the same period, most geriatric clinical studies used different criteria for normal aging dividing it into usual aging (when extrinsic factors accentuate the effects of aging) and successful aging (when extrinsic factors play a neutral role). Successful aging was defined as absence or low risk of disease, high functionality, and high engagement with life (10, 11).

According to Castle and coworkers (12), 16 years after its creation, SENIEUR protocol still proved to be methodologically viable because it was able to reveal immunological differences between "healthy elderly" (who fit the SENIEUR protocol) and "almost healthy elderly" (who nearly fit). However, stringent criteria are the SENIEUR protocol strength and weakness. Some studies showed that this protocol may exclude a significant proportion of the elderly living independently at home and leading an active life (12, 13).

A different approach to evaluate the immune system emerged from data obtained in immune longitudinal studies enrolling oldest-old subjects such as OCTO and NONA studies (14). They described octogenarians and nonagenarians who had mild chronic diseases in spite of their longevity and reported that morbidity did not significantly impact on the T-cell immune risk phenotype (15). In addition, an American centenarian cohort study revealed that 24% male and 43% women fit in the survivor morbidity profile (*i.e.*, centenarian patients who had a diagnosis of an age-associated illness prior to the age of 80) (16). Probably, the huge majority of them would not be considered healthy elderly by SENIEUR protocol. Since some experts consider centenarians as the best model to study human longevity (17), this data posed doubts on the fitness of the SENIEUR protocol for understanding longevity.

The idea of disease-free elderly subjects and the exclusion of any condition that might influence the immune system, as imposed by SENIEUR protocol, narrow the attention to a very exclusive group of healthy seniors (18) who are not representative of the elderly population. Apart from that, this protocol should be continuously updated as laboratory data and diagnosis mature and also does our knowledge about age-associated diseases. Now it is widely accepted that degenerative diseases usually start many years or even decades before they become clinically apparent, and immunological alterations are present much earlier than clinical symptoms (19–21). Pre-clinical diagnosis for some conditions (such as Alzheimer disease) are also already part of clinical and research practices. Therefore, as time passed and technological advances allowed early diagnosis of these pathological conditions, elimination of the 'multimorbidity noise' when examining immunosenescence became increasingly difficult.

Although our knowledge about diseases has increased during the last decades, no augment in life span and health span was observed by singly studying them. Aging still is the main risk

factor for chronic diseases. In order to have a better "bench-to-bedside" approach, it is important to be more inclusive, and aging should be studied in association with chronic conditions. Geroscience was created in this scenario as the intersection between the biology of age-related chronic diseases and the basic biology (22). As science developed so did the concept of what is normal aging. The presence of a disease says little about the impact it may have on an older person's life. Health cannot be viewed also as simply the absence of diseases. The World Health Organization defines Healthy Ageing "as the process of developing and maintaining the functional ability that enables wellbeing in older age" (7). From an immunological point of view, the concept of healthy aging has been proposed to describe the "individuals who reach advanced age free of inflammatory consequences of senescence" (23, 24). Unfortunately, there is no good biomarker for senescence which incorporates other elements strongly correlated with aging—autophagy, mitochondrial function, cellular senescence, and DNA methylation (25). In addition, all these mechanisms are linked in a complex and dynamic network to maintain homeostasis (25). Thus, a broader approach when studying the pace of senescence will certainly be more fruitful to understand the mechanisms that trigger or remodel the alterations brought about by aging itself.

Aligned with the concept of heathy aging and geroscience, our group use the clinical-functional categorization (26) classifying elderly into three categories (*i.e.*, robust, at risk of frailty, and frail). These three categories encompass 10 sub-categories. According to this categorization, individuals who are fully independent (*i.e.*, able to perform advanced activities of daily life), autonomous, do not have sarcopenia, mild cognitive impairment, frailty syndrome (27) or complex multi-morbidity are considered robust.

Finally, it is important to stress that, depending on the research question addressed by a particular aging study, the choice of a healthy elderly control group may vary but the inclusion and exclusion criteria for a patient to be classified as reference group should be clearly stated.

AGING, INFLAMMAGING, AND REMODELING

Although aging is a physiological process characterized by several changes in the organism as a whole, this review is focused on immunosenescence since the alterations in the function of the immune system impact on other organs and tissues (28). Immunosenescence can be described as a complex and multifactorial process influenced by genetic and microenvironment that results in gradual decrease of immunological activities including effector responses and their regulation (4, 28). Therefore, aging is associated with increased vulnerability to infectious and chronic diseases, and impairment of immune responses to vaccination (29–33). Investigations on the age-related changes occurring in the immune system of different populations (Sweden, Holland and Belgium) suggested that the immune parameters associated with mortality in the elderly are context-dependent (34). Thus, a major role in immunosenescence is played by the environmental conditions. We have conceptualized this main characteristic of immunosenescence by proposing the new concept of "immunobiography", defined as the combination of type, dose, intensity, and temporal sequence of antigenic stimuli that each individual is exposed throughout life (35). Owing to its memory and plasticity, the immune system is capable of adapting and recording all these immunological experiences. The immunological history of each individual is responsible for the capability in single persons to mount strong, weak, or no response to specific antigens, thus determining the large heterogeneity of immunological responses observed in the elderly (35).

Several immunological alterations associated with aging are well described in the literature (**Table 1**), and they heavily impact the T cell compartment (29, 36). Thymic involution is a hallmark of immunosenescence responsible for the early decline in the output of naïve T cells to the peripheral blood and, consequently, for the shrinking of the T cell repertoire (29, 30). Indeed, a universally observed aging-associated immunological alteration is the decrease of naive T cells (particularly CD8+ T cells) in the peripheral blood (37). Concomitantly, chronic stimulation of the immune system results in the increase of peripheral CD4+ and CD8+ memory T cells (38). Furthermore, the inverted CD4/CD8 ratio that was identified in aged individuals has been associated with increased frequencies of terminal memory T cells and of senescent exhausted lymphocytes (expressing PD-1, KLRG-1, CD57, TIM-3) with low proliferative capacity, defects in signaling pathways, and loss of molecules necessary for co-stimulation such as CD28 and CD27 (31, 32, 36). The aging-related shift in the bone marrow maturation of hematopoietic cells towards myelocytic differentiation (39) results in a decrease in naïve B cell production and an increasing oligoclonal B cell repertoire over a span of decades (64). The lower production of naive B and T cells and the alterations in their repertoire diversity and their interactions result ultimately in a poor ability to trigger effective responses against novel antigens (33, 40).

At the same time, it is known that senescent cells, in spite of the progressive loss of their activity and proliferative ability, develop a senescence-associated secretory phenotype (SASP) producing inflammatory cytokines such as IL-6, IL-8, IL-1, IL-18, and TNF-alpha (65, 66) which contribute to the inflammaging phenomenon (60).

The term inflammaging was proposed by Claudio Franceschi and coworkers (2) to name the chronic state of low-grade inflammation that is associated with aging (67). The continuous attrition caused by clinical and subclinical infections, as well as the persistent exposure to other non-infectious antigens (food, allergens, microbiota) has been correlated with chronic activation of the immune system and with the low-grade sterile inflammation that accompanies aging (41, 53). Inflammaging is characterized by the presence of high levels of pro-inflammatory cytokines such as IL-6, IL-1-beta, TNF-alpha, IL-8, IL-15, acute phase proteins (*e.g.* C-reactive protein) and can be identified in the elderly and super-elderly (centenarians) regardless of the degree of frailty (1, 5, 53). Although not fully established, some of possible causes of inflammaging include thymic atrophy, enhanced

TABLE 1 | Summary of some changes during immunosenescence.

Compartment	Overall changes	References
Adaptive immunity		
Decrease in Naïve cell number Decrease in IL-2 production Decrease in lymphoid number Decrease in CD27 expression Decline in antibody diversity (B cells) Increase in memory cell number Increase in regulatory T cell number Increase in CD8+CD28– T cells	Shrinkage of T cell receptor repertoire; Loss of immunological space; Less responsive to immune stimulation/infection and vaccination; Less efficient responses to stress; Decrease ability to cope with environmental challenges, such as reactivation of chronic and new infections; Accumulation of senescent cells in tissue and organs; Reduced proliferative capacity of T cells; Autoimmunity; Immune dysfunctions	(1–4, 24, 29, 30, 32, 33, 36–52)
Innate immunity		
Increase in cytokine production Increase in myeloid number Increase in NK cells Increase in the activity of certain signaling pathways, while other pathways are impaired Decrease or no change in phagocytosis Decrease in chemotaxis	Chronic progressive increase in the pro-inflammatory status resulting in inflammaging; Tissue damage and organ dysfunctions; Delayed wound healing; Loss of homeostasis; Modification of interaction with T cells; Predisposition to diseases and risk of frailty	(5, 6, 28, 31, 35, 39, 44, 51, 53–63)

IL, interleukin; Nk cells, Natural killer cells.

intestinal permeability, increased damage-associated molecular patterns (DAMPs), and the accumulation of senescent cells, with a consequent rise in the SASP (2, 29, 41) (**Figure 1**). In these circumstances, SASP presents as particularity the growth arrest, the resistance to apoptosis and a specific secretome (*e.g.* IL-8, TNF-alpha, IL-1-beta, IL-6, metalloproteinases, GM-CSF) that differs from inactive cells due to the preservation of metabolic activities (61, 65, 68). This phenotype can also induce DNA damage in neighboring cells by a paracrine effect and impacts on the microenvironment of the surrounding tissue impairing functioning, accelerating aging, and predisposing to age-related diseases (66, 69). Furthermore, extracellular vesicles (EVs) released from senescent cells spread pro-senescence signals that contribute to the propagation of SASP and inflammaging (70).

Interestingly, not all aged individuals have this profile, and studies on healthy centenarians and nonagenarians showed that these unique populations develop compensatory mechanisms called "immune remodeling" that allowed for the control of the deleterious effects of immunosenescence (42, 62). Data based on the stringent criteria proposed by SENIEUR protocol (9) have confirmed that senescence is not necessarily related to dysfunction of the immune system, but to a continuous immunological adaptation (42). Such process occurs heterogeneously among individuals, and it is well established that not all elderly have dysfunctional immunity, infections, chronic diseases, and frailty (24, 71, 72). Indeed, innate immune responses have been increasingly recognized for their adaptation to senescence and for their profound impact on health and longevity (24, 51). The immune remodeling is specially involved with the innate compartment. Despite the attenuation of some innate responses along the lifespan, there is a paradoxical rise in the activity of certain signaling pathways and in the production of cytokines (54). Studies have demonstrated that the cytotoxic capacity of natural killer (NK) cells is well-preserved in healthy elderly and centenarians suggesting these cells are important players in successful aging (36, 55).

Some studies also suggest that inflammaging is able to generate regulatory responses by cells with immunosuppressive phenotypes. Signals from SASP would lead to an increase in the expression and activity of the transcription factor Foxp3 in T cells and the expansion of regulatory T cells during aging (41, 56). In addition, regulatory B cells expressing IL-10 would control inflammatory effector T cells, thus aiding anti-inflammatory responses during immunosenescence (56).

Our group has studied Brazilian healthy individuals aged from 0 to 85 years and observed that the frequencies of pro-inflammatory and regulatory cytokine-producing innate and adaptive cells change during the aging process in an undulatory fashion. Although there is an increase in the frequency of cells that produce pro-inflammatory cytokines in the healthy elderly, a parallel rise in the frequency of regulatory cells could also be observed suggesting that a remodeling process takes place. IL-10-producing neutrophils and monocytes contribute to a balanced cytokine profile in these individuals (51).

In this sense, healthy immunosenescence can be seen as a result of the gradual adaptation of the organism to the deteriorative changes and the continuous stress that occurs over time (42). Indeed, according to this view, we have proposed that the body resources are continuously optimized and balanced, and successful immunosenescence consists of a potentially dynamic process of remodeling that depends on the individual's immunobiography (35). This lifelong remodeling process appears to be non-linear, as distinct fluctuations in the frequency of cytokine-producing cells throughout life was observed (51), mirroring the complex undulating changes of the blood proteome throughout life (43).

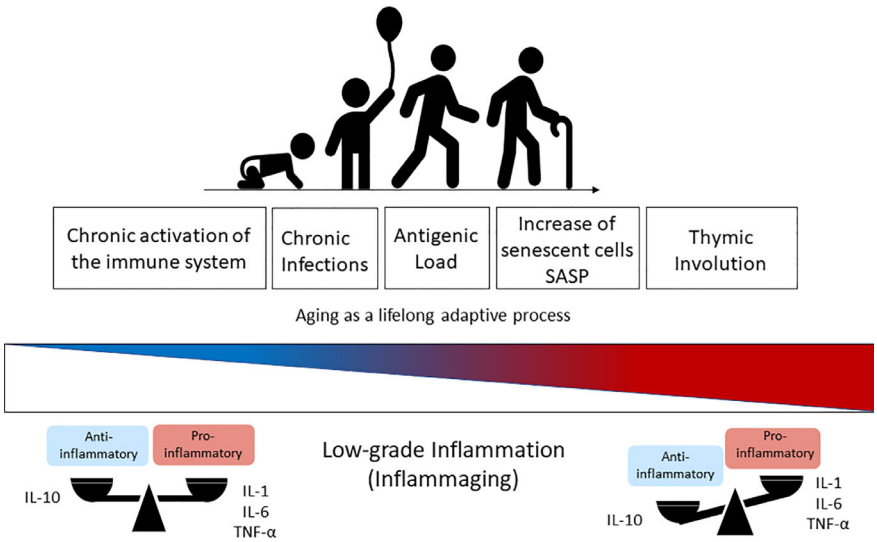

FIGURE 1 | Immunosenescence is a lifelong adaptive process that occurs naturally during aging due to the exposure to antigens. The chronic activation of the immune system through time changes the health span and the balanced profile of anti-inflammatory (IL-10) *versus* pro-inflammatory mediators (IL-1, IL6, TNF-α) found in the younger ages. This balance is lost progressively with aging and results in higher levels of pro-inflammatory cytokines in elderly people, which is known as inflammaging (low-grade inflammation) and driven by senescent cells (SASP phenotype) and thymic involution. IL-1, Interleukin-1; IL-6, Interleukin-6; TNF-α, Tumoral Necrosis Factor-alfa; SASP, Senescence-Associated Secretory Phenotype.

ANTIGENIC LOAD: STRESSORS OR IMMUNOLOGIC STIMULI?

Clonal mechanisms of antigen recognition can be identified only in vertebrate immune system, and they are known for being able to mount tolerance responses towards non-harmful self and quasi-self (food, microbiota) antigens and protective immune responses against dangerous antigens (toxic and infectious agents). However, the real immunological picture is not always that clear. In daily life, the immune system faces a complex mixture of misplaced and altered self-molecules resulting from damaged or dead cells, *i.e.* cell debris and organelles such as mitochondria components, that can be collectively designated as "garbage" (28, 63). The innate immune receptors for these stimuli are "degenerated", and on many occasions do not clearly distinguish them. Thus, innate responses can trigger not only protective responses towards non-self infectious agents but can also activate inflammatory responses towards quasi-self and endogenously produced self-components (28). Balanced responses (inflammatory and regulatory mechanisms) are essential to maintain the host health under infectious circumstances; however, both types of antigenic stimuli could activate immune cells. From an evolutionary point of view, immunosenescence could be a consequence of "garbage" accumulation and of stimulation by all antigenic stressors. Given the increase in average human life span, the immune system has to tackle an extensive variety of antigens along its lifespan (3, 28, 62). This is particularly relevant for T cells, and it results in the increase of effector and memory cells (4, 44) compromising the response to novel pathogens and to vaccination (45, 52). However, considering that not all immunological stimuli are harmful to the organism, distinct antigens might lead to different impacts on health outcome (being immunosenescence the most substantial one).

The capacity of the adaptive immune system to differentiate pathogenic microorganisms from beneficial ones, preserving symbiotic microorganisms and inducing regulatory mechanisms, can be a determinant of human survival (46, 47), but again the involved molecular mechanisms are far from being clear. Immunosenescence and, consequently, inflammaging, occur as a result of chronic exposure to different antigens, but it is also known that potential stressors, such as food protein and gut microbiota, can act as a key stimuli for the development of the immune system (73, 74).

ROLE OF MICROBIOTA AND DIETARY ANTIGENS IN THE DEVELOPMENT OF THE IMMUNE SYSTEM

The human body harbors trillions of microbial cells on the surface of the body (the skin and the gastrointestinal, respiratory, and urogenital tracts) (75, 76). These microbial populations, called collectively as microbiota, come to the highest density in the colon (gut microbiota) (75), and they are established since childhood (77–79). Millions of years of co-evolution created a mutualistic relationship between microbiota and human body; the microbiota improves many physiological functions of the host such as digestion and clearance of

potentially pathogenic microorganisms while receiving nourishment and habitat in return (80).

The gastrointestinal (GI) tract is the largest body surface that contacts the external environment with the function of food processing. It is constantly challenged by antigens from the lumen including antigens from the diet and microbiota that will be tolerated and antigens that have to be cleared such as pathogens and toxins (76, 80). Experiments in germ-free (GF) mice showed that early life colonization by the microbiota is critical for the full development of the immune system. Germ free animals have underdeveloped intestinal mucosal immune responses, unstructured spleen and lymph nodes, smaller mesenteric lymph nodes and Peyer's patches, an underdeveloped gut-associated lymphoid tissue (GALT), reduced frequencies of CD4+CD25+ Tregs as well as diminished levels of secretory IgA and serum IgG (81–84).

The most abundant phyla in human gastrointestinal tract are Firmicutes and Bacteroidetes, while Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia are subdominant divisions (85). The diverse collection of bacteria in the human gut microbiota contributes to several physiological functions by producing short chain fatty acids and vitamins (otherwise inaccessible to humans), regulating fat storage, promoting the differentiation of various cell types, protecting the host from colonization by pathogens, and creating tonic stimuli for the development/modulation of the immune system (76). On the other hand, any intestinal dysbiosis (disturbance in microbiota composition) is associated with the onset and/or aggravation of certain diseases including some autoimmune and allergic diseases, cancer, metabolic diseases, and bacterial infections (86–90). This crucial cross-talking between the human host and microbiota can be altered through dietary habits, influencing microbiota richness and diversity and potentially impacting intestinal barrier functions and the immune system (91, 92).

Food proteins also play a critical role in this context influencing the microbiota composition and creating a daily load of antigenic components. Some of us have previously shown that mice fed a balanced amino acid-based protein-free diet (Aa-fed) from weaning up to adulthood showed local and systemic abnormalities in their immune system even though they grew normally. Aa-fed mice had underdeveloped gut-associated lymphoid tissue (GALT), low levels of secretory IgA, serum IgG and IgA, low levels of type 1 cytokines and a predominant Th2 cytokine pattern produced by cells from lymph nodes and spleen resembling a neonate profile (93). Their immune response to infectious agents such as *Leishmania major* was retarded when compared to mice fed a control protein-containing diet probably due to their immature immunological status and to the poor Th1 responses they produce (73).

In addition to their tonic properties for the immune system, it is known that exposure to these luminal antigens generates a state of specific suppression of inflammatory responses known as "oral tolerance". Animals and humans usually tolerate the antigens that are present in their diets as well as their autochthonous gut microbiome (94, 95). Antigen presentation in such context would induce preferentially regulatory T lymphocytes (Tregs) producing IL-10 and TGF-beta with local and system modulatory effects. Tregs are major players in the tolerance response to the luminal antigens (95, 96), and oral tolerance can be seen as a process that evolved, as much as the digestive/absorptive process in the gut, to incorporate these materials as self-components.

As we age, it has been shown that changes in the composition and, remarkably, in the diversity of the microbiota are associated with health outcomes in the elderly, especially in the frailty context. Several studies have associated the gut microbiome with hallmarks of aging and immunity, including biomarkers of inflammation, immunosenescence, oxidative stress, and cardiometabolic health (89, 97–100). The gut ecosystem of centenarians differs equally from that of young adults and seventy-year old people (98). In centenarians, microbiota diversity is reduced with increased frequencies of pathobionts such as Fusobacterium, Bacillus, Staphylococcus, Corynebacterium and many members of Proteobacteriae. However, the increase of symbiotic species with reported anti-inflammatory properties, such as *Eubaterium limosum* and relatives, suggests that the composition of gut microbiota in centenarians undergoes a clear process of remodeling (98). Moreover, the gut microbiota of people characterized by extreme longevity, *i.e.* semi-supercentenarians (people who reached 105 years of age), show an increased capacity of xenobiotic degradation that likely contributes to their exceptional healthy aging (101). It is becoming clear that maintaining a health-associated microbiome is crucial to successful aging.

Interaction between dietary components and bacteria present in our microbiota also represents a source of interference with the immune system. The major products that result from bacterial fermentation of indigestible carbohydrates, also called "dietary fiber", in the colon are short-chain fatty acids (SCFAs), including acetate, butyrate, and propionate. SCFAs are involved in the maintenance of mucosal integrity as well as colonic homeostasis: they are able to regulate leukocyte function and to influence immune responses and disease risk by signaling through GPR receptors (GPR41 and GPR43) and by inhibiting histone deacetylase (HDAC) (80, 102).

Exposure of peripheral blood mononuclear cells and neutrophils to SCFAs blocked the pro-inflammatory nuclear factor-κB (NF-κB) and regulated the production of cytokines (such as TNF-alpha, IL-2, IL-6, IL-17, and IL-10) eicosanoids and chemokines (*e.g.*, MCP-1 and CINC-2) (91, 103–106). In this context, inhibition of HDACs by SCFA fosters an anti-inflammatory tolerogenic milieu indicating that the microbiota acts as an epigenetic regulator of body homeostasis (103).

Some dietary components, such as B vitamins and vitamins A, D, K, and E can be synthesized by the gut microbiota and have a key role in reducing inflammation, regulating energy metabolism, enzymatic functions important for gene expression and immune response regulation throughout the life course (91, 107, 108). Interestingly, both zinc deficiency and dysfunction of the immune system are accompanied by impaired immune responses and systemic low-grade chronic inflammation in aged individuals (109–112). Sodium chloride (NaCl) is a salt and a micronutrient

that also mediates immunological effects. In high concentrations, NaCl can induce alterations of gut microbiota composition, modifications of gut permeability, and inflammation in the gut mucosa, increasing the susceptibility to colitis development (113, 114). As an inflammatory stimulus, consumption of high-salt diets may interfere with the aging process.

Microbiota and dietary components are clearly innocuous natural antigens that promote immunological development at early age and regulatory immune responses throughout life. However, pathogens cannot be considered as tonic agents. Contacts with clinical and sub-clinical infections usually lead to inflammatory responses, and they may be considered as important stressors that continuously impinge on our immune system (48, 115).

HYGIENE HYPOTHESIS—INFECTIONS AS BENEFICIAL IMMUNOLOGICAL STIMULATION IN CHILDHOOD

The "Hygiene Hypothesis" and the "Old Friends Theory" advocate that childhood infections and microbiota provide immunoregulatory mechanisms that shape our immune system to cope with the continuous exposure of the body to antigens. The lack of contact with these infectious agents, due to high hygienic conditions (we became too "clean"), increases the incidence of atopic diseases, autoimmune and some chronic inflammatory disorders (116–119).

In the past few years, a large scientific effort has been focused on understanding how microbiome and parasites modulate the human immune system, especially how exposure to these antigens impacts on the incidence of inflammatory and age-related diseases (119–121). The scope of disorders affected by contact with different microbiomes and food components has been enlarged lately including allergies, autoimmunity, inflammatory bowel disease, celiac disease, food allergy, vascular disease, cancers, and inflammation-associated psychiatric disorders (117, 119, 122). However, there are pathogens such as respiratory syncytial virus (RSV) or rhinovirus that are not protective in any scenario and are usually associated with a high susceptibility to develop wheeze and asthma in children and adults alike (123, 124). In addition, the human microbiome is itself subjected to the influence of several related variables including microbial exposure, diet, lifestyle, medication, parasite infection, among others, and this network of influences may also be reflected in the immune system operation at steady state and in the onset of immune-mediated diseases such as allergy (125–129).

Therefore, the age when the contact with these antigens occurs is a determinant factor for the later immunological consequences they trigger. The antigenic load represented by food and microbiota as well as some parasitic antigens at early time in life has long lasting beneficial effects in the immune system contributing to the robustness of the regulatory immune mechanisms that operate in adults and old individuals (49, 130). Young adults still have a large repertoire of lymphocytes with high diversity and plasticity being able to mount proper immune responses when challenged by a variety of new antigens. In spite of that, it is not clear how durable are the effects of antigenic stimulation at this time point in life. On the other hand, the immune system of the elderly is less capable of properly dealing with new antigens since aging is associated with an increasing loss in repertoire diversity. At this late period of life, introduction of antigenic novelties, even if they are microbiota or dietary components, might represent a threat rather than a tonic regulatory stimulation (49).

The pleiotropy hypothesis of aging proposed by George Williams in 1957 presents an evolutionary perspective to interpret these discrepancies in immunological behavior when facing antigenic stimulation throughout life. It suggests that genes that evolutionarily resulted beneficial at young age became detrimental at old age, a period that was largely unpredicted by evolution. Such genes would be favored by natural selection by enhancing fitness early in life, a period when selection is stronger, even if they cause the aging phenotype to emerge (131). Today, it is generally accepted that antagonistic pleiotropy is common if not ubiquitous, implying that also a number of other molecular and cellular mechanisms of aging such as immunosenescence and inflammaging can be interpreted within such a conceptual framework.

GENETIC AND EPIGENETIC FACTORS AS MAJOR DETERMINANTS OF IMMUNOSENESCENCE

Aging is a natural phenomenon that affects individuals differently. While centenarians are clear examples of resilience against the detrimental effects of antigenic attrition during aging (132), some individuals present signs of aging and age-related diseases early in life. Studies on centenarians who present high levels of inflammatory mediators suggest that inflammaging is compatible with longevity (17, 132). These different performances of individuals facing aging and inflammaging may have strong genetic and epigenetic determinants (133, 134). The human lifespan is in part heritable; another part of the aging process is related to environmental factors such as injuries, lifestyle, socio-economic and education levels, and work activities (133). Heritability can increase from nonagenarians to centenarians (100+), semi-supercentenarians (105+), and supercentenarians (110+), and people who reach above 90 years of age appears to have stronger genetic basis for their longevity (135, 136).

Epigenetic factors mediate the relationship between the environment and the genome, and they are also involved in aging and age-related diseases (137). One of the most important epigenetic factors, DNA methylation, is known to influence the outcome of aging. DNA methylation is strongly related to unique individual environments, and only a small fraction of DNA methylated sites associates with familial factors (genetic or shared environment) (138). In elderly twins, for instance, a different profile of methylated CpGs is observed over time (133). Many of the

methylated genes are involved in the regulation of the immune system, especially of lymphocytes (139). Interestingly, the geographical location can alter DNA methylation patterns in family individuals supporting the hypothesis that inflammaging and immune modulation in aged individuals may vary among regions and countries.

Lifestyle and environmental factors are strongly involved in the basis of longevity and aging. Regarding dietary influences, animal studies on caloric restriction (CR) showed that mitochondrial-derived free radicals generated during ATP production are inducers of cellular senescence and aging. Indeed, caloric restriction in rodents is able to increase their life span by up to 50% indicating a correlation between oxidative stress in the mitochondria and life span (140). A randomized clinical study conducting CR during two years in people demonstrated that CR effectively controlled energy expended and oxidative stress, improving life expectancy (141). Caloric restriction and rapamycin treatment are also involved in anti-aging process, increase in lifespan, improvement of physiological functions, and reduction of pathology (137). On the other hand, a chronic inflammatory condition such as obesity is associated with decreased size of telomere length and also with increased oxidative stress in cells, leading to early aging and dementia (142, 143). Regular physical activity can be associated with decreased levels of oxidative stress and pathological inflammation. A recent study in twins revealed that sports are related with differences in telomere lengths between them (144). Another factor that impacts life expectancy is work behavior. People who work in night shifts or have irregular hours of work for more than 10 years had accelerated epigenetic age with differentially methylated CpG sites across their epigenome, including in genes for circadian rhythm (145).

There are genetic variations in certain proteins that became markers of longevity. One of them affects the uncoupling proteins (UCP1, UCP2 and UCP3) that belong to the family of mitochondrial transmembrane carriers and are regulators of the respiratory process in mitochondria. These proteins are able to decrease ATP-generation and ROS production by dissipating the proton gradient of the inner mitochondrial membrane resulting in the increased longevity observed during CR (146). Variations on sirtuin genes (*SIRT1*, *2*, and *3*) were also reported to influence the mitochondrial functionality and longevity (147, 148). *SIRT1* gene is involved in decreased oxidative stress and inflammatory response, and *SIRT3* is a mitochondrial deacetylase that reduces ROS production. Both genes are downregulated in the elderly and the activation of micro-RNA-9 (miRNA-9) can improve their functions resulting in decrease aging (149). Another study showed the involvement of micro-RNAs (miRNAs) in the development of the age-related Alzheimer's disease. Two miRNAs, mi-146b-5p and miR-15b-5p, were identified in a cohort as related to innate immune responses and regulation of cell cycle (150). A recent genome-wide analysis of miRNAs in centenarians and nonagenarians showed different clustering between the long-aged individuals and the younger controls. Cancer related proteins such as p53 and others were shown to be potential targets of these miRNAs indicating that tumor suppression and maintenance of genomic integrity are critical events during aging (151).

Genes related to cell cycle regulation and telomere length, such as *P21, FOXO3A, TERT, and TERC*, have also been described as associated with longevity (133). The cell cycle inhibitor *P21* or *CDKN1A* gene is induced by stress responses and inflammation during senescence, and it is implicated in the upregulation of several age-related genes (35, 152, 153). The *FOXO3* gene can have more than 100 SNPs, and some of them are associated with very long life span (154).

In fact, some individuals have more susceptibility to age-related diseases as dementia and cardiovascular diseases (134). Alzheimer's disease, diabetes mellitus, and cancer are common diseases among Western old people probably due to the presence of genes that predispose to those health conditions. On the other hand, some individuals from the same region are resistant to those aged related diseases probably because they lack other genes involved in disease development. The *APOE* gene, for instance, has different variants that are associated with high susceptibility for cardiovascular and Alzheimer's diseases (155–157). Another set of polymorphisms associated with age related diseases is at *FTO* (fat mass and obesity associated) gene, which are involved in increased morbidity and mortality due to increased adiposity and obesity in humans (156, 158). Polymorphisms (SNPs) in *FTO* gene have been recently described as associated with increased risk for Alzheimer's disease (159). *FTO* is also involved in cell cycle, and its silence prolongs G1 phase, reducing cell proliferation (160). SNPs in *SDC4* gene were investigated for their association with higher longevity in a cohort above 64 years old. *SCD4* encodes a transmembrane protein, Syndecan 4, that is associated with microglia activation during neuroinflammation. *SDC4* SNPs might have influence on lipid metabolism during aging, and *SDC4* gene SNP rs1981429 was negatively associated with longevity in the group between 64 and 85 years old. The same association is also observed for high triglyceride level and for low levels of LDL cholesterol. On the other hand, SNP rs2251252 seems to be associated with longevity and with high levels of LDL (161).

A simple conclusion from the genetic studies demonstrating a variety of polymorphisms involved in immunosenescence underscores the great importance of studying aging across distinct genetic backgrounds and distinct ethnical groups. A study conducted during 18 years in UK showed that the healthy condition of the offspring is associated with parental lifespan. In addition, the lower incidence of cardiovascular diseases, cancer, and reduced cognitive decline in certain populations is associated with higher parental and offspring survival (162). In a Spanish cohort, researchers observed that homozygosis in 192bp allele of *IGF-1* gene is a marker of healthy aging. Polymorphisms in this gene could be related to obesity and several derived conditions such as metabolic syndrome, cardiovascular diseases risk, cachexia and premature death (163). A study in centenarians from Italy showed five genes (*HRAS1, SIRT3, TH, INS*, and *IGF2*) associated with longevity. However the same result was not observed in individuals from

Germany (148). Therefore, different genes may be associated with longer life in distinct populations. Although centenarians usually display the same longevity of their families (164), they would show distinct ways to attain a longer and healthy life depending on their geographical location, genes and lifestyle.

THE ROLE OF INFLAMMAGING IN ENDEMIC AREAS FOR CHRONIC INFECTIOUS DISEASES

NK cytotoxicity has been described as a biomarker of immunological remodeling, healthy aging as well as longevity, and it seems to compensate for the changes/deficiencies occurring in other immune functions lost by lymphocytes during immunosenescence (42, 55, 62). Cumulative evidence in the last two decades identified a well-preserved NK cell activity in both healthy elderly individuals and centenarians (57–59, 165). Kaszubowsaka and coworkers demonstrated that the expression of TNF-alpha by non-stimulated cells was significantly higher in both $CD56^{dim}$ and $CD56^{bright}$ NK cells of aged individuals when compared to young ones. Moreover, $CD56^{dim}$ NK cells of the oldest were responsive to the IL-2 stimulation (59). As previously described, the increase of the inflammatory microenvironment associated with aging (6, 55, 166–168) may lead to degenerative and inflammatory chronic diseases (14, 20, 44, 50, 63). However, this immune profile can be important for an efficient response against infectious parasitic diseases, especially for individuals living in endemic areas.

In this context, in countries where the elderly population live in endemic areas for infectious diseases such as Chagas disease, leishmaniasis, and schistosomiasis, the presence of an inflammatory reactivity can favor these individuals against the constant challenges. Few studies on the effects of aging and specifically of inflammaging in Brazilian populations are available. Our groups are part of the few in the country working on the topic, and we have already examined the cytokine/chemokine profile of elderly from Belo Horizonte (51), Governador Valadares (89), and Bambuí (169, 170) in Minas Gerais State. Although we observed changes in the profile of pro-inflammatory *versus* regulatory cytokines throughout life in all these locations, they did not show a simple increase in inflamming-related cytokines.

It is true that life expectancy has increased in industrialized as well as in developing countries. Nevertheless, the medical challenges to deal with the aging population in these regions are very distinct. Considering that chronic infectious diseases are still prevalent in most developing countries (171), understanding the clinical outcomes of tropical diseases in elderly patients and how frailty is related to them will help to define healthy aging in different scenarios.

CHAGAS DISEASE

Although several studies have shown the importance of immune response in Chagas disease progression, the mechanisms underlying the severe forms of this disease are still elusive. The balance between inflammatory and modulatory cytokines towards an anti-inflammatory profile contributes to the control of the disease and to the development of its milder forms. Conversely, severe diseased patients with the cardiac form developed a Th1-specific immune response with inflammatory infiltrate and tissue damage (172–174). Our group studied elderly adults from an endemic area for Chagas disease analyzing the correlation between serum levels of cytokines and chemokines, *Trypanosoma cruzi* infection, and cardiac abnormality (175). When compared to healthy controls, Chagas disease patients had higher circulating levels of IL-1-beta, CXCL9, and CXCL10 and lower levels of CCL5 than healthy subjects. Interestingly and in contrast with control individuals, levels of CXCL9 and CXCL10 continuously increased with age indicating that these two chemokines are strong markers of immunosenescence in the elderly with Chagas disease (175).

LEISHMANIASIS

Visceral leishmaniasis (VL) is a neglected re-emerging chronic infectious disease in tropical and subtropical regions (176) where it is related to poor access to health care and poverty. Countries like Brazil have reported a high incidence of new cases annually (177) although it is not clear whether all cases result from recent infection or from reactivation of latent infection in patients that have chronic immunosuppressive conditions such as HIV (178) and organ transplantation (179). The rate of positive Leishmania skin test results in some areas of Brazil is extremely high in the elderly, and this might become a relevant geriatric issue (171). In Teresina, for instance, the capital of a state in the northeast Brazil, 50% of tested individuals were positive, and prevalence increased with age (180) suggesting that some of these individuals were experiencing a reactivation of a previous infection. Moreover, the overlapping of two chronic infections such as VL and HIV is reported to create an environment of persistent cellular activation inducing senescent/exhausted lymphocytes, affecting the generation of new T cells and accelerating immunosenescence (181, 182). Indeed, the thymus of patients living with HIV presents alterations in the lymphoid and stromal compartments as well as in the generation of the V-beta repertoire of T lymphocytes (183) that are typical of aging. In addition, HIV infection potentially contributes for the inflammatory immunopathogenesis of VL and, at the same time, impairs the effector immune responses to antigens, including Leishmania (184).

SCHISTOSOMIASIS

More than 230 million people are infected with schistosomiasis worldwide (185) causing a huge impact in the quality of life of affected individuals (186). Our group has studied individuals from schistosomiasis endemic areas in Minas Gerais State and

showed a clear correlation between well preserved mechanisms of innate immunity and the absence of infection in elderly subjects. We observed an increase in the frequency of IFN-gamma$^+$CD16$^+$NK cells in non-infected elderly individuals when compared to *Schistosoma mansoni* infected ones (187). Moreover, it was observed that non-infected elderly individuals present an increase in the frequency of the natural killer (NK) cells, macrophages, and dendritic cells expressing Toll-like receptors (TLR)-1, suggesting that, in endemic areas, remodeling of innate immunity mechanisms may have a protective role that could compensate for the aging-related decline in T-cell responses (188). In addition, the augmented frequency of T cells with a regulatory phenotype (Foxp3$^+$CD25$^+$CD4$^+$, LAP$^+$CD4$^+$, and IL-10$^+$CD4$^+$) observed in infected aged individuals from these endemic areas (when compared to non-infected ones) may have two consequences: they may hinder the development of protective immune responses but they also explain the absence of severe hepatosplenic clinical form of the disease during chronic infection in these individuals (189). Together, these results support the hypothesis that an inflammatory innate immune response in parallel with the decrease of regulatory mechanisms (as observed in non-infected individuals) can induce a protective immunity in elderly individuals from schistosome endemic areas. Although this can be seen as a desirable "protective profile", it also suggests that remodeling in these regions of high antigenic load (infectious agents) occurs at the expense of immune regulatory mechanisms which are important for controlling inflammaging.

ACCELERATED AGING

It is reasonable to believe that inflammaging could play a role in protective immunity in endemic areas for infectious disease. If one takes the premises of the hygiene hypothesis, exposure to infectious agents since childhood may induce both protective effector immune responses as well as robust life-long regulatory mechanisms that would prevent the spill-over effects of inflammaging causing degenerative diseases and frailty. However, inflammatory responses could have other consequences such as acceleration of the aging process itself. This may come as a price to pay for protective immunity. A hallmark of immunosenescence is the reduction in the output of naïve T and B cells and the increased frequency of memory and effector lymphocytes as a consequence of thymic involution. The continuous exposure to natural antigens (microbiota, food proteins, allergens) and the antigenic stress caused by clinical and sub-clinical infections may lead to inflammaging, degenerative diseases and frailty in senescence (190, 191). A wide range of age-related diseases including diabetes, auto-immune diseases, osteoporosis, sarcopenia, neurodegeneration, and atherosclerosis has a common inflammatory pathogenesis (53, 190). Therefore, it is expected that individuals exposed to a higher burden of antigen load would present accelerated immunosenescence, higher morbidity and mortality. If we also take into account the reduction in immunoregulatory mechanisms required in endemic areas to preserve protective immunity as reported earlier (189), and the fact that some components known to counteract cellular stress such as heat shock proteins are also diminished in the elderly (192), the accumulation of stress attrition during aging would be particularly deleterious. Although inflammatory responses mounted by individuals living in these areas are directed towards protective immunity, being infected or non-infected, the chronic exposure to infectious stressors may accelerate aging and predispose them to frailty (**Figure 2**).

After chronic and prolonged replication, cell senescence occurs as a natural part of the aging process but can potentially be accelerated in response to a variety of insults. Stress-induced premature senescence is a result of cytotoxic stimuli such as oxidative stress, proteasome inhibition or activation of *RAS*, and *Myc* oncogenes by tumorogenic agents (61, 69). These various stressors can induce cell cycle arrest, DNA damage, heterochromatin formation, increased senescence-associated beta galactosidase (SA-beta Gal) activity, expression of the cell cycle inhibitors, and the secretion of pro-inflammatory cytokines and proteases as part of the senescence-associated secretory phenotype (SASP) (65, 68). Senescent immune and non-immune cells are critical for the inflammaging phenomenon.

Some pathological conditions act as chronic stressors inducing premature senescence. Chronic Obstructive Pulmonary Disease (COPD) is considered a condition of accelerated lung aging, and senescent cells with shortened telomers have been identified in emphysematous lungs (193). Some reports also exist on the role of cancer and cancer treatment as stressors that could accelerate aging (194). On the other side of the spectrum, the most common natural process that accelerates epigenetic aging of blood cells is menopause (195).

Down syndrome is also associated with premature aging. Using DNA methylation (DNAmAge) as a measurement for biological age, Bacalini and coworkers identified an epigenetic signature of DS that sustains a link between developmental defects and disease phenotype, including premature aging (196). They found that methylated regions (DMRs) displayed a genome-wide distribution although they were enriched on chromosome 21 in genes involved in developmental functions, including neuronal (*NCAM1*), embryonic (*HOXA* family), and hematological (*RUNX1* and *EBF4*) development as well as regulation of chromatin structure (*PRMD8, KDM2B, TET1*). Interestingly, Biagi and coworkers reported alterations in gut microbiota of individuals with Down syndrome towards an overall immunomodulatory profile, when compared to that of healthy controls (197). This suggests that gut microbiome may counteract the genetic determined acceleration of immunosenescence in Down syndrome individuals.

Since infections are stressors and stimulators of immune cells, chronic infections can be potential stressors able to induce aging acceleration. In HIV infection this possibility has been already investigated. Growing evidence reveals a premature aging phenotype that accompanies HIV-infected patients (186, 187, 198). Thymic alterations in the development of T cell repertoire as well as increased frequency of senescent/exhausted T cells are part of the phenotype (182, 183). These patients are exposed to

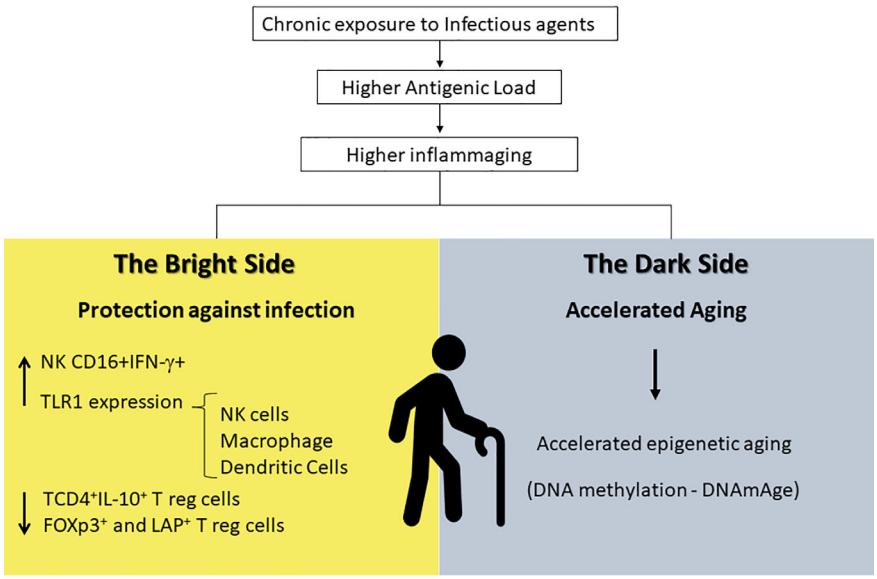

FIGURE 2 | Inflammaging is a distinct and exacerbated process in endemic areas that provides higher antigenic load and exposure to chronic infections. It seems to have a bright side resulting in protection against infectious antigens that can be explained by the increase in INF-γ-producing NK cells and in TLR-1 expression in innate immune cells (macrophages, NK, and dendritic cells) and by the decrease in regulatory T cells in general. At the same time, it has a dark side that might be responsible in accelerating aging due to epigenetic mechanisms. NK, Natural Killer; INF-γ, Interferon-gamma; TLR-1, Toll-Like Receptors-1.

several stressors including the virus itself, antiretroviral drugs, and very often, drug abuse. The fact that they suffer from a number of comorbidities commonly associated with frailty in the elderly such as diabetes, renal failure, atherosclerosis, neurological deficits, and osteoporosis also confirms the age acceleration hypothesis (198).

Other infectious diseases caused by virus might have an additional effect on aging and immunosenescence, especially in poor countries. In this context, human cytomegalovirus (CMV), a herpesvirus highly frequent worldwide, has a well-known impact in immunosenescence. Its prevalence is strongly associated with lower socioeconomic conditions and ethnicity, as verified by the National Health and Nutrition Examination Survey (NHANES) III conducted in the United States. A significant racial and socioeconomic disparity was found in CMV seroprevalence in children and young adults (199). The prevalence of CMV in German adults is 56.7% (200); it approaches 80% by the age of 70 years in Northern Europe (201); and it ranges from 8.7 to 99.2% in the MENA region (Middle East and North Africa) according to a systematic review (202).

The relevance of CMV in immunosenescence has been an important topic of discussion since the publication of OCTO and NONA studies, when it was defined as a part of the immune risk profile (IRP) and associated with increased mortality in older ages (14, 203, 204). Nevertheless, the BELFRAIL study conducted with very elderly individuals did not find a relationship between CMV infection and mortality in Belgium (205). When it comes to HIV patients, the presence of CMV co-infection seems to boost immunosenescence, not only by increasing late-differentiated CD8+ T cells regardless of chronological age, but also by promoting an accelerated telomere erosion in that same subset of cells (206).

The most pronounced effect of CMV in the immune system is memory inflation, a term used to describe the expansion of memory T cells with the accumulation of late-differentiated CD8+ effector cells (TEMRA) that re-express CD45RA, are considered senescent cells and increase with age (30, 207). Other possible contributions of the virus to senescence are the increase in inflammatory mediators and the elevated risk for age-associated morbidities, such as cardiovascular diseases, cancer, atherosclerosis, diabetes, and Alzheimer's disease (204, 206). Therefore, CMV infection seems to be a driving force accelerating immunosenescence due to its impact in T cell senescence and inflammaging. Epidemiological studies conducted in the US and in England showed a clear association between positive serology for CMV and cardiovascular disorders such as hypertension and ischemic heart disease (208–210). Cytomegalovirus can infect endothelial cells where they replicated and recruit neutrophils, monocytes, CD4, and CD8 cytotoxic T cells causing vascular damage (211, 212). The augment in the frequency of CD4+CD28− T cells, which are highly cytotoxic and producers of pro-inflammatory cytokines such as INF-gamma and TNF-alpha, seems to play a key role in the development of autoimmune and cardiovascular diseases (213). According to recent reports, increased levels of CD4+CD28− T cells are highly associated with CMV seropositivity, while aging lightly contributes to this change (214, 215). Furthermore, since CMV infection is linked to a range of cardiovascular and metabolic disorders, it might also influence negatively the clinical outcome of SARS-Cov-2 infection although positive serology for CMV alone has not been confirmed as an independent risk factor (216).

CMV-seropositive elderly individuals have a higher chance of developing age-associated chronic inflammatory diseases, but it remains unclear whether this latent infection has only a negative impact in the longevity (206). A study by Bajwa and coworkers (217) addressed the polyfunctionality of T cells (*i.e.*, several T-cell effector functions), a relevant quality for protection against virus and to vaccination, evaluating CMV-specific CD4+ and CD8+ T-cell responses to 19 different CMV target proteins in young and old volunteers. They showed that CMV specific T-cell polyfunctionality was not decreased in the healthy elderly, but it was reduced in the oldest-old group raising the question whether polyfunctional T cells in older people were necessarily associated with protection and longevity. Along the same line, Terrazini and coworkers (218) showed that most of CMV-specific CD4+ T cells have anti-inflammatory properties and may mediate a beneficial effect in aged individuals regarding cardiovascular disorders. The authors showed that CMV-specific induced regulatory CD4+ T cells (iTregs) are at high levels in older individuals, and they correlate with levels of CD8+ effector cells. A significant association between these CMV-specific T-cell subsets (CD4+ and CD8+), arterial blood pressure and vascular stiffness was found. Most of the iTreg cells expressed Foxp3; they suppressed antigen specific as well as non-specific proliferation and attenuated the inflammatory response as well as the cardiovascular pathology caused by CD8+ T cells.

Many studies have suggested that CMV effects in the immune system are age dependent. At early age, the chronic CMV infection might serve as a trigger to maintain the immune system in constant alert, enabling rapid recall responses and enhancing heterologous immune responsiveness, particularly prior to reproductive age (207, 219, 220). The inflammatory process triggered by this chronic infection can stimulate the maturation of the immune system and improve responses to homologous antigens. Additionally, there is evidence suggesting that infected young individuals present a better response to influenza vaccination (221). However, individuals that become seropositive at older ages were reported to have impaired response to vaccination (221–224). The cytokine storm triggered by CMV infection seems to compromise immune responses to influenza vaccine for instance (201). Therefore, from an evolutionary perspective, the detrimental effects of CMV infection during immunosenescence can be seen as a later consequence of its tonic role in immune responses earlier in life (220).

In endemic areas for chronic infections such as Chagas disease, leishmaniasis, schistosomiasis, and leprosy, individuals are usually exposed to infectious agents during their lifetime. In many regions in Brazil, such as the northeast of Minas Gerais, these diseases are a result of poor sanitary and economic conditions, and they co-exist increasing the burden for the immune system of the individuals who live there (189). Preliminary results from Ana Faria's group (D. Durso and coworkers, unpublished data) show that individuals from a city located in one of these areas, Governador Valadares in Minas Gerais, present an accelerated epigenetic aging phenotype as measured by DNA methylation (DNAmAge) as described by Horvath and coworkers (225).

Furthermore, we cannot rule out the possibility that the immunosenescence phenotype would be a risk factor for severe outcomes in viral infections such as COVID-19. An important characteristic of the SARS-Cov-2 infection is the pattern of high-risk groups reaching mainly individuals with underlying comorbidities such as diabetes and cardiovascular diseases and elderly people. In China, case-fatality rate was 0.4% in 40–49-year-old patients, 1.3% in 50–59, 3.6% in 60–69, 8.0% in 70–79 and reach 14.8% in >80-year-old patients (226). Similar findings were reported in Italy where case fatality rates were 12 and 20% among those aged 70–79 years and 80 years and older, respectively (227). Senescence-associated decline in immune function observed in aged people (inflammaging resulting from increased innate cytokine secretion, decline in effector and regulatory CD4+ T function and increased frequency of exhausted/senescent CD8+ T cells) may have a critical role in the development of lung and microcirculation damage and severe respiratory syndrome in SARS-Cov-2 infected elderly. This possibility has been speculated by few authors. Alterations such as lymphopenia have been identified as a tread linking COVID-19 and frail elderly. Indeed, these groups of individuals share a decline in the numbers of CD4/CD8 T cells but not of B cells (228). Others propose using biomarkers of biological age as predictors of disease severity by SARS-Cov-2 (229), and also that reversing immunosenescence would impact in the outcome of COVID-19 (230). Finally, it has been suggested that elderly with pre-existing but clinically silent CMV infection might be particularly susceptible to the severe COVID-19 since infection with cytomegalovirus is known to trigger the cytokine storm, reduction in naïve T-cell accumulation of terminally differentiated CD8+ T cells and impaired immune responses to vaccination (201). These propositions are worth further investigation.

CONCLUSIONS

The rapid aging of the population in developing countries is an unprecedent demographic phenomenon that is accompanied by the high prevalence of chronic infectious diseases among individuals who live there. This process represents a public health problem and a biological challenge. As part of the aging process, immunosenescence triggers several alterations in the immune system resulting in poor response to infection and increase in inflammation. However, inflammaging can be associated with remodeling mechanisms as the ones observed in healthy elderly. Although the concept of healthy aging has been proposed initially to describe the European individuals who reach advanced age free of the inflammatory consequences of immunosenescence, it is now clear that this concept must be broadened to encompass distinctions related to the role of inflammaging and remodeling according to genetic, epigenetic, environmental, and cultural scenarios in which the aging process takes place. Understanding these geographical differences in immunosenescence could provide a better understanding of age-related changes as well as their treatable effects. It will aid

in the prevention, diagnosis, and treatment of some age-related dysfunctions as well as infectious diseases in the elderly.

AUTHOR CONTRIBUTIONS

MAB helped in writing and organizing the first draft of the manuscript. FC-F, GS-N, GC, ES, ST, AT-C, OM-F, TM, NN, PB, and RS helped writing specific sections of the manuscript. CF helped with the original concepts and ideas as well as revising the writing of the manuscript and AMCF planned the original concepts, helped writing, and was in charge of the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Complex Role of Regulatory T Cells in Immunity and Aging

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The immune system is a tightly regulated network which allows the development of defense mechanisms against foreign antigens and tolerance toward self-antigens. Regulatory T cells (Treg) contribute to immune homeostasis by maintaining unresponsiveness to self-antigens and suppressing exaggerated immune responses. Dysregulation of any of these processes can lead to serious consequences. Classically, Treg cell functions have been described in $CD4^+$ T cells, but other immune cells also harbour the capacity to modulate immune responses. Regulatory functions have been described for different $CD8^+$ T cell subsets, as well as other T cells such as γδT cells or NKT cells. In this review we describe the diverse populations of Treg cells and their role in different scenarios. Special attention is paid to the aging process, which is characterized by an altered composition of immune cells. Treg cells can contribute to the development of various age-related diseases but they are poorly characterized in aged individuals. The huge diversity of cells that display immune modulatory functions and the lack of universal markers to identify Treg make the expanding field of Treg research complex and challenging. There are still many open questions that need to be answered to solve the enigma of regulatory T cells.

Keywords: regulatory T cells, immune homeostasis, diversity, autoimmunity, inflammation, aging

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INTRODUCTION

Immunological self-tolerance is the unresponsiveness of the adaptive immune system to self-antigens in primary lymphoid organs and further control of the activation, expansion and survival of self-reactive T and B cells in the periphery (1). The acquisition of immune tolerance is essential to avoid fighting one´s own cells and molecules. During thymic maturation of T cells somatic recombination leads to the expression of a distinct T cell receptor (TCR) on each individual T cell, which enables each T cell to recognize a specific antigen. The entirety of all T cell specificities is referred to as the repertoire. Binding of TCRs to self-peptide-loaded MHC molecules (self-pMHC) in the thymus leads to positive selection, and in a next step T cells binding self-pMHC with high affinity are eliminated by negative selection. Thymocytes can escape this clonal deletion by TCR gene rearrangement, which eventually changes the TCR affinity for self-pMHC. This central tolerance is not absolute as not all self-antigens are expressed in the thymus and due to the imperfect efficiency of the selection process. As a consequence, depletion of self-reactive T cells can

also occur in the periphery and many self-reactive T cells can enter a state of unresponsiveness called "anergy" (peripheral tolerance) (2).

Some decades ago it was shown that animals harbor self-reactive T cells but also T cells that were able to suppress the autoimmunity caused by these cells (3, 4). These suppressor cells were later called regulatory T (Treg) cells and the surface molecule CD25 was identified as the first marker for this $CD4^+$ T cell subset (5). It was not until 2003 that the transcription factor Forkhead box P3 (Foxp3) was described as a specific Treg marker by Rudensky's laboratory. They discovered that Foxp3 deficient mice developed a lethal autoimmune syndrome and lacked $CD4^+$ $CD25^+$ cells (6). However, not all suppressor/regulatory T cells express the Foxp3 transcription factor, and many of those other populations have been described even before the Foxp3 expressing T cells. In this review, we will focus mainly on Foxp3 expressing Treg, their mechanisms of suppression and their role in different contexts. Nevertheless, a short description of $Foxp3^-$ Treg cells is necessary to understand the complexity of the exquisitely regulated homeostasis of the immune system. Regulation of immune responses requires not only the control of autoreactive immune cells but also the termination of immune responses in order to avoid chronic activation of the immune system. It is important to understand how these mechanisms are regulated in order to modulate immune responses in different disease settings. For instance, we need to strengthen immune responses in chronic infection or cancer but, on the other hand, immune responses need to be dampened when there is unwanted immunological activity (e.g. in autoimmunity or graft rejection).

SUBSETS OF REGULATORY T CELLS

Naturally Arising $Foxp3^+$ $CD4^+$ Treg

Classically defined Treg are a subset of Foxp3 expressing $CD4^+$ cells which maintain peripheral tolerance by suppressing autoreactive $CD4^+$ cells that have escaped from negative selection in the thymus (7). Naturally arising $Foxp3^+$ $CD4^+$ Treg cells develop mostly in the thymus (tTreg) and require a relatively strong TCR signal which results in Treg cells having a repertoire enriched for self-antigen recognition (8). In the periphery, Treg can be generated upon TCR stimulation of naïve $CD4^+$ $Foxp3^-$ T cells in the presence of TGFβ and they are known as peripheral Treg (pTreg) (9). *In vitro*, Treg can also be produced from $CD4^+$ $Foxp3^-$ cells by mimicking *in vivo* conditions for pTreg generation (iTreg) (10). In contrast to tTreg, pTreg are likely generated upon exposure to non-self-antigens like allergens, food and microbiota (11).

It is important to keep in mind that whereas in mice Foxp3 expression is limited to Treg, many human $Foxp3^+$ T cells are more similar to conventional T cells (Tconv) than to Treg, and some activated non-suppressive Tconv express low levels of Foxp3. Miyara and colleagues defined three different human T cell populations based on the expression of Foxp3 and CD45RA: $Foxp3^{low}$ $CD45RA^+$ as resting Treg; $Foxp3^{high}$ $CD45RA^-$ as activated/effector Treg, and $Foxp3^{low}$ $CD45RA^-$ as non-suppressive cytokine-producing non-Treg (12). Thus, it is fundamental to combine Foxp3 expression with other Treg markers (CD45RA, CD127 (IL-7R), CD25) in order to identify and analyze these cells in humans (13).

$CD4^+$ $Foxp3^+$ T cells can modulate immune reactions in a direct or indirect fashion. One of the most studied direct suppression mechanisms is the production of the anti-inflammatory cytokine IL-10, which can inhibit phagocyte function, antigen presentation, co-stimulatory molecule expression, T-cell proliferation, and impairs the production of IL-2 and IFNγ. Treg-produced IL-10 promotes tolerance in the intestinal mucosa and defects in IL-10 signaling trigger inflammatory bowel disease in mouse and human (14, 15). In contrast, IL-10 can stimulate NK cell activity, B cell activation and isotype switching (16). Transforming growth factor-beta 1 (TGFβ1) signaling is associated with the development, stability and function of Treg. TGFβ1 antagonizes negative selection in the thymus, supporting early Treg development (17). In the periphery, it is essential for the differentiation of Treg from naïve $CD4^+$ Treg. TGFβ1 production by Treg and its autocrine signaling is required for Treg-mediated suppression, but several studies suggest that while it is not a major suppressor mechanism it might be needed under high inflammatory conditions (18). When $Foxp3^+$ $CD4^+$ Treg encounter effector T cells (Teff) and interact with them, one mechanism of suppression is the secretion of granzyme and perforin *via* exocytosis. By doing so, they can induce apoptosis in the target cells, e.g. in $CD4^+$ $CD25^-$ effector cells (19, 20).

Treg are able to indirectly turn down immune reactions by disturbing the optimal environment for immune responses by interfering with IL-2 availability, ATP/AMP balance, and the interface between T cells and DC. IL-2 is known for inducing and promoting T cell proliferation, but it also is involved in termination of T cell responses (21), since mice deficient in IL-2 or IL-2R suffer from a lymphoproliferative syndrome (22, 23). This negative effect on T cell activation happens indirectly by promoting the activation of anergic Treg, which then in turn suppress other T cells (24). Upon activation of naïve T cells, IL-2 is produced, which induces phosphorylation of STAT5 promoting Foxp3, Tbet and GATA3 expression and thereby the generation of Treg, Th1, or Th2 cells, respectively. At the same time, production of IL-17A and Bcl-6 and thereby differentiation towards Th17 or Tfh cells is inhibited (25, 26). Interestingly, high concentrations of IL-2 favor differentiation of effector T cells (27), whereas low IL-2 levels facilitate the production of memory T cell (28). Treg can interfere with these processes by modulating the amount of available IL-2. They suppress production of IL-2 by effector cells in a contact dependent manner *in vitro*. It has also been suggested that Treg can sense the source of IL-2 and migrate to the zones of immune activation where they "steal" IL-2 from other T cells promoting their apoptosis. This model remains controversial regarding *in vivo* studies since the source of IL-2 needs to be clarified (18).

Murine $CD4^+$ Treg express high levels of the two ectonucleotidases CD39 and CD73 which can convert ATP into non-toxic AMP and AMP into the immune suppressive

adenosine, respectively. In humans, co-expression of these ectonucleases is a rare event and most Treg express only CD39 which means they need to encounter $CD73^+$ cells in order to produce adenosine (29). Extracellular adenosine binds the A2AR receptor expressed by Treg increasing their frequency and promoting their immune modulatory function (30). In the presence of excessive inflammation and tissue damage, there is an increase of extracellular ATP, which is cytotoxic for many cell types. Extracellular ATP promotes death signaling through P2X7R engagement in sensitive Treg while some Treg survive and convert into effector Th17 cells upon exposure to extracellular ATP (31). An additional strategy of Treg to escape apoptosis and survive in such environments is the conversion of ATP into non-toxic metabolites, such as AMP. In addition, AMP can indirectly alter the expression of proinflammatory cytokines and promote the expression of inhibitory ones. This translates into a reduction of costimulatory molecules in DC (32), less activation of effector cells and a higher suppressive capacity of Treg (30).

Treg can disrupt the microenvironment in the immunological synapse provided by DC which is essential for T cell proliferation. In detail, Treg act by either reducing the limiting enzyme for glutathione (GSH) synthesis or by consuming extracellular cysteine which is needed for T cell cycle progression and DNA synthesis (33).

Treg are also capable of removing surface molecules from antigen presenting cells (APC) during the immunological synapse. They can engulf part of DC membranes containing pMHCII and co-stimulatory molecules which leads to abrogation of T cell priming (34). Moreover, the inhibitory molecule CTLA-4, which is constitutively expressed on Treg has been described to remove CD80/CD86 from the surface of antigen presenting cells during the immunological synapse (35).

Some Treg have developed specialized adaptations to their environment. As an example, VAT-Treg (visceral adipose tissue-Treg) express high levels of PPARγ in order to reduce insulin resistance associated with inflammation of fat tissue (36).

TABLE 1 | Different Treg subsets identified in human and/or mouse.

Cell population	Human	Mouse	Reference
$CD4^+$ $CD25^+$ $Foxp3^+$	✓	✓	(5, 6)
$CD4^+$ $Foxp3^{low}$ $CD45RA^+$	✓	✗	(12)
$CD4^+$ $Foxp3^{high}$ $CD45RA^-$	✓	✗	(12)
$CD4^+$ $Foxp3^-$ $IL10^+$ (Tr1)	✓	✓	(37)
$CD4^+$ $Foxp3^-$ $TGFβ^+$ (Th3)	✓	✓	(38)
$CD4^+$ $Foxp3^-$ $IL35^+$ (iTr35)	✓	✓	(39)
$CD4^+$ $Foxp3^-$ $IL10^+$ $TGFβ^+$ (Treg of B cells)	✗	✓	(40)
$CD8^+$ $Foxp3^+$ and/or $CD28^-$ and/or $CD25^+$	✓	✗	(41–43)
$CD8^+$ $CD45RA^+$ $CCR7^+$ $Foxp3^+$	✓	✗	(44)
$CD8^+$ $CD45RC^{low/-}$	✓	✓	(45)
$CD8^+$ $CD122^+$	✗	✓	(46)
$CD8^+$ HLA-DR^+	✓	✗	(47)
$CD8^+$ HLA-E^+	✓	✗	(48)
$CD8^+$ Qa-1^+	✗	✓	(48)
γδ T cells	✓	✓	(49)
NKT	✓	✓	(50)

Treg cells are categorized based on CD4 or CD8 surface markers expression.

Non-Classical $CD4^+$ Treg

As already mentioned above, there are other types of regulatory T cells that do not fit the phenotype of the classically defined $CD4^+$ $Foxp3^+$ Treg cells (**Table 1**). Many of them share the mechanisms of suppression of Treg described above, but some use different strategies.

Type 1 regulatory T cells (Tr1) are a population of $CD4^+$ $Foxp3^-$ cells expressing high levels of the anti-inflammatory cytokine IL-10. They are generated in the mucosa-associated lymphoid tissue (MALT) when naïve $CD4^+$ cells encounter IL-10 produced by APC. Tr1 cells control T cell responses in infection and autoimmunity and they have been shown to produce higher levels of IL-10 than $Foxp3^+$ Treg (37). Another subset of adaptive Treg are the Th3 cells, which are a unique population of T helper cells induced by oral tolerance to non-self-antigens. Th3 cells produce high concentrations of TGFβ and moderate amounts of IL-10 (38). In contrast to Tr1 cells which do not express Foxp3, some Th3 cells are TGFβ-induced-$Foxp3^+$ cells (51). Collison and colleagues described another inducible type of Treg (iTr35) which do not secrete IL-10 or TGFβ, but instead IL-35, an inhibitory member of the IL-12 pro-inflammatory family. This cytokine suppresses T cell responses and expands Treg by inducing the conversion of conventional T cells into suppressive $Foxp3^-$ regulatory T cells (iTr35). iTr35 cells are highly suppressive and stable *in vivo*, they are key mediators of infectious tolerance and can contribute to Treg-mediated tumor progression (39).

Similar to other components of the immune system, B cells are involved in the expansion and generation of Treg. In the periphery, naïve B cells can convert $CD4^+$ $CD25^-$ cells into $CD4^+$ $CD25^+$ $Foxp3^-$ Treg (Treg-of-B cells) in a cell-cell contact dependent manner. These Treg-of-B cells express molecules characteristic for Treg, such as IL-10, TGFβ, CTLA-4, PD-1, LAG-3, GITR, ICOS, and OX40. They exert their suppressive function *in vivo* and *in vitro* in antigen-specific and antigen-independent manners, utilizing IL-10-mediated as well as other suppressive mechanisms (40).

$CD8^+$ Regulatory T Cells

$CD8^+$ T cells were first described to exert immunosuppressive functions more than 40 years ago, but the lack of specific markers and therefore the difficulties in isolating this population limited this area of research (52). Interest in $CD8^+$ Treg cells increased again after the revival of the concept of T-cell-mediated immunosuppression in the mid-1990s and the study of $CD4^+$ Treg cells. In the following years the immunosuppressive properties of $CD8^+$ cells were independently characterized by several groups (53).

Foxp3 is preferentially expressed in $CD4^+$ $CD25^+$ cells in mice and it is barely detectable in $CD8^+$ T cells. The expression of the transcription factor HELIOS or the surface marker CD122 or the lack of CD28 on the surface are used to identify $CD8^+$ Treg in mice (46). In contrast, human $CD4^+$ as well as $CD8^+$ T cells are able to express Foxp3, but levels of this transcription factor are substantially higher in $CD4^+$ compared to $CD8^+$ cells (54). In contrast to $CD4^+$ regulatory T cells, there is no reliable marker to

define $CD8^+$ Treg cells, but they are rather identified by their immunosuppressive function.

Firstly isolated from rats, $CD8^+$ $CD45C^{low/-}$ Treg cells suppress the proliferation of $CD4^+$ T cells and their differentiation into a Th1 phenotype. They produce IL-4, IL-10, and IL-13 and express CTLA-4 and Foxp3 (45). Sorted human $IFN\gamma^+$ $IL\text{-}10^+$ $CD8^+$ $CD45RC^{low/-}$ Treg are more potent suppressor cells than the rest of the $CD8^+$ $CD45RC^{low/-}$ Treg and blockage of IFNγ abrogated their suppressive activity in a model of allogenic cardiac transplantation (55).

$Foxp3^+$ $CD8^+$ cells are rarely detected in human blood but they are found in human tonsils where they express high levels of CTLA-4 and CD45RO but only little CD127 and CD69. Tonsillar $Foxp3^+$ $CD8^+$ Treg are $CD25^-$ and express pro-inflammatory cytokines like TNFα, IFNγ, and IL-17 (41). Foxp3 expressing $CD8^+$ T cells have also been found in blood of HIV-infected individuals showing an activated (HLA-DR, Ki-67, and PD-1 expression) and senescent ($CD57^+$ $CD28^-$) phenotype (56). Several studies show that $CD8^+$ $Foxp3^+$ Treg can be induced under certain conditions. *In vitro* stimulation of peripheral blood mononuclear cells (PBMC) with anti-CD3 mAb has been shown to induce $CD8^+$ $CD25^+$ $Foxp3^+$ T cells, which were able to suppress proliferative responses to *Staphylococcal* enterotoxin B (SEB), and that the inhibitory effect was partially depending on CCL-4, TNF, and IL-2 (43). $CD8^+$ $CD28^-$ $Foxp3^+$ cells can be generated *in vitro* after multiple rounds of stimulation of PBMC with allogenic or xenogenic APC. They are believed to tolerize APC by up-regulating the inhibitory receptors immunoglobulin-like transcript 3 (ILT-3) and 4 (ILT-4) and down-regulating costimulatory molecules such as CD58 and CD86 (42). Upon suboptimal TCR stimulation in the presence of IL-15, $CD8^+$ $CCR7^+$ T cells express Foxp3 (they become $CD8^+$ $CD45RA^+$ $CCR7^+$ $Foxp3^+$) and acquire immunosuppressive functions. They prevent $CD4^+$ T cells from responding to TCR stimulation by directly interfering with the TCR signalling cascade and not by the usual suppression mechanisms mediated by IL-10, TGFβ, or CTLA-4 (44). In addition, these $CD8^+$ Treg release exosomes carrying NADPH oxidase 2 (NOX2), which are taken up by $CD4^+$ T cells and inhibit their proliferation *in vivo* and *in vitro* (57).

Most studies investigating non-Foxp3 expressing $CD8^+$ Treg in humans describe them as $CD8^+$ $CD28^-$ cells, although $CD8^+$ $CD28^+$ Treg can be generated *in vitro*. Mechanistically, $CD8^+$ $CD28^-$ Treg act by i) influencing CD80/CD86 surface expression of DC leading to inhibition of $CD4^+$ T cell responses, or ii) secretion of IFNγ and IL-6 cytokines or iii) secretion of the anti-inflammatory cytokine IL-10 (54). In addition, $CD8^+$ $CD28^-$ Treg express high levels of the IL-2 receptor CD122 and this has been used as a marker for the characterization of these $CD8^+$ $CD28^-$ Treg. On the other hand, a population of $CD8^+$ $CD28^+$ Treg cells expressing the chemokine receptor CXCR5 has been identified, which are capable of suppressing B cell responses and antibody production by inhibiting follicular helper T (Tfh) cell-mediated B cell differentiation (58). They also exert strong antitumor activity and their presence is associated with favorable prognosis in follicular lymphoma patients (59).

In mice and humans, $CD8^+$ Treg have been described to preferentially recognize the non-classical MHC class I molecules Qa-1 (mouse) or HLA-E (human) which are orthologous genes. These non-classical MHC class I restricted populations have the property to recognize TCR, MHC or heat shock protein derived peptides (i.e. Qdm, HSP60sp) presented by Qa-1 or HLA-E (48). $CD8^+$ Treg exert a cytotoxic effect against antigen–activated $CD4^+$ T cells, and this function depends on the expression of the MHC–Ib molecule Qa–1 in mice (54).

Whereas $CD8^+$ $CD45RO^+$ $CCR7^+$ T cells are found in blood and have no suppressive function, $CD8^+$ $CD45RO^+$ $CCR7^+$ $IL\text{-}10^+$ suppressive cells are found intratumorally in ovarian cancer patients and they are believed to be induced by plasmacytoid DC (60).

In 2014, a novel population of $CD8^+$ Treg characterized by the expression of HLA-DR was identified in human peripheral blood and umbilical cord blood. $CD8^+$ $HLA\text{-}DR^+$ cells suppress in a cell-to cell contact dependent manner, which involves CTLA-4 (47). Within the $CD8^+$ $HLA\text{-}DR^+$ Treg cells the $CD28^+$ subpopulation shows higher suppressive capacity compared to their $CD28^-$ counterparts and also expresses higher levels of the checkpoint inhibitory molecules CTLA-4, TIM-3, PD-1 and LAG-3 (61). Similarities have been found between $CD8^+$ $HLA\text{-}DR^+$ and $CD4^+$ $Foxp3^+$ Treg with regards to the expression of TIGIT, the chemokine receptors CCR4 and CCR5, the low expression of IL-7R (CD127) and a memory and effector-like phenotype. In addition, after polyclonal TCR stimulation, $CD8^+$ $HLA\text{-}DR^+$ Treg cells increase IFNγ and TNFα expression suggesting that they are not exhausted cells despite the fact that they express PD-1 (62).

$CD4^-CD8^-$ Regulatory T Cells

Gamma-delta T cells (γδT) are the first T cells to develop in the thymus upon gene rearrangement which generates different TCR chains (γδ) than the more abundant αβT cells during fetal ontogeny. In contrast to αβT cells, γδT cells do not undergo thymic TCR selection (63). γδT cells represent a small T cell population (3–5% in human peripheral blood) and are able to interact with different immune cell types such as other T cells, B cells, DC, NK cells, monocytes/macrophages, and granulocytes. In some cases, they exert an anti-inflammatory effect. These regulatory γδT cells have been studied in different contexts and are associated with immunosuppression in pregnancy, inflammation, allergy and cancer. Similarly to αβT cells, γδT cells produce TGFβ and IL-10 together with variable expression of the transcription factor Foxp3 (49).

Natural killer T (NKT) cells are a special subset of T cells that co-express NK cell surface receptors (NK1.1/CD161) with the semi-invariant T-cell receptors (TCR), which consist of an invariant TCR α chain paired to a limited number of TCR β chains. Like all T cells, they are generated in the thymus, but most of the NKT cells do not express CD4 or CD8 on their surface (64). Upon activation, NKT cells produce large amounts of Th1 (including IFNγ and TNFα) and Th2 (IL-4, IL-10, and IL-13) cytokines enabling them to act as powerful regulators of the immune system (50). Under certain conditions, NKT cells can

exert potent suppressor functions by shifting from Th1 to Th2 responses both in human and mouse. Their main target cells of suppression are tumor cells, pathogen-activated T cells and APCs (65).

ROLES OF TREG IN DIFFERENT SCENARIOS

Treg constitute a group of phenotypically distinct subsets that can reside in lymphoid and in non-lymphoid organs where they exert diverse functions. The main non-lymphoid tissues where Treg can be found are the visceral adipose tissue (VAT), the intestine, skin and muscle. In these four tissues Treg are important regulators of inflammation and fibrosis and contribute to tissue repair (66). As already mentioned, Treg participate in numerous processes in which they adapt to the environment in order to eventually maintain tissue homeostasis. Some situations such as pregnancy, organ transplants or the common presence of bacteria in the intestine, require tolerance against foreign antigens for which Treg activity is essential. If Treg activity is too low, there can be a failure in self-tolerance leading to the development of autoimmune diseases. On the other hand, it can be hypothesized that if Treg are over-active, they may favor the progression of neoplasic malignancies. During pregnancy, the allogenic nature of the fetus (harboring maternal and paternal antigens) requires tolerance from the mother's immune system in order not to be rejected. Treg and other immune cells create a tolerogenic environment whose composition changes throughout gestation. At least five different $Foxp3^+$ Treg subtypes have been identified during different stages of pregnancy. In addition, other minor Treg subsets such as $CD4^+$ $HLA\text{-}G^+$ $Foxp3^-$ (which inhibit NK, $CD4^+$ and $CD8^+$ T cells), Tr1, Th3, γδT cells, $TIGIT^+$ T cells, and $CD8^+$ Treg have been detected in the decidua and/or peripheral blood of pregnant women (67).

Barrier tissues are constantly exposed to dietary, environmental, and commensal microbiota antigens and therefore immune homeostasis and tolerance need to be ensured *via* Treg or Teff antigen-specific repertoires in these tissues (68). In intestinal tissues, this tolerance is achieved by the cooperation of different immune populations including $Foxp3^+$ Treg and Tr1 cells. Dysregulated intestinal responses to dietary antigens or commensal microbiota frequently lead to immunological disorders in humans such as celiac disease, food allergy, and inflammatory bowel disease (69). Encounter with commensal microbiota generates pTreg rather than anti-microbial effector cells. These pTreg use site-specific TCRs different than the ones that facilitate tTreg development in the thymus, implying that many colonic Treg arise by means of antigen-specific driven pTreg development (70).

Several reports highlight Treg functional deficiencies in autoimmune diseases, but the underlying molecular mechanisms are still unknown. One of the major limitations of studying human autoimmunity is the lack of validated experimental assays and the discrepancies between *in vitro* and *in vivo* experiments (71). Also, there is little consensus for Treg identification (i.e. agreement on the markers used for their identification), which makes the comparison between different studies almost impossible (13). Treg dysfunction in autoimmune diseases can be grouped according to different factors (68): i) Genetic disease like germline mutations in the Foxp3 locus. The development of the severe immune dysregulation, polyendocrinopathy, enteropathy, and X-linked (IPEX) syndrome is due to point mutations and microdeletions in the Foxp3 gene that impair Treg function (72). ii) The abrogation of Treg promoting signals. The disruption of the IL-2/IL-2R pathway dysregulates thymic development and peripheral homeostasis of Treg. In a murine model of Type 1 diabetes, pancreatic Treg die showing a decreased expression of the IL-2R CD25 and of the anti-apoptotic protein Bcl-2 (73). iii) The presence of Treg destabilizing factors. Overexpression of IL-6 and TNFα can interfere with Foxp3 expression and consequently alter Treg/Teff balance. In the presence of TGFβ, IL-6 enhances RORγt expression, which induces Th17 generation *via* STAT-3, and represses at the same time Foxp3 expression. In the case of rheumatoid arthritis, high IL-6 levels are related to a preferential development of Th17 cells over Treg in the periphery (74, 75).

In addition to maintaining immune homeostasis in the lymphoid tissues, Treg are recognized as regulators of non-immunological processes. Treg are present in healthy tissues and upon tissue injury, they promote tissue regeneration in an amphiregulin-dependent manner (76). In a model of influenza virus infection, Treg-induced tissue repair is triggered in response to the inflammatory mediators IL-18 and IL-33, but not by TCR signaling, which is required for their suppressive function (77).

Increased immune suppression contributes to cancer onset and tumors promote the generation of an environment that allows tumor cells to escape from immune responses. Cancer cells develop immunosuppressive mechanisms such as expression of anti-inflammatory mediators and recruitment of suppressive leukocytes, such as Treg, myeloid derived suppressor cells (MDSC), tolerogenic DC and tumor-associated macrophages (TAMs) (68). In recent years it has been found that many tumors are enriched in Treg cells, indeed, a high Treg/Teff ratio correlates with poor prognosis (78). Treg may facilitate cancer progression *via* suppression of effector cells that otherwise would attack the tumor. Turnis and colleagues showed enrichment of IL-35-secreting Treg cells in tumors and demonstrated that Treg-derived IL-35 promotes T cell exhaustion in the tumor microenvironment (79). In fact, different tumor models benefit from Treg depletion leading to an increased anti-tumor response (80–82), but not all tumors benefit from the presence of Treg cells. In addition, $CD4^+$ $CD25^+$ Treg collaborate with $CD8^+$ $CD28^-$ Treg cells within different tumors so that the immunosuppressive activity of these tumor infiltrating Treg cells may be predominant (83). In multiple myeloma patients, $CD8^+$ $CD57^+$ lymphocytes show an activated phenotype ($HLA\text{-}DR^+$ and Fas^+) and can inhibit the suppressive effect of Treg as well as antibody production (84). However, the effect of Treg depends on the tumor site, molecular subtype and

tumor stage (85). Interestingly, Foxp3$^+$ tumor infiltrating Treg were associated with better prognosis in colorectal cancer (86). These cells were later shown to not be fully suppressive and to display some inflammatory T cell features (87). In human follicular lymphoma, high amounts of intratumoral Treg were related to positive outcome (88, 89) whereas high levels of circulating Treg correlated with a negative prognosis (90). All these discrepancies indicate that the presence of intratumoral or circulating Treg may depend on the nature of the tumor, the tumor microenvironment itself and the functionality of the supposedly suppressive Treg.

Low responsiveness and reduced proliferation of virus-specific T cells during chronic viral infection is associated with the expansion of Treg. In acute and chronic murine retroviral infection models, depletion of Treg decreases viral load and restores the activity of virus-specific cytotoxic CD8$^+$ T cells (91, 92). On the other hand, it has been found that the role of Treg depends on the disease stage in tuberculosis patients. Treg expand and delay immune responses in initial phases but they counter-regulate excessive inflammation later in the chronic phase (93). In a mouse model of chronic infection with Friend retrovirus (FV), vaccination with a calcium phosphate nanoparticle-adjuvant, which efficiently reactivated CD8$^+$ T cells, in combination with a transient ablation of Treg enhances anti-viral immunity (94).

Germinal centers (GC) are transient structures in peripheral lymphoid organs where B cells develop and differentiate into antibody secreting plasma and memory B cells. Upon activation by follicular dendritic cells, B cells proliferate and interact with primed antigen-specific Tfh cells in order to be fully activated and differentiate into antibody-secreting plasmablasts (95). Recently, a new Treg subset has been described in GC called follicular regulatory T cells (Tfr). These Tfr cells control Tfh-driven GC responses preventing induction of autoreactive and foreign antigen-specific antibodies (96). They control IgG and IgE responses to vaccines, allergens and autoantigens, and have a critical immunoregulatory function before GC formation (97). The relevance of these Tfr in the control of antibody production has drawn the attention of several studies which investigate these cells as new targets for immunotherapy.

TABLE 2 | Immunological changes during aging.

Immune organ/cell	Changes in aging
Thymus	↓ bone marrow progenitors
	↓ sex hormones
	↓ thymic ephitelial cells
	↑ adipose tissue
APC	↓ antigen processing capacity
	↓ antigen presenting capacity
	↓ chemotaxis, cytokine production and signal transduction
Neutrophils	↓ chemotaxis, cytokine production and signal transduction
B cells	↓ somatic hypermutation
	↓ Ig class switch
	↓ number of plasma cells
T cells	↓ TCR repertoire
	↑ effector-memory cells
	↑ DNA damage accumulation

A summary of the most prominent changes in the thymus as well as in different immune cells is described according to their main function.

TREG HOMEOSTASIS AND FUNCTION IN AGING

The severity of many infections increases with age and many of the vaccines currently used are less effective in older compared to younger adults. This is due to changes that the immune system undergoes over time leading to dysregulation the adaptive and, to a lesser extent, innate mechanisms (**Table 2**). In old age, the capacity of APC to process and present antigens to T cells declines (98) and chemotaxis, cytokine production and signal transduction upon antigen recognition are impaired in these cells as well as in neutrophils (99, 100). In addition, B cells show reduced somatic hypermutation and class switch and the number of plasma cells is decreased which leads to lower antibody production (101). The T cell compartment is skewed towards effector/memory like T cells, shows a reduced TCR repertoire and also accumulates DNA damage in aged individuals (102). In this review, we aim to describe the processes relevant for Treg, but extensive reviews on general immunosenescence can be found elsewhere (103–105).

During aging, the thymus undergoes a gradual reduction in size and function together with changes in its architecture (106). The activity of key factors for thymus functionality is also modified. Less bone marrow progenitors reach the thymus, the flow of sex hormones is diminished, thymic epithelial cell (TEC) number decreases and there is an increase in adipose tissue (107). TEC are part of the structural environment necessary to support the normal differentiation of thymocytes. TEC-mediated thymic involution results in reduced cellularity capable of maintaining normal thymocyte differentiation (108). Despite the decline in thymic cellularity, no blockade of thymocyte differentiation is observed and thymic function is maintained proportional to the reduced size (109). Adipocytes are not only responsible for anatomical changes within the thymus but they actively contribute to thymic involution. They produce higher levels of negative factors for thymic maintenance (e.g. IL-6, sex hormones and steroids) and thereby transmit suppressive signals to TEC reducing thymopoiesis and cellularity (107). During aging, the overall endocrine profile changes with an extra-thymic reduction of sex hormones and growth hormones, and an increase in glucocorticoid levels (110). Estriol and chorionic gonadotropin positively affect Foxp3 expression and increase Treg frequency. Steroids and glucocorticoids can enlarge Treg populations in the periphery stimulating their function (111). In fact, Treg lacking the glucocorticoid receptor lose their suppressive function turning into Th1-like, IFNγ-producing cells in a murine colitis model (112).

In the periphery, the number of recent thymic emigrants (RTE) is decreased in aged mice and humans which implies a lower contribution of thymic cells to the pool of total T cells in the body (113–115). In mice, it has been observed that the production of Treg cells declines more and faster than conventional T cell production.

This is attributed to the inhibitory effect of recirculating Treg cells on the differentiation of new Treg when the former revisit the thymus (116). This accumulation of antigen-specific Treg reduces clonal diversity which translates into a skewed aged Treg pool which can suppress only certain T cells while leaving the rest unaffected. This might favor some proinflammatory cells to remain active in aged hosts (111).

Aging and age-related diseases are associated with profound changes in epigenetic patterns. Treg are subject of epigenetic modifications that are altered during aging. It has been shown that hypomethylation of CpG sites upstream of the Foxp3 enhancer correlates with a higher suppressive function of Treg from aged mice (117).

In addition, pTreg differentiation also decreases with age. It has been observed that naive conventional T cells from aged mice differentiate less into pTreg *in vivo* and *in vitro* compared to their young counterparts (118). Despite less thymic and peripheral Treg differentiation, Treg accumulate with age, which could be explained by the loss of the pro-apoptotic protein Bim rendering these cells apoptosis-resistant compared to Treg from young individuals (119). Loss of Bim or overexpression of Bcl-2 lead to Treg accumulation, but at the same time to reduced suppressive capacity in a model of murine colitis (120). On the other hand, *in vitro* studies show that Treg from young and old adults have the same suppressive capacity (121). Others describe that old Treg are better suppressors than Treg from young adult mice due to a higher IL-10 production from the older ones (117). Despite many discrepancies among different studies, the overall data suggests that Treg function remains unchanged or is even enhanced in the elderly. Aged individuals are more prone to develop infections and neoplastic malignancies which would agree with enhanced Treg function, whereas they are also more susceptible to develop autoimmunity due to Treg dysfunction (122).

In the periphery, total T cell numbers remain unchanged with age maintaining an adequate pool of circulating T cells including Treg. In peripheral blood mononuclear cells (PBMC) from aged humans an increased proportion of activated Treg ($Foxp3^{hi}$ $CD45RA^{-}$) and a low but detectable resting Treg population ($Foxp3^{low}$ $CD45RA^{+}$) are present. Both populations show suppressive potential but the activated Treg die after exerting *in vitro* suppression (12). It has been observed that there is an accumulation of $CD25^{low}$ $Foxp3^{+}$ Treg in old age and this correlates with lower IL-2 levels. The IL-2 receptor (IL-2R), consist of the subunits IL-2Rα (CD25), IL-2Rβ (CD122) and the common gamma chain (CD132), and is expressed on T and B cells, DC and steady state NK cells. $CD25^{low}$ $Foxp3^{+}$ Treg upregulate the surface molecule CD122, which is also part of the IL-15 receptor, suggesting that IL-15 might support $CD25^{low}$ Treg in old age (123). Tr1 cells also accumulate in aged mice in an IL-6 dependent manner and are able to produce large amounts of IL-10 (37). Follow-up studies show that these IL-10 producing cells manifest a Tfh phenotype and that they are involved in immune suppression in old age (124). Van der Geest and colleagues studied different $CD4^{+}$ T cell populations in healthy individuals and observed that the proportion of naive Treg cells declined with age whereas the memory Treg compartment and the memory Treg/Teff ratio increased. This accumulation of memory Treg in old age is associated with poor responses to influenza vaccination (125). Tfh cells from aged mice express high levels if IFNγ and IL-10 and an environmental increase in TGFβ levels in old mice favors the development of Treg (126). After vaccination in old age, impaired differentiation of Tfh cells followed by a suboptimal T cell priming is observed with a consequent poor GC B cell expansion. This is thought to be due to not only lower T cell activation but also to an accumulation of Treg which negatively affect the GC reaction (127). Along these lines, another study in aged patients showed that non-responder individuals to influenza vaccination have a higher frequency of Treg as well as a higher inflammatory status compared to responders (128). Depletion of Treg with anti-CD25 treatment prior to influenza infection protected against lethal viral challenge in aged mice (129). In another study, responders to influenza vaccination showed a higher frequency of Treg compared to non-responders, together with an elevated frequency of early differentiated $CD4^{+}$ T cells and lower proportion of memory $CD4^{+}$ cells (130). Actually, a detailed study in the context of inflammation showed that, upon secondary activation, memory Treg could not undergo pronounced recall expansion as conventional $CD4^{+}$ T cells do (131). These findings emphasize the importance of the accumulation of memory Treg during aging and their role in the ineffectiveness of vaccination in the elderly.

Similar to $CD4^{+}$ cells, the $CD8^{+}$ T cell pool also loses naïve precursor cells but the contraction of the naïve $CD8^{+}$ T compartment is greater than the one for $CD4^{+}$ T cells (132). In human and mouse, naturally occurring $CD8^{+}$ $Foxp3^{+}$ Treg numbers increase with age (133, 134) whereas inducible $CD8^{+}$ $CD45RA^{+}$ $CCR7^{+}$ human (135) and $CD8^{+}$ $CD44^{+}$ $CD62L^{+}$ $CCR7^{+}$ mouse (43) Treg cells decline in old age. The increase of $CD8^{+}$ $Foxp3^{+}$ $CD28^{-}$ Treg is consistent with the higher overall numbers of $CD8^{+}$ $CD28^{-}$ T cells in aged individuals (136). Despite the fact that $CD8^{+}$ $HLA\text{-}DR^{+}$ Treg accumulate with age, the expression of CD28 in these cells remains unchanged. $CD8^{+}$ $HLA\text{-}DR^{+}$ Treg cells lose suppressive activity and decrease the production of checkpoint inhibitory molecules in aged individuals (61).

Together with $CD4^{+}$ $CD25^{+}$ Treg frequencies observed in the elderly, $CD8^{+}$ Treg accumulation has been suggested to contribute to the decline in adaptive immune responses in the aging process (137). The capability of human $CD8^{+}$ $CCR7^{+}$ cells to differentiate into Foxp3-expressing cells negatively correlates with age (44). It has been shown that expression levels of Foxp3 and CD45RA in $CD8^{+}$ $CCR7^{+}$ Treg are lower in older individuals compared to young, and this may suggest a diminished suppressive capacity of these cells potentially contributing to the development of autoimmune diseases in the elderly (138). In contrast, the ability of aged naturally occurring $CD8^{+}$ Treg to suppress the proliferation and cytokine production of effector $CD4^{+}$ T cells remains similar between younger and elder individuals (137). Dysfunction of $CD8^{+}$ and $CD4^{+}$ Treg cells in old age could support age-associated subclinical inflammation referred to as "inflamm-aging" (139, 140), which is associated

with increased levels of oxygen radicals, IL-15, TNFα and IFNγ (141, 142).

Taken together these data suggest that $CD4^+$ Treg as well as $CD8^+$ Treg accumulate in aged individuals. Dysregulation of immune homeostasis is involved in the development of different diseases in old age and a connection with increased numbers of Treg has been demonstrated for several conditions (**Figure 1**).

DISCUSSION

The findings discussed above show that the field of regulatory cells is in continuous expansion. The more we try to define it, the more complex becomes the diversity of different cells (T, B, and NKT cells) that might be involved. Initially described as suppressor cells, Treg have demonstrated to be able to regulate several processes with the final aim of preserving immune homeostasis. To date, there are numerous types of Treg described in mouse and human (**Table 1**) and a lack of consensus to establish the right markers to define these populations make of their characterization a matter of controversy. The promiscuity of Foxp3 expression as a *bona fide* marker of Treg is a good starting point to discuss the huge diversity of Treg identified so far. There is not a single marker distinct for Treg but a combination of some of them (Foxp3, CD25; GITR; CTLA-4, IL-10, etc) together with their suppressive activity could help to identify this elusive type of cells. Furthermore, most of the studies on human Treg are based on circulating cells in the blood, which may not represent the real landscape in a given tissue.

Treg cells are extremely versatile. They exert various effector functions and use a number of molecular mechanisms depending on the tissue and the health or disease context. Dysregulation of Treg function in one or another direction can unchain a huge variety of ailments (**Figure 1**). Indeed, modulation of Treg activity is a main target for anti-tumor therapies, treatment of autoimmunity or the avoidance of graft rejection in transplantation, for instance. The danger of targeting Treg to treat or prevent a certain disease is that the consequence could promote the development of another one. As an example, therapies for autoimmune diseases, which enhance Treg activity in order to dampen the autoreactive immune components, have the potential to increase the risk of tumor development as the suppressed immune cells might be unresponsive to malignant cells.

The level of complexity increases further when we add the factor age. In the elderly, the immune compartment is altered due to thymic involution and dysregulation of several immune processes. Most of the studies show that there is an accumulation of antigen-experienced Treg with age, but discrepancies exist regarding the hypo- or hyper-activity of these cells. This difference in Treg accumulation may not only be age-dependent but also context-dependent (**Figure 1**). In some cases, this results in increased responsiveness of aged individuals to Treg targeted therapies compared to young ones, as it is the case for anti-PD1 therapy in melanoma patients (143).

The vast diversity of Treg in different tissues and the lack of molecular markers to clearly define Treg subtypes makes this area of study highly complex. The clear increase in immune regulatory cells in aging is offering the scientific community plenty of opportunities to learn the context-specific roles of Treg

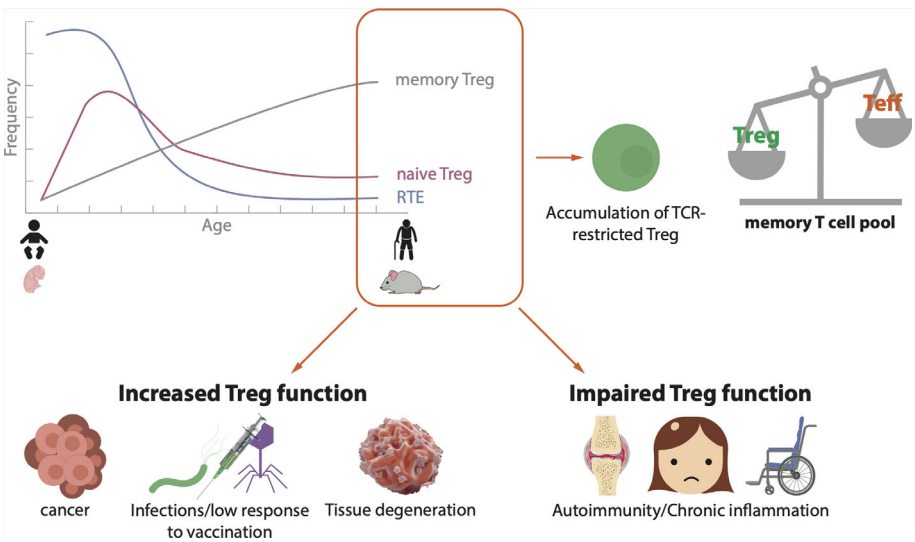

FIGURE 1 | Schematic landscape of Treg in the elderly. During human and mouse aging, there is a decreased efflux of recent thymic emigrants (RTE) to the periphery, a reduction of naïve Treg and an accumulation of antigen experienced memory Treg. As a consequence, TCR-restricted Treg accumulate and the pool of memory T cells is skewed towards memory Treg over effector/memory T cells. This imbalance on T cell homeostasis is related to the development of different diseases. In old age, an increase in Treg function may lead to the progression of tumors, chronic infections or tissue degeneration, whereas an impaired Treg function may cause autoimmunity and/or chronic inflammation.

cells in immunosenescence, advancing the concept into a more tailored tissue/disease/Treg-specific point of view.

AUTHOR CONTRIBUTIONS

LRR, FLM, RW, and BW wrote and discussed the manuscript, and all authors agree to be accountable for the content of the work. All authors contributed to the article and approved the submitted version.

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REVIEW
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A Phenomic Perspective on Factors Influencing Breast Cancer Treatment: Integrating Aging and Lifestyle in Blood and Tissue Biomarker Profiling

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Breast cancer is the most common malignancy among women worldwide. Over the last four decades, diagnostic and therapeutic procedures have improved substantially, giving patients with localized disease a better chance of cure, and those with more advanced cancer, longer periods of disease control and survival. However, understanding and managing heterogeneity in the clinical response exhibited by patients remains a challenge. For some treatments, biomarkers are available to inform therapeutic options, assess pathological response and predict clinical outcomes. Nevertheless, some measurements are not employed universally and lack sensitivity and specificity, which might be influenced by tissue-specific alterations associated with aging and lifestyle. The first part of this article summarizes available and emerging biomarkers for clinical use, such as measurements that can be made in tumor biopsies or blood samples, including so-called liquid biopsies. The second part of this article outlines underappreciated factors that could influence the interpretation of these clinical measurements and affect treatment outcomes. For example, it has been shown that both adiposity and physical activity can modify the characteristics of tumors and surrounding tissues. In addition, evidence shows that inflammaging and immunosenescence interact with treatment and clinical outcomes and could be considered prognostic and predictive factors independently. In summary, changes to blood and tissues that reflect aging and patient characteristics, including lifestyle, are not commonly considered clinically or in research, either for practical reasons or because the supporting evidence base is developing. Thus, an aim of this article is to encourage an integrative phenomic approach in oncology research and clinical management.

Keywords: breast cancer, tumors, clinical response, biomarkers, immunosenescence, lifestyle, exercise, physical activity

INTRODUCTION

Breast cancer is the most common form of cancer affecting women worldwide, with around two million new cases each year (1). Breast cancer is the second most common form of cancer overall and the fifth most common cause of cancer-specific death (2). Men diagnosed with breast cancer account for 1% of all malignancies and represent 1% of all cases of breast cancer worldwide (3). The risk of developing breast cancer is influenced by many factors, including age, age at first birth, parity, breast feeding, menopausal status, physical activity level, body composition, and hereditary factors (e.g., mutations in key genes, such as BRCA1) (4). Treatment for breast cancer has improved over the last four decades and can consist of a combination of traditional and more advanced interventions including surgery, chemotherapy, radiation therapy, hormone therapy, small molecule therapy, immunotherapy and other targeted approaches (such as mTOR inhibitors) (5). Although most treatments are very effective, the clinical profile and characteristics of each patient are unique and tumor heterogeneity—even among patients with the same TNM (T: tumor, N: node, M: metastasis) staging—results in patient-to-patient variation in clinical outcomes. This patient-to-patient variation in clinical outcomes might partly be due to genetic factors, including germline mutations (e.g., BRCA1/2 or P53) or polymorphisms in genes encoding drug metabolizing enzymes and transporters (e.g., DPYD, TPMT or UGT1A1, involved in 5-fluorouracil, mercaptopurine or irinotecan metabolism, respectively) (6–8). Although these factors can be assessed, a challenge that remains is predicting which patients will respond optimally to different treatment options, and to stratify patients to provide the best care (9). Difficulties in managing heterogeneity in the clinical response exhibited by patients emphasizes the need to consider other factors when measuring and interpreting predictive and prognostic biomarkers in breast cancer.

Biomarkers are molecular, histological, radiographical or physiological characteristics that can be measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapy (10). From a clinical perspective, a biomarker could be described as an objective observation of the medical state of a patient, which can be assessed accurately and reproducibly (11). To be reliable, biomarkers need to be sensitive and specific. Sensitivity refers to the ability of the biomarker to correctly identify patients with a disease from the whole population, and specificity refers to the ability of the biomarker to correctly identify people without the disease (12, 13). Molecules linked with the presence of cancer are often referred to as tumor biomarkers or tumor antigens, where antigens are molecules containing sites that are recognized by, and interact with, components of the immune system. Neoantigens are antigens that are generated by somatic mutations in the tumor, whereas tumor-associated antigens can also be found in healthy tissues, usually at lower levels (14). Many classical tumor biomarkers are proteins, and they can either be located on the cell surface, in the intracellular space or secreted into body fluids by cancer cells or other local cells in response to the tumor(s) (2, 15). Further, many tumor biomarkers are shared among different cancers with only a few biomarkers being disease specific (2).

Tumor biomarkers can be categorized based on their role and time of assessment, including diagnostic, monitoring, predictive or prognostic biomarkers (16, 17). Diagnostic biomarkers, for example, show utility in early phases of disease, as they confirm the presence of a tumor, whereas biomarkers used for monitoring disease become more relevant following diagnosis and during treatment, as their serial measurement gives real-time information of disease status (16). In this review, our focus is largely on predictive and prognostic biomarkers, given their utility in establishing the clinical response to treatment. Predictive biomarkers assess the response or lack of response to a specific form of therapy, while prognostic biomarkers can reflect the natural course of the disease and thus can assess clinical outcomes in the absence of therapy (18). When interpreting any type of biomarker, the specific endpoint of analysis should also be taken into consideration (19). Examples include calculations of progression-free survival or objective response rates. The increasing relevance of biomarkers in the management of cancer has led to the development of a number of agencies who support and advise on the clinical use of biomarkers, including the American Society of Clinical Oncology (ASCO) (20–24), the American National Academy of Clinical Biochemistry (NACB) (25), the European Group of tumor markers (EGTM) (5, 26), the European Society of Medical Oncology (ESMO) (27, 28), the National Institute for Health and Care Excellence (NICE) and the National Comprehensive Cancer Network (NCCN). Guidelines have also been produced with the validation steps needed for biomarkers to reach the clinics, including evaluation of confounding factors, analytical and clinical validation, demonstration of clinical utility and regulatory approval (29). Further, a biomarker registry has been created to compile data from ongoing, completed but not yet published, and completed studies, as well as those with negative results, serving as a useful tool for further analyses or for the design of new biomarker studies (30).

The search for new cancer biomarkers continues, and once measurements become established, there is often further validation and refinement, including the assessment of other biomarkers simultaneously, to improve the sensitivity or specificity of tests. An additional step, often not undertaken either for practical reasons or because the supporting evidence base is developing, is understanding whether cancer biomarkers are influenced by broader factors, including the characteristics of patients and their lifestyle. If it could be established, that factors such as age, physical activity level, or body composition, influence the concentration or characteristics of a given biomarker, then accounting for these inter-individual patient-centric factors, might improve the clinical utility of that measurement[1]. Given that first; some biomarkers indicate the severity of disease and are secreted or expressed by tumor cells

[1]It should be considered that unless the relationship between a biomarker and disease is causal, then a change to biomarker concentration might not necessarily reflect a prognostic (or predictive) change.

during active disease (31–34), and that second; factors, such as exercise, physical activity or body composition, are known to influence disease progression (35–40), then it is conceivable that there is interaction. Indeed, the effects of age, exercise, and adiposity, on the composition and function of cells, tissues and organs, is well established, and there are a variety of mechanistic links with disease (41, 42). In turn, the composition and characteristics of tissues that are both local and distant to tumor sites, could influence the measurement of cancer biomarkers and also disease progression directly.

The first part of this article summarizes current and emerging breast cancer biomarkers that are measured in tumors or in blood (see **Tables 1A**, **1B** and **Table 2**). The second part of this article summarizes the effects that aging, exercise or physical activity, and adiposity can have, on the cellular composition and function of a variety of cells and tissues, including tumors. In places, links between these broader characteristics of patients and overall cancer risk, disease progression, and treatment outcomes are highlighted. In summary, the overall aim of this article is to encourage an integrative phenomic approach in oncology research and clinical management.

MEASUREMENTS IN TUMORS

Estrogen Receptor (ER)

Estrogen receptors (ERs) are nuclear steroid receptors that operate as transcriptional regulators of several cell processes, such as proliferation and differentiation, in response, primarily, to estrogen (45). There are two forms, ER-alfa and ER-beta, and the majority of ER-positive tumors express the alfa form (49, 120). ER expression is measured by semiquantitative immunohistochemistry in formalin-fixed paraffin-embedded tumor biopsies (46). ER expression has proven importance as a prognostic and predictive factor by identifying which patients will respond to hormone therapies (e.g., aromatase inhibitors, tamoxifen and other ER antagonists) informing treatment decisions, and providing an estimate of overall survival (2). For almost 50 years, many studies have confirmed both the prognostic and the predictive value of ER measurements (31, 43) and ER status is used widely in clinics after diagnosis. For example, a study analyzed data from 4478 breast cancer patients across seventeen cancer registries in six European countries, to determine the influence of hormone receptor status on survival (121). Comparing ER status and relative survival over 5 years, it was found that women who had been classified as ER positive had better outcomes (90% survival, 95% CI: 88–92) compared to ER-negative counterparts (77% survival; 95% CI: 73–78). Among ER-positive women, tamoxifen treatment was associated with a 10% decrease in relative excess risk of death compared to women not treated with tamoxifen. Although the majority of studies examining ER have focused on the alfa form, some reports have shown prognostic value of the beta isoform, even in ER-alpha negative tumors (44, 122). Attention has been directed more recently to mutations in the gene that encodes the ER—so called ESR1 mutations—because they have been associated with resistance to endocrine therapy, especially in metastatic settings (62).

Progesterone Receptor (PR)

Progesterone receptors (PRs) are also nuclear steroid receptors that govern processes such as proliferation and differentiation in response, primarily, to progesterone. There are two isoforms, PR-alpha and PR-beta, which regulate different genes (50). PR expression is measured by semiquantitative immunohistochemistry in formalin-fixed paraffin-embedded tumor biopsies. In healthy breast tissue, both isoforms are expressed equally, but some studies have shown a dysregulation of this balance in breast cancer (47). A large literature base supports the use of PR status for predicting clinical outcomes. For example, a study defined both clinical utility and cut off points of immunohistochemistry for PR status measurement in a 'test' group of 1235 cases of primary breast cancer receiving endocrine therapy. This study then confirmed clinical utility for successful therapeutic outcomes in an extra 'validation' group of 423 breast cancer patients who underwent mastectomy and were randomized to either 5 years of adjuvant tamoxifen treatment or no adjuvant treatment (123). Analysis of formalin-fixed samples from the 423 patients showed that PR was a strong and significant predictive factor of both improved disease-free and overall survival (HR = 0.546, P = 0.0034; HR = 0.595, P = 0.0040 respectively). The PR-alpha/PR-beta ratio has also been suggested to influence responsiveness to hormone therapies, with some studies showing that a high ratio of PR-alpha to PR-beta expression is linked to tamoxifen resistance (48). Combined with information from assessing ER-status, it is known that tumors expressing both ER and PR respond best to endocrine therapies (49).

Human Epidermal Growth Factor Receptor 2 (HER2)

Human epidermal growth factor receptor 2 (HER2; also known as c-erbB-2, due to the encoding gene, or HER2/neu, due to its discovery in neuroblastoma rat models (51)), is an epithelial growth factor oncoprotein, localized in the cell membrane and involved in communication among cells for proliferation, differentiation and survival signalling (2). HER2 is commonly measured in formalin fixed sections of tumor tissue, by immunohistochemistry, but also by Fluorescence In Situ Hybridization (56). HER2 status is most commonly used to identify patients eligible for treatment with HER2-targetting therapies such as trastuzumab, also known as herceptin (22, 52). HER2 status has prognostic and predictive value, in part, due to the effectiveness of HER2-targetting therapies. However, HER2 positivity and overexpression has been associated with worse prognosis and reduced disease-free and overall survival in the absence of HER2-targetting treatments (124). In addition, HER2 expression has been associated with resistance to endocrine therapy, especially tamoxifen (53, 57, 125, 126) but has been linked with the success of other chemotherapy regimens. For example, a study including 638 patients with ER and/or PR negative tumors and axillary lymph node

TABLE 1A | Measurements made in tumours: singleplex/duplex/quadruplex assays.

Biomarker or test	Type	Detection technique	RTCs, meta-analyses and other studies	Reviews, and consensus papers	Recommendations	Used clinically
ER	Singleplex	Semiquantitative IHC, FFPE	(31, 43–44)	(2, 45, 46)	ASCO (20): treatment decisions EGTM (26): treatment decisions ESMO (27, 28): treatment decisions NACB (25): treatment decisions; prognosis (combined biomarkers)	Yes
PR	Singleplex	IHC, FFPE	(47–48)	(49, 50)	ASCO (20): treatment decisions EGTM (26): treatment decisions ESMO (27, 28): treatment decisions NACB (25): treatment decisions; prognosis (combined biomarkers)	Yes
HER2	Singleplex	IHC, FISH, sequencing; FFPE	(51, 52–53–55)	(56, 57)	ASCO (20): treatment decisions EGTM (26): treatment decisions ESMO (27, 28): treatment decisions NACB (25): treatment decisions; prognosis if combined	Yes
UPA and **PAI-1**	Duplex	ELISA, fresh/frozen tissue, FFPE	(32, 58–59)	(60, 61)	ASCO (20): prognosis newly diagnosed (node–) ASCO (62): treatment decisions (adjuvant therapy ER+HER2–node–) EGTM (26): prognosis (combined biomarkers); prediction (adjuvant therapy ER+HER2–node–) ESMO (27, 28): prognosis (node–/+); treatment decisions (combined biomarkers; early disease) NACB (25): prognosis; further evaluation for treatment decisions	*Note (A)*
P53	Singleplex	IHC, TTGE/sequencing, cDNA microarrays; FFPE	(63, 64–65)	(66, 67)	ASCO (20, 22): insufficient data for treatment decisions EGTM (5): insufficient data ESMO (27, 28): not mentioned NACB (25): prognosis (conflicting results) 2007 3rd international workshop on TP53: prognosis	No
Ki-67	Singleplex	IHC, RT qPCR fresh/frozen tissue, FFPE	(68–69)	(70, 71)	ASCO (20): insufficient data for prognosis ASCO (62): not recommended for treatment decisions EGTM (26): prognosis if combined ESMO (28): prognosis ER+HER2–, treatment decisions (combined biomarkers; adjuvant therapy) NACB (25): not mentioned International Ki67 BC working group (72): guidelines on use	Yes
D cathepsin	Singleplex	IHC (FFPE); immunoenzymatic or radiometric assays (tumour lysates); Western Blotting	(73–74)	(75, 76)	ASCO (20): insufficient data for prognosis/ prediction EGTM (5): insufficient data ESMO (27, 28): not mentioned NACB (25): prognostic (node–; conflicting results)	No
PSA	Singleplex	IHC (FFPE), ELISA (tumour cytosolic extracts)	117	(77–78)	ASCO (20, 22): not mentioned for BC EGTM (5, 26): Not mentioned for BC ESMO (27, 28): Not mentioned for BC NACB (25): not mentioned for BC	No
IHC4	Quadruplex (ER, PR, HER2 and KI67)	IHC, FFPE	(79, 80)		ASCO (22): Not recommended for treatment decisions EGTM (5, 26): not mentioned ESMO (27, 28): not mentioned NACB (25): not mentioned 2018 NICE DG34 guidelines: not recommended for treatment decisions in early ER+HER2-node- (uncertain analytical validity)	

TABLE 1B | Measurements made in tumours: multiplex assays.

Biomarker or test	Type	Detection technique	RTCs, meta-analyses and other studies	Reviews, and consensus papers	Recommendations	Used clinically
TILs	Multiplex	Microscopy, ICC, flow cytometry, gene expression; blood, fresh/frozen tissue, FFPE	(81–82)		ASCO (22): insufficient evidence for treatment decisions EGTM (5, 26): not mentioned ESMO (28): prognosis, not treatment decisions NACB (25): not mentioned 2014 International TILs working group (83): guidelines/recommendations 2019 St Gallen Consensus: prognosis	No
Oncotype DX	Multiplex (21 genes)	RT PCR, FFPE	(84–85)		ASCO (20, 22): prognosis/prediction (adjuvant therapy, tamoxifen) EGTM (26): prognosis/prediction (adjuvant therapy, tamoxifen ER+HER2– ESMO (28): prognosis/prediction (adjuvant therapy; combined biomarkers) NACB (25): prognosis/prediction (adjuvant therapy; combined biomarkers) 2018 NICE DG34 guidelines: treatment decisions in early ER+HER2-node- (adjuvant chemotherapy)	Yes
Mammaprint	Multiplex (70 genes)	Microarray, fresh/frozen tissue, FFPE	(86–87)		ASCO (23): prognosis/treatment decisions EGTM (26): prognosis/treatment decisions (adjuvant therapy; invasive) ESMO (28): prognosis/prediction adjuvant therapy; combined biomarkers) NACB (25): not mentioned 2018 NICE DG34 guidelines: not recommended for treatment decisions in early ER+HER2-node- (not cost effective) FDA approved	Yes
Prosigna	Multiplex (50 genes)	Microarray, FFPE	(88–89)		ASCO (22): treatment decisions (adjuvant therapy, combined biomarkers, ER+HER2–node–) EGTM (26): prognosis/ treatment decisions (adjuvant therapy, combined biomarkers, ER+ HER2–) ESMO (28): prognosis/prediction (adjuvant therapy, combined biomarkers) NACB (25): not mentioned 2018 NICE DG34 guidelines: treatment decisions in early ER+HER2-node- (adjuvant chemotherapy) FDA approved	Yes
Endopredict	Multiplex (8 genes)	RT PCR, FFPE	(90–91)		ASCO (22): treatment decisions (adjuvant therapy ER+HER2–node–) EGTM (26): prognosis/treatment decisions (adjuvant therapy, combined biomarkers, ER+ HER2–) ESMO (28): prognosis/prediction (adjuvant therapy, combined biomarkers) NACB (25): not mentioned 2018 NICE DG34 guidelines: treatment decisions in early ER+HER2-node- (adjuvant chemotherapy) Not FDA approved but approved for use in Europe	Yes
Rotterdam signature	Multiplex (76 genes)	Microarray, fresh/frozen tissue	(92–93)		ASCO (20): insufficient data EGTM (26): insufficient data ESMO (28): not mentioned NACB (25): not mentioned Not commercially available	No

ASCO: American Association of Clinical Oncology; BC: Breast Cancer; Chemo: chemotherapy; EGTM: European Group of Tumour Markers; ELISA: Enzyme Linked Immunosorbent Assay; ER: Estrogen receptor; FDA: Food and Drug Administration; ESMO: European Society of Medical Oncology; FISH: Fluorescence In Situ Hybridization; FFPE: formalin-fixed paraffin-embedded tissue; HER2: Human Epidermal Growth Factor Receptor 2; ICC: Immunocytochemistry; IHC: Immunohistrochemistry; NACB: American National Academy of Clinical Biochemistry; NICE: National Institute for Health and Care Excellence; PR: Progesterone receptor; PSA: Prostate Specific Antigen; RT PCR: reverse transcription Polymerase Chain Reaction; RT qPCR: Quantitative reverse transcription Polymerase Chain Reaction; TILs: Tumour Infiltrating Lymphocytes; TTGE: temporal temperature gradient gel electrophoresis; UPA and PAI: Urokinase plasminogen activator and Plasminogen Activator Inhibitor 1. Notes: (A) Not widely used as fresh or freshly frozen tissue is required.

involvement, showed that patients with HER2 overexpression benefited from chemotherapeutic regimens where anthracycline-based drugs such as doxorubicin were added, compared to HER2 negative patients. The 10-year disease free survival of HER2 positive patients increased from 26% to 41% when treated with doxorubicin, whereas survival did not change in the HER2 negative group (40 vs. 41%) (54). In another study, 442 women with node positive breast cancer were randomized to three different doses of adjuvant chemotherapy, combining cyclophosphamide, doxorubicin and fluorouracil. Women with tumors overexpressing HER2 (≥50% overexpression) benefited the most from high doses of chemotherapy, compared to those with little or no expression of HER2 (55).

Urokinase Plasminogen Activator (uPA) and Plasminogen Activator Inhibitor 1 (PAI-1)

Urokinase plasminogen activator (uPA) is a serine protease that converts plasminogen into plasmin, which has a key role in degradation of extracellular matrix-components, leading to release of growth factors implicated in migration and invasion (60, 61). The proteolytic activity of uPA is regulated by inhibitors such as plasminogen activator inhibitor 1 (PAI-1). Given the role of uPA in metastasis, PAI-1 was once thought to be protective, but studies have shown that this inhibitor is also associated with tumorigenesis, likely by preventing apoptosis (58) or enhancing angiogenesis (32). Simultaneous measurement of both molecules has been shown to have better prognostic and predictive value compared to measuring them separately (127). Both uPA and PAI-1 are commonly measured in parallel with enzyme-linked immunosorbent assays (ELISA) in extracts of the primary tumor, and general reference cut off levels are 3 ng/mg and 14 ng/mg respectively. uPA and PA-1 levels have prognostic value in breast cancer patients regardless of menopausal status (128) and node status (129, 130), and high levels of both markers have been significantly associated with shorter overall and disease-free survival. A prospective randomized control trial showed that uPA and PAI-1 levels also had predictive value, identifying lymph-node negative breast cancers with better responses to adjuvant chemotherapy consisting of cyclophospamide, methotrexate and 5-fluorouracil (CMF) (131). In this study, breast cancer patients were stratified into either a high-risk or low-risk group, depending on whether they had high or low levels of uPA and PAI-1, respectively. Among the high-risk group, patients receiving chemotherapy had a 44% decrease in the relative risk of disease recurrence compared to those who did not receive treatment (RR = 0.56, 95% CI: 0.25–1.28). Similar findings have been reported in other studies (59), and future studies need to confirm clinical utility with other more commonly used treatment regimens (132).

Tumor Protein 53 (P53)

Tumor protein P53 is a nuclear protein involved in cell cycle regulation that also acts as a tumor suppressor, binding to DNA in the presence of damage and triggering either DNA repair pathways, checkpoint arrest or apoptosis (66). In tumors, one or both alleles of P53 are commonly deleted and/or mutated (63), and this can result in non-functional P53, which, unable to detect DNA damage, contributes to tumorigenesis. Overexpression of mutated versions of P53 can promote tumor formation due to oncogenic gain-of-function activity (67). Traditionally P53 status is examined by immunohistochemistry in formalin fixed paraffin blocks, which is useful for identifying overexpression. However, given the importance of identifying specific mutations, Temporal Temperature Gradient Gel Electrophoresis, with sequencing of aberrant migrating bands to determine the nature of mutations, or cDNA microarrays are now more common. Overexpression of P53 protein and some mutations have been linked with poor prognosis and shorter survival (64, 133–137). For example, there was a significant reduction in disease free survival over 5 years among 700 women with node-negative breast cancer exhibiting tumors that were positive for a mutated version of P53. Disease free survival probability at 5 years was 80% for P53 negative tumors, 72% for P53 positive tumors with low expression, and 58% for P53 positive tumors with high expression ($P < 0.05$) (133). Some studies have supported the predictive value of P53 for treatment outcomes, as certain mutations (e.g., stop codons, point or deletion mutations, in regions like the zinc-binding domain) have been associated with resistance to some forms of chemotherapy (e.g., doxorubicin, tamoxifen, 5-fluorouracil and mitomycin, or cyclophosphamide, methotrexate and 5-florouracil) or radiotherapy (138–144). Other studies on the other hand, have shown better responses to certain chemotherapy regimens (e.g. paclitaxel, or epirubicin and cyclophosphamide) among patients with mutations in P53, such as deletions, transversions or transitions in exons 4, 6, 8 or 10 (65, 145).

Ki-67

Ki-67 is a nucleic protein that is a marker of proliferation expressed at higher levels during mitosis (70). It is commonly assessed by immunohistochemistry, typically using the MIB-1 antibody (71), although examining gene expression using RT qPCR provides comparable results (68). High Ki-67 expression in tumor tissue is associated with poorer outcomes (146–149). For example, a metanalysis of 12,155 breast cancer patients showed that, in the overall population, Ki-67 expression was associated with decreased overall (HR 1.95, 95% CI: 1.70–2.24; $P < 0.001$) and disease-free survival (HR 1.93, 95% CI: 1.74–2.14; $P < 0.001$) (146). Similar results have been shown by other studies, examining patients undergoing endocrine therapy (150). On the other hand, some studies have shown that positive responses to certain forms of therapy can be predicted with high Ki-67 scores, such as some chemotherapy combinations (e.g., docetaxel, fluorouracil and epirubicin) in ER positive tumors (151) or addition of adjuvant chemotherapy to endocrine therapy in HER2 negative tumors (152). However, other studies have not been able to prove predictive value of Ki-67 (69, 153). The International Ki-67 in Breast cancer working group reviewed the available evidence base and provided guidelines for the accurate measurement of this marker (72).

D Cathepsin

D cathepsin is a lysosomal aspartyl protease that breaks down intracellular and endocytosed proteins in most mammalian cells

TABLE 2 | Measurements made in blood: singleplex and multiplex assays.

Biomarker or test	Type	Detection technique	RTCs, meta-analyses and other studies	Reviews, and consensus papers	Recommendations	Used clinically
CEA	Singleplex	ELISA, plasma/serum	(94–95)	(2)	ASCO (20, 21): monitor treatment (combined biomarkers, metastatic) EGTM (5): prognosis (combined biomarkers, early recurrence) ESMO (27, 28): not mentioned NACB (25): monitor treatment (combined biomarkers)	Occasionally
CA 15.3 & CA 27.29	Singleplex (one or the other)	ELISA, plasma/serum	(33, 96–97)		ASCO (20, 21): monitor treatment (combined biomarkers, metastatic) EGTM (5): prognosis (combined biomarkers, early recurrence) ESMO (27, 28): not mentioned NACB (25): monitor treatment (combined biomarkers)	Occasionally
MCA	Singleplex	ELISA, plasma/serum	(98, 99)		ASCO (20, 22): Not mentioned (favor: CA15.3 & CA 27.29) EGTM (5): Not mentioned (favor: CA15.3 & CA 27.29) ESMO (27, 28): not mentioned NACB (25): not mentioned	No
Circulating HER2	Singleplex	ELISA, plasma/serum	(100, 101–102)		ASCO (20, 22): insufficient evidence prognosis/ treatment EGTM (5, 26): Not mentioned ESMO (27, 28): Not mentioned NACB (25): Potential: prognosis/treatment/ prediction/monitoring (undergoing evaluation) FDA approved	No
Circulating PSA	Singleplex	Immunoassays, serum	(103, 104)	(105–106, 107)	ASCO (20, 22): not mentioned for BC EGTM (5, 26): Not mentioned for BC ESMO (27, 28): Not mentioned for BC NACB (25): not mentioned for BC	No
ctDNA	Multiplex	PCR or sequencing techniques, blood	(108, 109)	(107)	ASCO/CAP (24): complementary to genomic tests (metastasis), insufficient evidence (early-stage/monitoring/recurrence) EGTM (5, 26): not mentioned ESMO (27, 28): not mentioned NACB (25): not mentioned FDA approved (PIK3CA mutation test) (110)	No
CTCs	Multiplex	Microscopy, flow cytometry, RT-PCR, blood	(34, 111–112)	(113)	ASCO (20, 22): insufficient evidence for treatment decisions EGTM (5, 26): not mentioned ESMO (27, 28): not mentioned NACB (25): prognosis/monitoring (advanced disease, undergoing evaluation) FDA approved (CellSearch assay) (114)	No
Circulating Immune cells	Multiplex	Flow cytometry, blood	(115–117)	(118, 119)	No	No

ASCO: American Association of Clinical Oncology; BC: Breast Cancer; CAP: College of American Pathologists; CEA: Carcinoembryonic Antigen; CTCs: Circulating Tumour Cells; ctDNA: Circulating tumour DNA; EGTM: European Group of Tumour Markers; ELISA: Enzyme Linked Immunosorbent Assay; FDA: Food and Drug Administration; ESMO: European Society of Medical Oncology; HER2: Human Epidermal Growth Factor Receptor 2; HE4: Human epididymis protein; MCA: Mucin-like carcinoma associated antigen; NACB: American National Academy of Clinical Biochemistry. PSA: Prostate Specific Antigen.

(75) and is involved in remodeling processes in mammary tissue (76). D cathepsin can be assessed by immunohistochemistry in formalin fixed paraffin embedded tumor samples, or immunoenzymatic assays and radiometric immunoassays in breast tumor lysates or by Western Blotting. Some studies have indicated that D cathepsin has prognostic value in primary breast cancer. For example, in an analysis of 2810 cytosolic extracts of breast tissue by radiometric immunoassay, it was shown that tumors with high levels of D cathepsin had significantly poorer relapse-free and overall survival regardless of node or menopausal status (73). In addition, dividing D cathepsin levels into four quartiles (Q1: 0–33, Q2: > 33–47, Q3: > 47–70, and Q4: > 70 pmol/mg protein) an association was shown between patients in the higher quartiles with early relapse and

death. Relapse-free survival probability at 10 years in the group with highest D cathepsin levels was 36% compared to 55% among the group with the lowest levels. In addition, overall survival probability was 43% in the group with the highest levels compared to the 63% in the group with the lowest levels. Although other studies have shown similar results (154–156), the prognostic value of D cathepsin has not been fully established and is not used routinely. However, some studies have shown associations with treatment outcomes, as patients with higher levels seem to benefit from tamoxifen-based therapies (157, 158) but other studies show no impact (74, 159).

Prostate-Specific Antigen (PSA)

Prostate-specific antigen (PSA) is a serine protease with chymotrypsin-like activity which is normally released from the prostate into seminal fluid to increase sperm motility. PSA is most commonly considered to be a serum biomarker for the diagnosis, prognosis and progression of prostate adenocarcinomas. However, PSA is also produced by other tissues, including the breast, and PSA has received attention in breast cancer (103). PSA can be detected by different methods, such as immunoassays in tumor cytosolic extracts, or immunohistochemistry and studies have shown prognostic value in breast cancer (77, 160). For example, a study of 174 breast cancer patients measured PSA in samples of tumor cytosol and found that PSA positive tumors correlated with early disease stage, smaller tumors and estrogen receptor positivity (77). Moreover, patients with PSA-positive tumors showed a significantly lower risk of relapse and death. However, other studies have not been able to confirm independent prognostic value for PSA (161, 162). Studies have also linked PSA to treatment outcomes. For example, in an analysis of tumor cytosol from 434 patients with breast cancer that had recurred who were treated with tamoxifen, a significant association was shown between high PSA and poor treatment response, as well as poor progression-free and overall survival ($P < 0.001$) (78). Further research is needed to confirm the clinical utility of PSA in breast cancer.

IHC4

Immuno-HistoChemical-4 score (IHC4) is a four-parameter immunohistochemistry test that measures the ER, PR, HER2 and Ki-67 in formalin fixed paraffin embedded tumor samples. In 2011, the ATAC trial (Arimidex, Tamoxifen, Alone or in Combination) examined the prognostic value of combining those four immunohistochemistry markers among 1125 ER positive breast cancer patients in comparison with another multiparameter test—Oncotype DX, or Genomic Health Recurrence Score—covered in the next section (79). A prognostic model and a combined score, the IHC4 score was computed. Results showed independent prognostic value of each of the immunohistochemical markers, and a prognostic value for the IHC4 score that was comparable to that of Oncotype DX (although IHC4 score was slightly more prognostic for distant recurrences). In turn, the IHC4 score was subsequently examined and validated in an additional group of 786 ER positive patients. High levels of the adjusted IHC4 score were shown to be a strong prognostic factor for negative outcome (HR = 4.1, 95% CI: 2.5–6.8). Other later studies have confirmed the utility of IHC4 to identify ER positive breast cancer patients that have a low risk of recurrence (80). However, the IHC4 test needs further validation and investigation in large randomized trials before it can be used routinely in clinical practice.

Tumor Infiltrating Lymphocytes (TILs)

Tumor infiltrating lymphocytes (TILs) reflect the immune response to the presence of a tumor (81). Most studies have focused on the predictive value of T cells, but many other immune cell subtypes are present within tumors, including natural killer cells, B cells and, despite the common name referring to "lymphocytes", macrophages have also received attention (163). TILs can be detected by several methods including immunocytochemistry, flow cytometry, gene expression and semiquantitative histological evaluation by light microscopy (164). The frequency of TILs varies among the different breast cancer subtypes, and TILs are typically most abundant in the most aggressive forms, such as basal-like (ER–PR–HER–) and HER2-positive tumors (165). Studies have shown that infiltration of some lymphocyte sub-types, such as cytotoxic CD8+ T cells and helper CD4+ T cells, B cells and dendritic cells, are associated with good prognosis and therefore longer survival. However, studies have also shown that the infiltration of other cells, including regulatory T cells, neutrophils, and tumor-associated macrophages (TAMs) with an M2-like (alternatively activated) phenotype are associated with worse prognosis (82, 164, 166–168). Studies have examined the predictive value of TILs in the context of treatment outcomes, showing significant associations between high frequencies of TILs and positive responses to anthracycline-based chemotherapy (166), or to chemotherapy combined with trastuzumab (81). TILs have received a lot of attention in research settings, and studies have interpreted results in a variety of ways, including examining the presence or absence of cell subtypes, and also their relative abundance. The international TILs working group meeting in 2013 produced guidelines for these assessments, yet further work is required for routine clinical use (83).

Oncotype DX

Oncotype DX, developed by Genomic Health (California, USA; now part of Exact Sciences, Wisconsin, USA), is a multiparameter RT-PCR assay that simultaneously measures the expression of 21 genes in formalin-fixed paraffin embedded tumor samples. The panel of genes includes 16 cancer-related genes, such as HER2 and ER, and others implicated in proliferation and invasion, and also 5 genes for reference (84). Based on the relative expression of each gene, a recurrence score is computed classifying patients into three risk categories: low (recurrence score lower than 18), intermediate (recurrence score between 18 and 30), and high (recurrence score above 30). The assay was designed to predict risk in lymph node negative, ER positive breast cancer patients treated with tamoxifen. The prognostic value of the Oncotype DX recurrence score has been extensively validated. High scores are associated with

shorter relapse-free and overall survival in both lymph node positive and lymph node negative patients (84, 85, 169–172). The predictive value of this test is best demonstrated by identifying patients with ER positive tumors who would benefit most from adjuvant chemotherapy, regardless of node involvement (170, 172). A study tested the 21-gene recurrence score assay in tumor samples from the phase III trial SWOG-8814, which included lymph node negative ER-positive breast cancer patients treated with either tamoxifen alone or with chemotherapy consisting of cyclophosphamide, doxorubicin and fluorouracil prior to tamoxifen (172). The study confirmed the significant prognostic value of the assay in the tamoxifen alone group as shown by previous studies. The study also showed a significant improvement in survival from the addition of chemotherapy to tamoxifen in the high-risk score group, but a lack of benefit from chemotherapy in low-intermediate score groups. In the high risk group, the 10-year estimates for percentage of disease-free survival were 55% for chemotherapy and tamoxifen vs. 43% for tamoxifen alone (P = 0.033), and for overall survival, 68% for chemotherapy and tamoxifen vs. 51% for tamoxifen alone, (P = 0.027), and for breast-cancer specific survival, 73% for chemotherapy and tamoxifen vs. 54% for tamoxifen alone (P = 0.033). On this basis, Oncotype DX and similar platforms are routinely used to help decision-making for the use of adjuvant chemotherapy in ER-positive breast cancer. Further research is needed to evaluate the use of Oncotype DX among ER negative patients.

MammaPrint

Mammaprint, developed by Agendia (Amsterdam, Netherlands), is a multi-parameter microarray-based technique that simultaneously measures the expression of 70 genes in either fresh or frozen tumor tissue or formalin fixed paraffin embedded samples, which produces a recurrence score. In turn, patients are classified as either low risk with a good prognosis signature, or high risk with a bad prognosis signature. Several studies have confirmed the clinical utility of this test to identify patients with better or worse outcome (86, 173, 174), and to inform clinical decisions over whether to treat patients with adjuvant chemotherapy following surgery (175, 176). One of these is the MINDACT study (Microarray in Node- Negative Disease may Avoid ChemoTherapy), a prospective randomized trial, which was conducted with 6693 patients diagnosed with early breast cancer. In this study, the recurrence risk calculated by Mammaprint and referred to as genomic risk was compared with risk calculated by an online decision-making tool for clinicians that was available at the time (Adjuvant!Online; referred to as clinical risk) (87, 176). Patients were assigned as being low or high risk with both scores. There were 1550 patients with high clinical risk (determined by the online tool) and low genomic risk (determined by MammaPrint) (176). After randomization to receive adjuvant chemotherapy or not following surgery, the difference in survival was small: 1.5% lower among patients not receiving chemotherapy. Indeed, the 5-year survival rate without distant metastasis was 95.9% (95% CI: 94.0–97.2) among those receiving chemotherapy compared to 94.4% (95% CI: 92.3–95.9) among patients who were not treated with chemotherapy. Thus, MammaPrint is a useful tool for informing treatment decisions.

Prosigna

Prosigna (also called PAM50 gene signature) is a 50-gene microarray-based technique developed by Nanostring technologies (Seattle, USA) for use with fresh and frozen tissue or formalin fixed paraffin embedded samples. The test classifies tumors in one of four subtypes: Luminal A, Luminal B, HER2-positive or Basal-like (88). The test provides a Risk of Recurrence score (ROR), where low scores (ROR<40) categorize patients as being low risk, and high scores (ROR>60) categorize patients as being high risk. Several studies have validated the prognostic value of Prosigna in postmenopausal women with ER-positive early breast cancer. For example, a study of 1478 women from the ABSCSG-8 trial who were being treated with tamoxifen or tamoxifen and anastrozole, showed that the ROR score from Prosigna has significant prognostic value (177). This study showed that the Luminal A subtype presented a lower ROR score after 10 years compared with Luminal B, emphasizing the utility of this multiparameter test for predicting the risk of distant recurrence. Other studies have analyzed the utility of Prosigna for therapeutic prediction. For example, a randomized controlled study—the DBCG89D trial—among patients with early breast cancer treated with either CMF (cyclophosphamide, methotrexate and fluorouracil) or CEF (cyclophosphamide, epirubicin and fluorouracil) undertook Prosigna assays on samples from 686 patients and studied associations with distant recurrence, time to recurrence and overall survival (89). The results showed that patients from the HER2 subtype presented a significant benefit from anthracycline-based (epirubicin) chemotherapy, in comparison with patients from the luminal subtypes, as the time to distant recurrence was significantly longer in the HER2 subtype treated with CEF. Further, the benefit of CEF therapy over CMF was associated with higher ROR scores.

Endopredict

Endopredict is an 8-gene RT polymerase chain reaction developed by Sividon Diagnostics (Koln, Germany, now part of Myriad Genetics). The test is usually used with formalin fixed paraffin embedded samples, and, combined with tumor size and nodal status, it can predict the clinical risk of distant recurrence ten years after diagnosis by assigning a score (either low or high). Endopredict is normally used as a prognostic test for patients with early breast cancer, who are ER-positive and HER2-negative (90, 178) however other studies have demonstrated its utility to inform treatment decisions. A retrospective comparative analysis of five large clinical trials (GEICAM/9906, GEI-CAM 2003/02, ABCSG-6, ABCSG-8 and TransATAC trials) analyzed a total of 3746 women, who were treated with either adjuvant endocrine therapy alone or endocrine therapy plus chemotherapy, and determined the ability of Endopredict to estimate the 10-year distant recurrence free interval rates (91). The results showed that women who received chemotherapy in addition to endocrine therapy and those who had higher Endopredict scores, showed significantly lower distant recurrence after 10 years compared to those who only received endocrine therapy.

Rotterdam Signature

The Rotterdam signature is a multi-parameter microarray-based technique that analyses tumor expression, in fresh or frozen tissue, of 76 genes involved in cell death, cell cycle, proliferation, immune response, survival, cell to cell signaling, DNA replication and repair. These genes do not overlap with Oncotype DX or Mamma-Print. The Rotterdam signature—so called due to its development at the Erasmus Medical Centre in Rotterdam—was designed for lymph node negative breast cancer patients, to predict metastatic disease over a period of five years. The model was validated in 171 breast cancer patients and showed a significant difference of 40% between good and poor prognosis groups for distant-metastasis-free survival at 60 months and a difference of 27% between groups for overall survival. This test could predict distant tumor recurrence regardless of age, menopausal status and tumor size, and could identify patients with a better prognosis who could avoid adjuvant systemic therapy (92). Later studies validated the Rotterdam signature in a large cohort of node negative breast cancer patients, including those from the TRANSBIG trial (network of TRANSlational research by the Breast International Group) (93, 179). Further research is needed for this index to be used regularly in routine practice.

Summary of Genetic Profiling Tests

Despite the value of the genetic profiling platforms described above (i.e. Oncotype DX, MammaPrint, Prosigna, Endopredict and Rotterdam Signature) to inform treatment decisions, these tests fail to predict recurrence in a fraction of patients, particularly in those with luminal subtypes (180). Thus, new or improved tools are needed to accurately predict recurrence and avoid undertreatment and overtreatment.

MEASUREMENTS IN BLOOD

Carcinoembrionic Antigen (CEA)

Carcinoembrionic antigen (CEA) is a cell surface glycoprotein which is a 641 amino acid polypeptide chain that can be released into blood by tumor cells. It is the most widely used tumor biomarker in clinical settings and for several cancers, particularly carcinomas of the bowel (2). This biomarker, normally assessed by Enzyme-linked Immunosorbent Assay (ELISA) in plasma or serum, has also received a lot of attention in breast cancer, as studies examining its prognostic value have shown that high levels are associated with poorer outcomes (94, 96, 181). For example, in a prospective study that measured pre-operative CEA levels in serum among 2062 breast cancer patients, it was shown that high levels of CEA (>5 μg/L) in 12.7% of the patients correlated with nodal involvement and larger tumors (96). In addition, an elevated CEA level was present in 56.3% of patients exhibiting cancer recurrence. Furthermore, CEA was found to be an independent prognostic factor for both disease free and overall survival regardless of node status. In addition, high CEA was associated with a high probability of metastasis, as all patients with >7.5 μg/L had recurrences during the follow up time. Other studies have examined the predictive value of CEA and high levels have been associated with poorer responses to therapy in patients with advanced disease. For example, in a study of 232 breast cancer patients with recurrent tumors following mastectomy, an increase of >2 ng/ml after the second cycle of the therapy correlated with shorter progression-free survival compared with those with lower/stable levels: 6.7 vs. 17.7 months, respectively ($P < 0.001$) (95). Furthermore, high CEA was associated with bone metastases. Despite these promising results, further studies are required before CEA is used widely in clinical practice.

CA 15.3 and CA 27.29

CA 15.3 and CA27.29 are mucin-like glycoproteins that belong to the MUC1 family. Mucins (MUCs) are heavily glycosylated, high molecular weight glycoproteins with an aberrant expression profile in various malignancies. The names 15-3 and 27.29 refer to the specific monoclonal antibodies used for detection. CA 15.3 is most commonly used although CA 27.29 has been shown to have comparable utility (182). These biomarkers are normally measured by ELISAs, but also other commercially available kits, based on radio-, enzyme- or chemi-luminescence. Studies examining CA 15.3 have shown that high levels of this protein are associated with worse outcomes and shorter survival (97, 100, 183). For example, one study in 2004 recruiting 600 newly diagnosed breast cancer patients showed that increased levels of CA 15.3 prior to surgery (>30 units/L) were associated with shorter overall survival [hazard ratio (HR) = 2.16, CI, 1.55–3.03, $P < 0.0001$], regardless of the type of adjuvant treatment administered (183). Another study prospectively measured pre-operative serum levels of CA 15.3 in 2062 breast cancer patients (96). It was shown that high levels of CA 15.3 (>30 kU/L) in 19.6% of the patients correlated with nodal involvement and larger tumors. In addition, CA 15.3 was a significant prognostic factor for disease free survival in the absence of CEA. Furthermore, rising CA 15.3 assessed with serial blood samples also predicts poor outcomes. Studies have also confirmed the predictive value of these biomarkers with several types of cancer treatment, including chemotherapy. A retrospective study examined CA 15.3 for predicting response to treatment in 73 patients with locally advanced breast cancer and found that elevated levels prior to administering of primary chemotherapy were significantly associated with poor clinical and pathological response (33). Furthermore, if the elevated levels of CA 15.3 were sustained following treatment, this appeared to be an independent predictor of recurrence ($P = 0.007$). Another study with 232 breast cancer patients who had recurrent tumors following mastectomy, analyzed the associations between CEA and CA 15.3 and the response to therapy (95). This study found that increased levels of CA 15.3 (an increase of >15 U/ml) after the second cycle of therapy correlated with shorter progression-free survival compared with normal levels: 7.7 vs. 17.3 months, respectively for CA 15.3 ($P < 0.0001$). Furthermore, elevated levels also correlated with metastases in the bones. Current evidence does not justify the use of CA 15.3 and CA27.29 for monitoring responses to therapy.

Mucin-Like Carcinoma Associated Antigen (MCA)

Mucin-like carcinoma associated antigen (MCA) is another measurement of MUC-1. Some studies have measured MCA with other circulating markers, such as CA 15.3, CEA and Tissue Polypeptide (TPA) (98). Testing two different cut off values for MCA (11 U/ml and 15 U/ml), it has been shown that MCA is more sensitive than CA 15.3, CEA or TPA (68 vs. 32%, 10%, 26% for cut off 11 U/ml and 53 vs. 32%, 16%, 42% for cut off 15 U/ml) but, less specific than CEA and CA15.3 (42% for cut off 11U/ml or 73% for cut off 15 U/ml vs. 96% and 97% respectively). Changes to MCA levels have been related to tumor response to therapy in metastatic patients and elevated pre-surgical levels seem to be associated with lower disease-free survival. For example, a study recruiting 548 participants consisting of 148 primary breast cancer patients, 150 with metastatic breast cancer, 50 patients with benign disease, and 200 participants with no clinically evident disease, showed an association between higher pre-surgical levels of MCA with lower disease-free survival, which appeared to be most significant in those with no nodal invasion. Also, in the metastatic breast cancer subgroup, decreases in MCA levels positively correlated with therapeutic response in 82% of the patients (99). However, few studies have evaluated the prognostic and predictive value of MCA individually, precluding its use clinically.

Circulating HER2

The extracellular domain of human epidermal growth factor receptor 2, also known as extracellular circular domain or ECD, can undergo proteolytic cleavage and can be released into blood, and is commonly measured by ELISA. High levels of circulating HER2 levels have been associated with worse outcomes and poorer survival, therefore measurement of this protein is a useful prognostic marker (100, 101). For example, it has been shown that higher levels of circulating HER2 were associated with a 50% reduction in overall survival in metastatic breast cancer patients compared to lower levels (10.1 months, 95% CI: 5.2–13.6 vs. 20.2 months 95% CI: 15.0–28.6, $P < 0.001$) (100). Some studies have also shown utility of this marker to monitor cancer recurrence (184), and the predictive value has been shown by studies showing that patients with high circulating ECD levels, which were sustained through treatment, benefited less from trastuzumab. For example, in a study of 175 breast cancer patients from the GeparQuattro trial, a >20% decrease in circulating HER2 throughout the course of treatment was associated with a 60% chance of pathologic complete response compared to patients where this decrease was not achieved through therapy (185). Almost identical results have also been shown with the response to lapatinib treatment (102).

Circulating PSA

After being secreted by breast cancer cells, PSA likely accumulates in the tumor microenvironment and eventually reaches peripheral blood. PSA has been measured in serum from breast cancer patients, primarily using immunoassays, and some studies have shown prognostic utility of this biomarker in breast cancer management (103, 105, 186). However, other studies have not been able to demonstrate prognostic value, despite promising results when PSA is measured in tissue samples from the tumor (187, 188). In general, using circulating PSA as a biomarker for breast cancer among women remains a challenge, as PSA levels are very low compared to men, and often undetectable (106). Although more sensitive assays are being developed (104), further research with large cohorts of patients is required before this marker is used routinely in breast cancer management.

Circulating Cell-Free DNA (ctDNA)

Apoptotic and necrotic cells can secrete fragments of DNA into blood, referred to as cell free DNA or cfDNA. If it can be confirmed that this DNA has come from cancer cells, then this measurement is better known as circulating tumor DNA (ctDNA). ctDNA is present at a very low concentration in plasma and enables non-invasive serial assessments of tumor characteristics including, assessing point mutations and DNA methylation in key genes (107). ctDNA is assessed *via* next generation sequencing or PCR-based assays. Recently, a ctDNA assay measuring 110 alpha catalytic subunit of phosphoinositide 3-kinase (PIK3CA) mutations in HER2-negative breast cancer patients has recently obtained FDA approval (110). Indeed, studies have confirmed the utility of ctDNA to monitor metastatic disease. For example, a prospective study examined plasma from 30 breast cancer patients to compare ctDNA levels, circulating tumor cells and CA 15.3 levels (108). Using digital PCR and targeted deep sequencing, somatic mutations or structural variants in PIK3CA and TP53 genes were screened for, identified and quantified at different timepoints. It was shown that the concentration of PIK3CA and TP53 mutations in plasma significantly positively correlated with increases in tumor burden, with high levels reflecting progressive disease in 89% of the cases and being associated with shorter overall survival ($P < 0.001$). Furthermore, it was suggested that ctDNA analysis could be predictive of therapeutic response earlier than CA 15.3 and circulating tumor cells. Other studies have shown that measurement of ctDNA can identify mutations linked to resistance to certain treatments, such as anti-HER2 therapy, and therefore predict treatment failure (109). However, further research is needed *via* more high-quality prospective studies, and standardized methodology, before it is used routinely in all clinics.

Circulating Tumor Cells (CTCs)

Circulating tumor cells (CTCs) can be found at very low frequency in blood and just a few CTCs per 10 ml of blood can predict an aggressive primary tumor or metastasis (20). CTCs are a heterogeneous group of cell types, such as epithelial tumor cells, epithelial-to-mesenchymal cells and cancer stem cells (29). Given their low frequency, enrichment procedures and highly sensitive assays are required to measure them, and CTCs can be quantified *via* microscopy, flow cytometry or using RT-PCR (113). One of the widely used techniques is the CellSearch Assay, which has had FDA approval for prognostic and predictive use in metastatic breast cancer (114). CellSearch

identifies circulating epithelial tumor cells, defining the CTC phenotype as EpCAM+ (Epithelial cell adhesion molecule), Cytokeratins (8+, 18+, and/or 19+), DAPI+ and CD45−, and only counts intact cells (intact cell >4 microns). Other methods are used in research settings, including flow cytometry, RT-PCR, gene expression arrays, and Fluorescence In Situ Hybridization. A study recruiting 99 metastatic breast cancer patients, enumerated CTCs using CellSearch after the second cycle of chemotherapy and showed that patients with ≥5 CTCs per 7.5 ml of blood exhibited reduced overall survival (8.7 months vs. 38.5 months, P < 0.001) and reduced progression-free survival (3 months vs. 9.4 months, P = 0.001) compared with patients who had < 5 cells per 7.5 ml of blood (34). In addition, the clinical benefit rate was also considerably lower (44 vs. 77%, P = 0.0051). Similar results were obtained in another prospective study, with metastatic patients before they started a new line of treatment (111). Finally, some studies have shown that CTCs can predict early relapse after neoadjuvant chemotherapy and shorter overall survival (189) and can predict treatment outcomes (112). Further validation studies and standardization is required for integration in clinics.

Immune Profiles

The phenotype and function of immune cells, as well as the T cell repertoire and diversity in blood, have been examined for predictive and prognostic utility in the context of breast cancer. While an individual's immune profile prior to a cancer diagnosis might influence clinical outcomes, cancer itself and/or treatment of the disease might exacerbate immunosenescence, changing immune profiles, leading to poor outcomes (190). In a study of 88 breast cancer patients with metastasis treated with cyclophosphamide or paclitaxel based chemotherapy regimens, extensive immunophenotyping was conducted in peripheral blood using flow cytometry (115). It was shown that among patients treated with paclitaxel, higher frequencies of naïve CD4+ or CD8+ T cells (CD45RA+CD95−CD27+CD28+) were associated with worse prognosis, as they correlated with shorter breast cancer specific survival (CD8+: 28.7 vs. 12.6 months, HR = 0.32 95% CI: 0.15–0.67, P = 0.0028; CD4+: 29.4 vs. 15.1 months, HR = 0.45 95% CI: 0.22–0.91, P = 0.027). In these patients, however, higher frequencies of CD11c+ dendritic cells were linked to better outcomes (13.4 vs. 25.3 months, HR = 4.60 95% CI: 1.23–17.1, P = 0.023). In the cyclophosphamide-treated group, CD14+ monocytes were also associated with good prognosis. Another study of 89 women with metastatic breast cancer showed that a CD8+CD28− cells were significantly increased compared to age-matched healthy women, and the frequency of these cells negatively correlated with progression free survival. The median survival was on average 2 months less (P < 0.001) among patients with high frequencies of CD8+ CD28− cells (≥24.0% of the CD8+ T cell pool) compared to patients with a lower frequency (<24.0%) (191).

Some studies have examined whether the capacity of T cells to recognize tumor-associated antigens is a predictive or prognostic factor in breast cancer, and in turn, whether other aspects of immunosenescence influence this response. For example, the frequency of regulatory T cells and Myeloid derived suppressor cells (MDSCs: Lin−CD14+HLA-DR−) and HER2-specific T cells were examined among 40 patients with breast cancer prior to treatment (192). Patients exhibiting HER2-reactive T cells with a lower frequency of MDSCs had a 100% rate of survival after 5 years, compared to 38% of patients without HER2-reactive T cells with higher frequencies of MDSCs (P = 0.03). Furthermore, patients without HER2-reactive T cells and with higher levels of regulatory T cells had a 50% chance of survival compared to 100% survival of patients who mounted an anti-HER2 response with lower frequencies of regulatory T cells (P = 0.03). This survival advantage appeared to be independent of metastases (192). Moreover, T cell receptor diversity and clonality was studied in a group of 26 breast cancer patients. It was shown that HER2-positive patients displayed greater highly expanded clone ratios among the CD8+ T cell repertoire and that greater heterogeneity during chemotherapy was associated with a better clinical response (116).

Finally, there is concern that the overall immune profile of individuals, especially those exhibiting signs of immunosenescence, could influence the effectiveness of some immunotherapies (193) such as the monoclonal antibodies atezolizumab and avelumab for treating breast cancer by targeting PD-L1 (Programmed death ligand 1). This ligand can be expressed by tumors and other local cells (e.g., fibroblasts, endothelial cells, antigen presenting cells, myeloid derived suppressor cells) and inhibit tumor infiltrating T cells and NK cells which express PD1. Perhaps counter-intuitively, although PD-L1 is generally expressed at low levels (around 10%) on tumor cells, it has been shown that expression level positively correlates with a higher pathological complete response rate to neoadjuvant chemotherapy (117). However, PD-L1 expression appears not to be a good predictor of the response to PD-L1 targeting therapies (118). Taken together, these findings emphasize the importance of a strong anti-tumor immune response, hence the development of anti-PD1 therapies which target T cells and NK cells directly, such as pembrolizumab (118). Indeed, the capacity to mount a strong anti-tumor response is likely to be influenced by the characteristics of the *patient* such as immunosenescence but also the characteristics of the *tumor* given that tumor mutational burden is a strong predictor of the effectiveness of anti-PD1/PD-L1 treatment (118, 119).

THE RELEVANCE OF AGING AND LIFESTYLE FOR CANCER BIOMARKER PROFILING AND DISEASE PROGRESSION

Aging Influences Tissues and Blood

Aging is a temporal and progressive decline in the integrity of different physiological systems in an organism, consisting of tissue-specific changes characterised by processes such as inflammation and cellular senescence (41). These changes affect the functional properties of most cells, tissues and organs. One feature of aging is a gradual accumulation and redistribution of adipose tissue and a change to its cellular

composition (194). The accumulation of adipose tissue is prominent within the abdominal cavity, but ectopic deposition also occurs around organs and within skeletal muscle (195, 196). Aging contributes to dysfunction of adipose tissue, characterised by changes to the tissue microenvironment at structural and cellular levels, resulting in abnormal secretions derived predominantly, from adipocytes and resident immune cells (197). Changes to the tissue include adipocyte hypertrophy, hypoperfusion, hypoxia, impaired insulin signaling, and accumulation of macrophages with a pro-inflammatory phenotype and infiltration of other inflammatory immune cells, such as sub-populations of T cells. In turn, adipose tissue dysfunction contributes toward a change in physiology at a local level (e.g., effects on the surrounding tissues, which could include, tumors for example) but also at a systemic level (e.g., low-grade inflammation and insulin insensitivity). Aging is also associated with a decline in muscle mass, muscle strength and changes to the myokinome (198–200). This muscle secretome consists of many cytokines and other soluble mediators produced by skeletal muscle in response to contractions during exercise. These so-called "exercise factors" are released into the circulation and exert endocrine or paracrine functions in other cells, tissues or organs, which has relevance for disease risk and progression (201). Interleukin-6 (IL-6) is the most well-characterized myokine and its roles when secreted from muscle are considered to be positive rather than pro-inflammatory, and include promoting glucose uptake, insulin sensitivity, lipolysis and fatty acid oxidation. However, in other contexts IL-6 is considered a mediator of inflammation, and so this cytokine is sometimes referred to as being pleiotropic; whereby depending on the context and the site of production, it can be pro- or anti-inflammatory (202, 203).

Inflammation is a self-limiting process which consists of a complex network of chemical signals triggered in the presence of damage for healing purposes, upon infiltration of pathogens as part of an immune response, or due to adipose tissue dysfunction (204). Inflammation can directly affect pathogens, such as by C-Reactive Protein activating complement (205), interferons limiting viral replication or by stimulating other immune processes, including attracting immune cells (206). The term inflammaging refers to the sustained low-grade inflammation that is characteristic of aging, and consists of higher levels of cytokines, such as IL-6 and TNF-alpha, increased levels of glucocorticoids and decreased levels of insulin-like growth factor 1 (207). Inflammaging has also been associated with deregulation of the complement pathway and increased activation of coagulation processes (208). Inflammaging leads to, or is part of, the age-associated decline and functional deterioration of immune competency, referred to as immunosenescence (209). The most accepted hallmarks of immunosenescence are lower numbers of naïve T cells and higher numbers of memory T cells, particularly within the CD8+ T cell pool (210). Sustained antigenic stimulation due to viral infection, especially Cytomegalovirus (CMV), drives these changes among T cells, but some cells accumulate with age *per se* (211), or as a result of other infections or perhaps even sub-clinical malignant transformation (212–214). Further, aging leads to impaired function of neutrophils, dendritic cells and natural killer cells, and increased frequencies of regulatory T cells and myeloid-derived suppressor cells (215). Most of these changes are very evident and well established in blood, but research characterizing inflammatory and immunological processes in tissues is limited.

Although it is likely that key mechanistic links between aging, cancer risk and tumor progression feature within inflammatory and immunological processes, it is important to emphasise that aging affects the structure and function of almost all aspects of physiology (41). In principle, a positive development in cancer care would be to incorporate measurements of aging into routine clinical tests and decision making to provide an estimate of a patient's biological age. Despite the quest for a single and easily measured biomarker of aging, a range of blood and tissue biomarkers would need to be assessed. Aside from inflammatory and immunological parameters, assessing age-associated changes to a variety of body systems might be recommended, including the cardiovascular system (e.g., blood pressure, homocysteine), metabolic health (e.g., cholesterol, glucose, leptin), the central nervous system (e.g., amyloid β42, Tau), the hypothalamic pituitary axis and sympathetic nervous system (e.g., cortisol, DHEA, IGF-1, adrenaline, noradrenaline) (216). In addition, a number of genetic markers have been proposed, such as particular alleles of apolipoprotein E, polymorphisms in the gene encoding angiotensin-converting enzyme, mutations in mitochondrial DNA, telomere length, and many epigenetic changes (216–218). Recent emphasis has been placed on measuring the accumulation of senescent cells with aging. For example, by assessing DNA damage pathways and cyclin-dependent kinase inhibitors (e.g., $p16^{INK4a}$), characterizing a senescence-associated secretory phenotype and apoptosis resistance, or determining morphological changes, such as lysosome accumulation (e.g *via* beta-galactosidase activity) or plasma membrane disturbances (e.g., caveolin-1 upregulation) (219). Finally, it might be recommended that a panel of aging biomarker measurements are interpreted alongside integrated whole-body measurements of physical functioning and frailty (e.g., sit-to-stand tests, walking tests, muscle function tests) (220).

Aging Influences Tumor Progression and Cancer Outcomes

Given the constellation of changes that happen over the life course as time elapses, both chronological and biological aging are associated with increased cancer risk. Older people are more likely to get cancer, the majority of cases occur in people over 65 years of age (221). Given that life expectancy has significantly increased in the last century (222), around 30% to 40% of patients with breast cancer are over 70 years of age (223), and yet this population is underrepresented in clinical trials (224). Older age is associated with faster disease progression, and more complications, including treatment resistance (225). Indeed, menopausal status has a very strong influence on breast cancer risk, tumor characteristics, and disease progression (226).

Although poor outcomes among older adults might be influenced by late/delayed diagnosis and undertreatment, a variety of other age-associated mechanisms likely contribute, of which some, interact with inflammation.

Deregulation of normal inflammatory processes is characteristic of aging, including a sustained release of pro-inflammatory cytokines, which can damage cells, and lead to an accumulation of damaged cells in tissues, which could conceivably progress into a malignancy (215, 227). Moreover, reactive oxygen species released by neutrophils in inflammatory settings can also damage cells, by oxidizing proteins, lipids and DNA (228). Once a tumor has developed, the levels of some cytokines have been associated with worse outcomes among patients. This is the case of IL-6, for example, as high serum levels appear to be linked with higher rates of metastasis and shorter survival in breast cancer patients (229, 230). Indeed, mechanistic studies have implicated IL-6 treatment resistance. For example, an *in vitro* study of drug-sensitive and drug-insensitive breast cancer cell lines showed that IL-6 was present at a high concentration in the media of drug-insensitive cells, but absent in the media of drug-sensitive cells (231). In addition, pre-treatment of drug-sensitive cells with IL-6 for 10 days caused an 8–10 fold increase in the resistance to the chemotherapeutic agent doxorubicin, and when drug-sensitive cells were transfected to constitutively express the IL-6 gene, drug resistance was shown to be 70-fold higher as compared with the drug-sensitive cells. Thus, it is conceivable that inflammaging could be one explanation for the treatment resistance that is sometimes seen among older people.

While several cytokines have well-established pro-tumor effects (e.g., IL-1, IL-4, IL-6) and can be produced by tumors directly in an autocrine manner (232), not all cytokines contribute toward pro-tumor processes. Indeed, many cytokines may elicit anti-tumor effects, including IL-2, IL-12, IL-15, IL-21, IFN-alfa and Granulocyte-Macrophage Colony-Stimulating Factor GM-CSF (233). Some of these cytokines have anti-inflammatory roles and can interfere with cancer progression, either by enhancing anti-tumor immunity—stimulating certain immune cells—or by exerting direct anti-proliferative or pro-apoptotic actions on tumor cells directly (234). These properties have been explored in cytokine-based immunotherapy trials, either as monotherapy or in combination with other therapeutic agents (235). IL-2, for example, promotes survival, expansion and differentiation of activated NK and T cells, and its use in immunotherapy is approved for the treatment of metastatic disease in renal cell carcinoma and melanoma (236). IFN-alfa has been shown to exert anti-proliferative, pro-apoptotic and anti-tumor activity on cancer cells, and is approved to treat Hairy cell leukemia, AIDS-related Kaposi's Sarcoma, Chronic Myelogenous Leukemia, Malignant Melanoma and Follicular lymphoma (237). However, challenges remain with these therapies, including short half-life of the cytokines, low response rates and frequent adverse events with high doses (238). However, it is conceivable that in older adults who might exhibit lower basal levels of IL-2 or IFN-alfa, or might have an impaired capacity to produce these cytokines (239, 240), these individuals might exhibit a greater risk of cancer and poorer anti-tumor responses. Indeed, the shift to a pro-inflammatory phenotype is well-known with aging (241) and some evidence shows this profile is reversed in extremely old populations, termed 'anti-inflammaging' (242, 243).

More broadly, other aspects of an aging immune system have been linked with unexpected hospitalisations during chemotherapy and limited effectiveness of some treatments—in particular immunotherapies—among older people (244–246). It is thought these effects might be partly attributed to the reduction of the naïve T cell pool, as this translates into an impaired ability to recognise and eliminate malignant cells. In addition, the senescence associated secretory phenotype (SASP) that some cells in aging tissues adopt, characterized by aberrant production of a range of cytokines, growth factors, proteases, and chemokines, could also play a role in tumorigenesis and progression (247). Finally, studies have shown that other markers of immunosenescence, including high frequencies of CD8+CD28− T cells, regulatory T cells, and myeloid-derived suppressor cells are associated with shorter survival (191).

Aging Influences Cancer Biomarker Profiles

Evidence shows that the levels and characteristics of some cancer biomarkers, that are routinely measured in tissues and in blood, can be influenced by aging, which could affect the interpretation of clinical measurements and treatment outcomes. For instance, cross-sectional studies have shown that simple biomarkers measured in plasma, which are implicated in cancer risk and disease progression, can be influenced by aging (and also other factors that change with aging, including physical activity and body composition). For example, 77 cancer and inflammatory biomarkers were assessed in plasma from 1005 individuals from the Northern Sweden Population Health Study, and the influence of 158 inter-individual factors, was assessed (248). The results showed that 18 factors including age had a significant influence on the levels of one or more of 52 of the 77 biomarkers (248). In another study, plasma IGF-1 and serum IGFBP-3 were assessed in samples from 364 women with intraepithelial neoplasia or early invasive breast cancer and compared to 376 unaffected women (249). Women with early breast cancer had 21% higher IGF-1 and 19% higher IGFBP-3 than unaffected women, however IGF-1 levels were negatively associated with age (and also BMI) across all groups (249). Similar relationships have been shown with other biomarkers, for example, preoperative serum levels of CEA were shown to significantly positively correlate with age at diagnosis and menopausal status (250).

Some of the strongest evidence of aging influencing cancer biomarkers comes from studies that have considered the menopause. For example, differences in tumor characteristics were examined among 428 pre- and post-menopausal women (251). Compared with post-menopausal women, pre-menopausal women had significantly larger tumors (21% of pre-menopausal women had tumors of >5cm of diameter vs. 12% of post-menopausal women, $P = 0.047$). In addition, pre-menopausal women were more likely to have lymph node

metastasis (77% of pre-menopausal women had positive axillary lymph nodes vs. 56% of post-menopausal women, P < 0.001) and more likely to have a positive expression of estrogen and progesterone receptors (ER: 56% of pre-menopausal women had positive expression vs. 44% of post-menopausal women, P = 0.002. PR: 52 vs. 41%, respectively, P = 0.014). Finally, pre-menopausal women had tumors with a greater proliferative capacity as shown by the higher likelihood of KI-67 positivity (33% of pre-menopausal women were KI-67 positive vs. 22.8%, of post-menopausal women, P = 0.017). Post-menopausal women, on the contrary, had significantly higher likelihood of expression of HER2 (pre-menopausal women: 2% vs. post-menopausal women: 19%, P = 0.038). Menopausal status also influences treatment decisions, and post-menopausal women were significantly more likely to have breast conserving surgery (P = 0.004), chemotherapy (P = 0.007), radiotherapy (P = 0.008), and endocrine therapy (P = 0.025) than pre-menopausal women. These results highlight important differences in breast tumors depending on menopausal status, which translate into differences in treatment and outcomes. However, other studies have suggested that age itself may be a stronger determinant of biological and etiological heterogeneity in breast tumors than menopausal status (252).

Aging in general is associated with particular molecular subtypes of breast cancer and a differential expression of some tumor biomarkers. For example, a study evaluated several makers by immunohistochemistry in different subtypes of invasive breast cancer among two groups (162 women ≤40 years and 100 women ≥50 years) (253). The results showed that Triple Negative Breast Cancer and HER2 subtypes were more common among young women. Furthermore, young women were more likely to have ER-negative tumors overall (253). In this work, tumor size and characteristics (ER, PR, HER2, Ki-67 and p53) were also compared (253). tumors from younger women were found to be significantly larger than those from older women; approximately 1.03 cm larger on average (P = 0.01). In addition, there was a significant quantitative differential expression of the tumor biomarkers on the basis of age. Younger women presented with lower expression levels of ER and PR (25% lower for ER, P < 0.01 and 10% lower for PR, P = 0.03), and higher levels of Ki-67 and P53 overexpression (10% higher for Ki-67, P = 0.01 and 13% higher for P53, P < 0.01) compared with women in the older group. Another study evaluated the influence of both age and menopausal status on several prognostic biomarkers in 1226 patients with operable primary breast cancer (254). Patients were divided into four groups: ≤40 years, premenopausal >40 years, postmenopausal <75 years and ≥75 years. The results showed that youngest patients had a worse prognosis, which improved with increasing age. Younger patients had the highest infiltration of TILs (P < 0.001), greatest p53 and Ki-67 expression (both P = 0.01) and the lowest expression levels of ER (P < 0.001). Finally, ER was also influenced by menopausal status, as expression level was higher in postmenopausal women compared to pre-menopausal counterparts (P < 0.001). Similar results have been found in larger studies (255). For example, by assaying 3800 tumor samples, significant inverse correlations with age and biomarkers of tumor growth and genetic instability (e.g., Ki-67 and p53 positivity) and growth factor receptor over expression (e.g., ErbB2+ or EGFR+) were shown (all P = 0.05), and among ER+ tumors, ER expression was significantly positively correlated with age (P < 0.0001). Likewise, a potential age-related association between HER2 and PR was evaluated in a study that examined 1104 ER positive tumors (divided into two age groups, 173 women of ≤45 years and 931 women of >45 years). There was an inverse relationship between HER2 and PR only in the group of women >45 years old (P = 0.001) (256).

There is an increasing interest on how factors such as age can affect TILs. A study examined TILs in young (35–45 years), middle-aged (55–65 years) and older (>70 years) patients with luminal B (ER+PR+HER2−) breast cancer (257). TILs were phenotyped using CD3, CD4, CD5, CD8, CD20, CD68 and FOXP3 with immunohistochemistry. The results showed that increasing age was associated with a decrease in the overall percentage of stromal TILs in biopsies (P = 0.025). In addition, age had a significant effect on the composition the tumor/immune infiltrate, including a lower density of certain immune cells identified using CD3, CD5, CD8 and CD20, which was significant in all tumor regions (P < 0.042). The proportions of CD8+ TILs also decreased significantly with age in all tumor regions (P < 0.0001). However, the distribution patterns of TILs across each tumor region did not differ with age. Likewise, another study quantified the abundance of the immune cell infiltrate (B cells, CD4+ and CD8+ T cells, neutrophils, dendritic cells and macrophages) in tumors using transcriptome datasets. It was shown that there were no significant differences in the frequency or composition of TILs between age groups (young group: <40 years, old group: ≥40 years), but high levels of TILs, and in particular, CD8+ T cells, were associated with better clinical outcomes (P < 0.04) in women under 40 years of age (258).

Other studies have examined whether the multi-parameter molecular profiling tests, including IHC4, Oncotype Recurrence Score (RS) and Prosigna Risk of Recurrence Score, are influenced by age (259). Data from 940 women in the transATAC trial was split across three age groups (group 1: ≤59.8 years, group 2: 59.8–68.2 years and group 3: >68.2 years). The results showed that the prognostic performance of all molecular scores significantly differed with age, with the lowest scores among older patients. For example, for both IHC4 and Oncotype RS, their prognostic value appeared to be strongest in the lowest age group or group 1 (IHC4: group 1 HR = 3.01, 95% CI: 1.99–4.53, vs. group 2: HR = 1.67, 95% CI: 1.23–2.26 vs. group 3: HR = 1.64, 95% CI: 1.25–2.15. Oncotype RS: group 1: HR = 2.16, 95% CI: 1.62–2.87 vs. group 2: HR = 1.39, 95% CI: 1.16–1.66 vs. group 3: HR = 1.38, 95% CI: 1.11–1.73). However, Prosigna had the most prognostic value in women between 60 and 68 years or group 2 (group 1: HR = 3.87, 95% CI: 2.21–6.78 vs. group 2:HR = 4.51, 95% CI: 2.87–7.10 vs. group 3: HR = 1.83, 95% CI: 1.28–2.60). The influence of age on other more recent biomarkers, including CTCs and ctDNA has also been examined. For example, one

study has reported a significant positive association between older age and ctDNA positivity among 31 primary breast cancer patients scheduled for neoadjuvant chemotherapy (260).

An Active Lifestyle Is Associated With Better Cancer Outcomes

In addition to the robust evidence linking a physically active lifestyle with a reduction in breast cancer risk (261), studies are beginning to show that both exercise and physical activity are beneficial during cancer treatment and in the years after. The terms "exercise" and "physical activity" are sometimes used interchangeably, and there is an important distinction that has implications for the recommendations made in a cancer setting. For example, the term "physical activity" includes leisure-time, occupational, home-based and transport-related activities, some of which, might be undertaken as normal activities of daily living. The term "exercise" refers to a component of physical activity (within the leisure-time domain) and comprises physical activities that are planned, structured, repetitive and undertaken for the purpose of improving or maintaining components of physical fitness and/or sporting performance (262). In many studies, individuals are referred to as being "active" or "inactive" and these terms infer that individuals undertake (or fail to undertake) a defined level of physical activity (e.g., such as the recommendations published by the World Health Organization). Overall, patients with cancer are advised to lead a lifestyle that is as active as symptoms allow, whether this is through structured exercise or being physically active *via* activities of daily living, and specific guidelines have been developed for all stages of disease (263–265). For example, in general, patients are recommended to undertake around 150 min of moderate-intensity physical activity each week, which if achieved in a structured way, could be in bouts of around 30 min on 5 days of the week. Alternatively, recommendations also promote around 75 min of vigorous physical activity per week and advise supplementing this aerobic exercise with strength training on at least two days of a week. These recommendations are largely based upon those advocated by the World Health Organization and other bodies for the general population (266). However, very recently, more specific recommendations have been developed for patients with cancer, focusing in particular, on structured exercise training (267). For example, unique recommendations have been made for patients with complications (e.g., metastases) and for targeting particular side-effects and symptoms of disease and treatment (e.g., anxiety, fatigue, lymphedema, physical function) (267). For example, to counter fatigue, aerobic exercise training at moderate intensity for at least 12 weeks, exercising for 30 min three times a week has been recommended. Whereas for other complications, such as lymphedema, supervised resistance exercise training in a progressive manner two or three times per week is recommended.

Aside from the distinction between structured exercise and physical activity, many studies have shown that leading a physically active lifestyle generally brings about benefits, but studies that have employed structured and supervised exercise training provide the strongest evidence. Benefits include limiting treatment toxicity and alleviating cancer-related symptoms such as fatigue, anxiety, depression, and improving quality of life (QoL), mood and self-esteem (268, 269). For example, a randomized and controlled trial investigated the effects of exercise training on QoL and cardiorespiratory fitness among 53 postmenopausal breast cancer survivors (270). Women were either assigned to an inactive control group (n = 28) or were asked to exercise on cycle ergometers three times per week for 15 weeks (n = 25). Exercise was shown to increase overall QoL by 9.1 points compared to 0.3 points from the control group (mean difference, 8.8 points; 95% CI: 3.6–14.0; P = 0.001). Further, exercise also increased peak oxygen consumption by 0.24 L/min, whereas this decreased by 0.05 L/min in the control group (mean difference, 0.29 L/min; 95% CI: 0.18–0.40; P < 0.001). Moreover, a meta-analysis investigated effects of exercise interventions on QoL, social functioning, and physical functioning of breast cancer survivors in 18 trials (exercise group = 602 participants; control group = 603 participants) (271). The pooled effect confirmed that exercise significantly improved QoL (SMD = 0.35; I2 = 61%; 95% CI: 0.15–0.54; P = 0.0004), social functioning (SMD = 0.20; I2 = 16%; 95% CI: 0.08 to 0.32; P = 0.001), and physical functioning (SMD = 0.32; I2 = 32%; 95% CI: 0.20–0.44; P < 0.00001). Remaining active during cancer treatment has also been shown to improve clinical outcomes (268) and to enhance the efficacy of various cancer treatments (272). Other studies have shown that high levels of physical activity are associated with improved survival and lower levels of cancer recurrence (35–37). The mechanisms underlying these observations have not been proven, however likely explanations include exercise and physical activity influencing the effectiveness of treatment and modulating the properties of tumors both indirectly and directly.

An Active Lifestyle Might Lead to Better Cancer Outcomes Due to Improved Chemotherapy Completion Rates

Patients who remain active during the period when they receive chemotherapy are more likely to tolerate a greater dose and complete their treatment (273, 274). For example, a study evaluated the potential benefits of aerobic and resistance exercise among 243 breast cancer patients undergoing adjuvant chemotherapy (273). Patients were randomly assigned to either supervised resistance exercise (n = 82), supervised aerobic exercise (n = 78) or usual care (n = 82), for a median of 17 weeks. Chemotherapy completion rate was assessed as the average relative dose intensity (RDI) from the originally planned regimen, and it is known that patients who receive an RDI of >85% have better outcomes. It was shown that patients in the resistance exercise training and the aerobic exercise training groups had better completion rates when compared to the usual care group, although this was only statistically significant for the resistance exercise regimen (RDI =84.1% control group vs. RDI= 89.8% resistance exercise group; mean difference=5.7%; 95% CI: 0.4–11.0; P < 0.033). Another study with a comparable group of breast cancer patients (n = 230) also compared usual care with

two exercise regimens: a low intensity home based regimen and a moderate-high intensity supervised regimen combining aerobic and resistance exercises during a period of chemotherapy treatment (274). This study evaluated chemotherapy and trastuzumab completion rates and found that moderate-high intensity exercise improved completion rates, as a significantly lower number of patients in this group required chemotherapy dose adjustments compared to other groups (12% moderate-high intensity vs. 34% low-intensity vs. 34% usual care, $P < 0.002$). In addition, a smaller percentage of patients in the moderate-high intensity group required a delay or termination of trastuzumab therapy compared to the other two groups (6% moderate-high intensity vs. 24% low-intensity vs. 28% usual care). It is worth highlighting that the home-based exercise was not supervised and was of lower intensity, whereas the most effective intervention employed exercise that was supervised and of moderate intensity. Generally, supervised exercise, and activities that are more demanding, elicit more robust effects.

Exercise and Physical Activity Influence Cancer Biomarker Profiles

There is a need for further research examining whether exercise and physical activity influence cancer biomarker profiles. Most evidence in support of this concept shows that broader factors, which are not necessarily cancer-specific, but are linked to clinical outcomes, including immune competency, inflammation, and metabolic health, can change among patients who modify their lifestyle (190). For example, a systematic review of 45 articles, including a variety of observational studies and randomized control trials of different designs, summarized the effects that physical activity in general can have among cancer survivors on biomarkers (275). This analysis included the HEAL (Health, Eating, Activity and Lifestyle) study, an observational prospective cohort study of 746 breast cancer survivors. It was concluded that regular physical activity can lead to immunological benefits (e.g., natural killer cell cytotoxicity, increased T cell proliferation), positive changes to proteins involved in insulin-signaling pathways (e.g., C peptide, insulin-like growth factors) and decreases in systemic inflammation (e.g., C-Reactive Protein, serum Amyloid A). Similar conclusions were drawn by a pooled analysis of three randomized controlled trials examining the influence of resistance exercise on factors that have been linked to poor cancer prognosis, including C-reactive protein, IL-6, IL1-beta, insulin-like growth factor binding proteins, leptin, serum amyloid A, adiponectin and TNF-alpha (276). Post-menopausal breast cancer survivors were allocated to either 1 year of resistance exercise consisting of two 1 hour supervised classes and one 45-minute home-based session each week ($n = 109$) or to a control group who undertook stretching and relaxation exercises ($n = 106$). It was shown by each trial that resistance training reduced systemic inflammation and improved insulin signaling.

A limited number of studies have examined the effects of exercise and physical activity on cancer-specific biomarkers. For example, a study of 15 females with breast cancer investigated the influence of 8 weeks of aerobic exercise training on serum levels of CEA and CA 15.3 (277). Participants exercised three times a week, at a light-to-moderate intensity. The results showed that participants exhibited a significant reduction in their BMI, body fat percentage, and body mass ($P = 0.0001$) and there was a trend for a decline in the levels of CA 15.3 ($P = 0.091$). There was no significant change in CEA. Another study, examined whether 12 weeks of structured exercise affected CEA among 54 healthy elderly women (70–77 years), randomized to different groups, varying on the frequency of exercise undertaken (278). The results showed that CEA significantly decreased in all groups with the largest decrease (percentage change: $-59 \pm 5\%$) among women who exercised 2–3 days per week.

Exercise and Physical Activity Affect Tumors Directly and Indirectly

Exercise and physical activity lead to changes in tumor characteristics, including angiogenesis and enhanced tumor blood perfusion (due to an increase in tumor blood vessel density, function and maturity, which leads to reductions in intratumoral hypoxia), impaired growth and increased immune cell infiltration (279–283). These changes are clinically relevant as they may enhance the efficacy of some therapies, such as chemotherapy or immunotherapy, by facilitating the delivery of drugs to the tumor, and increased tumor vascularization and blood perfusion could facilitate immune-surveillance and processes such as reactive oxygen species production by some immune cells and treatments (272).

For example, a study in 50 athymic female mice evaluated the effects of 6 weeks voluntary wheel running on breast cancer growth and progression (279). Half of the mice were allocated to an active group with access to a running wheel and the other half were a control group with no access to a running wheel. Mice were implanted with human breast cancer cells on the first day of the study. During the intervention, tumor growth was monitored, as well as several markers of tumor blood perfusion, hypoxia, vascularization and angiogenesis. After 6 weeks, although no statistically significant differences were found between the groups for tumor growth or survival, access to a running wheel changed many tumor charactetistics. The active group exhibited increased intratumoral vascularization and blood perfusion, but also an increase of hypoxia-inducible factor 1 (HIF-1). In this study, mice were athymic and therefore lacking T cells, which may explain why tumor growth and overall survival was not affected. Indeed, even more encouraging results have been shown by another study of a very similar design but with immunocompetent animals. Mice allocated to a voluntary exercise condition were compared to a control group ($n = 11–12$ per group) and it was shown that the exercise group had a significantly lower tumor growth rate ($P < 0.012$), higher tumor apoptosis ($P = 0.048$), greater microvessel density ($P = 0.004$) and increased tumor vessel maturity, as determined by colocalization of CD31 with desmin (281). However, different to the previous study, intratumoral hypoxia was significantly reduced in the active group compared to the control group ($P = 0.012$). Most importantly, this study examined interaction between exercise

and treatment. Tumor bearing mice were allocated to either recieve no treatment, exercise only, cyclophospamide only, or exercise combined with cyclophosphamide (n = 17 per group). It was shown that the combination of exercise and cyclophosphamide had the most striking impact on slowing tumor growth, providing initial evidence that exercise and the adaptations that may follow, improve the delivery of chemotherapy to tumors.

Similar to an improvement in the delivery of drugs to sites where they are needed, physical activity may also enhance the ability of immune cells to migrate to tumors. For example, one study examined a number of cancer models in mice, including breast cancer. Mice were randomized to four weeks of voluntary wheel running, or to a non-running control group prior to tumor cell inoculation. Additional groups were designed to examine questions related to the timing of exercise relative to tumor formation (284). Overall, physical activity resulted in a significant accumulation of tumor infiltrating immune cells, including natural killer cells, CD3+ T cells and dendritic cells, which appeared to be mediated, at least among natural killer cells, by IL-6 and epinephrine. Physical activity was also linked with an upregulation of pathways associated with inflammation in the tumor (e.g., increased gene expression for IL-1-beta, IL-6, TNF-alpha) and immune function (e.g., increased gene expression of NKp46, NKG2D, CD68, CD209, CD8, CD74, FoxP3). Other studies have shown that reduction of hypoxia can also facilitate the infiltration of these immune cells is tumors in mice (285), and given that exercise has been shown to reduce tumor hypoxia, this might be another exercise-induced mechanism that facilitates the homing of immune cells to tumors. However, although some tumor infiltrating lymphocytes may have a beneficial role (e.g., CD8+ T cells) in tumor control (286), other cells, such as myeloid derived suppressor cells could have the opposite effect promoting tumorigenesis, tissue-destruction and metastases (287).

There are likely to be many other characteristics of tumors that could be affected by physical activity or exercise, but the effects on treatment and clinical outcomes may remain unknown. For example, one study has indicated that exercise reduces oxidative stress in breast tumors, as shown by 3-fold lower levels of 8-oxo-dG—a marker of oxidative damage to DNA—when examining tumors from a group of mice that had access to a running wheel compared to controls (288). It has also been hypothesized that physical activity and exercise may counter the dysregulated energy metabolism of cancer cells, which is characterized by high glucose uptake and glycolysis (289). Studies in rats injected intraperitoneally with the carcinogen 1-methyl-1-nitrosourea showed that rats with free access to running wheels exhibited less cancer incidence and a lower average number of tumors per rat compared to controls (290). The exercising rats also showed changes in blood levels of hormones and growth factors involved in glucose metabolism, as reductions in plasma insulin, insulin-like growth factor 1 (IGF-1) and leptin were shown. In support, breast cancer bearing mice undergoing 7 weeks of endurance exercise training studied showed that in addition to a reduction in tumor mass, there was also a significant decrease in the levels of tumor lactate compared to untrained controls (291). Exercise training also resulted in significant changes in the levels of some enzymes that are essential for sustaining a glycolytic phenotype of tumor cells. For example, lactate dehydrogenase isoforms A and B, and monocarboxylate transporter 1 were decreased in tumors from trained mice, which, in combination with lower lactate production, could contribute to slower tumor progression. Indeed, excess of lactate anaerobic metabolism in cancer cells has been associated with poorer activation, infiltration and function of immune cells within the tumor (292). Therefore, these metabolic findings support the positive impact of exercise in enhancing anti-cancer immunity that may improve treatment outcomes.

Despite some very advanced studies with animal models, mechanistic research with human participants examining the effects of exercise on tumor characteristics and clinical outcomes is limited. Indeed, most mechanistic insight in human settings is limited to review articles, which summarize that better clinical outcomes among more active patients, are likely to be linked to mechanisms related to metabolic growth factors, inflammation, immune function, myokines and adipokines (293). Indeed, some understanding of how exercise and physical activity can affect tumors directly comes from studies that have incubated cancer cell lines with human serum collected before and after exercise. For example, a study collected serum from breast cancer survivors before and after a 6-month exercise training intervention (i.e. to examine chronic effects of exercise) and before and after a 2 hour bout of exercise (i.e. to examine acute effects) (294). Breast cancer cell lines were grown in human serum for 48 hours and the effects on viability was examined. Serum samples collected before and after the exercise training intervention provided evidence of a reduction in systemic inflammation shown by lower IL-6 and TNF-alpha post-intervention, but these serum samples had no anti-growth effect on the breast cancer cell lines. However, serum samples collected immediately after an acute bout of exercise—which, as expected, exhibited a high concentration of adrenaline, noradrenaline, lactate and IL-6—reduced the viability of the breast cancer cell lines by approximately 9% (294). Subsequent work showed that breast cancer cells grown in this acute-exercise-conditioned serum were 50% less tumorigenic when implanted into mice, due to adrenaline and noradrenaline activating the Hippo signaling pathway, and subsequent phosphorylation of the YAP protein, reducing the expression of genes associated with proliferation (295).

Prospective cohort studies with patients are ongoing, such as the AMBER study, which is examining relationships between physical activity and health related fitness with treatment outcomes among 1500 newly diagnosed breast cancer patients (296). Physical activity is measured objectively using wearable devices, cardiorespiratory fitness is assessed directly, along with body composition using dual x-ray absorptiometry, and clinical measurements such as lymphedema and fatigue are also being recorded. However, most importantly, molecular measurements in tumors will be interpreted alongside clinical outcomes,

with follow up at 1, 3 and 5 years. Among the very few studies which have investigated the relationship between exercise and treatment outcomes with cellular and molecular measurements, is a randomized clinical trial of 20 breast cancer patients undergoing neoadjuvant chemotherapy (297). One group underwent a standard period of doxorubicin and cyclophosphamide treatment, whereas another group received this chemotherapy with supervised aerobic exercise training. Exercise reduced systemic inflammation, but increased some angiogenic factors, including proangiogenic factor placenta growth factor (PLGF). In addition, circulating endothelial progenitor cells increased, which might contribute toward tumor vessel normalization and the reduction of hypoxia, shown by animal studies. However, this study was unable to examine whether exercise improved the clinical response to chemotherapy due to power.

Other human studies provide more indirect evidence of exercise-induced mechanisms that might benefit patients with cancer. For example, it is very well established that acute bouts of exercise cause a transient lymphocytosis and a subsequent lymphocytopenia in the hours after, whereby lymphocytes with strong tissue-migrating and effector capabilities, migrate to peripheral tissues searching for antigens (298). This effect is particularly marked among T cells and natural killer cells, and is thought to represent immunosurveillance, that may even facilitate the detection and elimination of tumors (42, 298–300). The concept that regular exercise might bolster aspects of immune function has been shown by a randomized and controlled trial in breast cancer survivors (301). Participants were randomized to either aerobic exercise training for 15 weeks three times per week (n = 25), or an inactive control group (n = 28). The results showed that regular exercise increased cytotoxic activity of natural killer cells. Other indirect effects of exercise shown in human studies that might benefit patients with cancer might be brought about by interaction with age-related processes, such as immunosenescence and inflammaging. For example, exercise training or remaining physically active throughout life might prevent, limit, delay or even reverse some aspects of immunosenescence (190, 299, 302). A potential mechanism is limiting the expansion of late-stage differentiated T cells by exercise mobilizing these cells to peripheral tissues, where they are exposed to apoptotic signals, followed by a mobilization of hematopoietic cells and trafficking to the thymus, stimulating development of naïve T cells (190). This hypothesis is supported by several observational studies, including a comparison of 125 regular cyclists (55–79 years), 75 age-matched older adults and 55 young adults who did not exercise regularly (303). Cyclists exhibited many features of a less-aged immune system, including lower proportions of late-stage differentiated T cells, high frequencies of B cells, lower levels of IL-6, and higher levels of the thymoprotective cytokine IL-7 (303). In support, another study has shown that higher levels of directly measured cardiorespiratory fitness are associated with lower frequencies of late-stage differentiated T cells and higher frequencies of naïve T cells (304). Finally, it is well established that regular exercise and physical activity can counter inflammation, and perhaps over a lifetime, this effect limits inflammaging (42, 299, 300). For example, a study of 3075 participants aged 70–79 years reported lower levels of inflammatory markers, including IL-6, TNF-alpha and CRP, among those who performed higher levels of exercise (305).

Adiposity Is Associated With Poor Cancer Outcomes

Overweight and obesity are characterised by excess accumulation of adipose tissue and are commonly been defined using Body Mass Index (BMI), of between 25–30kg/m^2 or more than 30kg/m^2 respectively (306). Being overweight or obese is associated with an increased risk of developing breast cancer, and these associations are strongest in postmenopausal women (307, 308). However, a higher BMI and/or higher percentage body fat are measurements that have also been associated with worse clinical outcomes among women diagnosed with breast cancer, including worse prognosis, higher risk of recurrence, and lower overall and disease-specific survival (38–40). For example, a metanalysis showed that there appears to be a linear relationship between BMI and mortality beginning from 20 kg/m^2 when assessed before diagnosis and up to 12 months after (40). Moreover, obesity also appears to have an impact on the effectiveness of some treatments. A pooled study compared data from 8 prospective trials of breast cancer patients treated with neoadjuvant chemotherapy and found that high BMI negatively influenced the response to anthracycline-taxane based treatment, and was significantly associated with lower rates of pathological complete response (309). High BMI was also associated with shorter disease-free survival and overall survival independently of pathological complete response in luminal-like tumors and in triple negative breast cancer. In addition, obesity has been linked to the development of tumor metastases (310) and recurrence (311). For example, in a study of 1250 HER2 positive breast cancer patients it was shown that in the ER negative subgroup of patients, obese individuals were more likely to develop distant metastases at 5 years (33.4%, 95% CI: 22.1–50.5) than those in the overweight (17.9%, 95% CI: 12.3–25.9) or under/normal weight (17.5%, 95% CI: 13.8–22.4) groups (310). However, not all studies evaluating the influence of overweight and obesity in cancer settings have reported worse outcomes compared to lean counterparts: this phenomenon has been named the "obesity paradox" as some studies reported that people with a high BMI responded better to therapy than expected or had better survival rates (312). As an example, a prospective study of 88 metastatic breast cancer patients on palliative chemotherapy analyzed the impact of BMI on survival and treatment response over a follow up period of 40 months (313). It was shown that a greater proportion of overweight patients were most responsive to treatment (56%) followed by obese patients (30%) compared to a smaller proportion in the normal weight group (15%) (313). Moreover, patients with a BMI ≥25 kg/m^2 survived for longer (19 months) in comparison with patients who had a BMI < 25 kg/m^2. However, it is worth considering that this study has a relatively small sample size and it may not have adequately controlled for

other potentially influencing factors, such as tumor type, receptor status, extent of disease, cardiovascular risk, etc.

Adiposity Could Be Associated With Poor Cancer Outcomes Due to Undertreatment

It has been suggested that in the past, obesity has been linked with undertreatment, where the dose of some chemotherapies has been adjusted to the ideal body mass of a patient, or arbitrarily capped at a body surface area of 2.0 m^2. For example, a retrospective cohort study compared treatment patterns among overweight, obese, and patients of a normal weight, in a total of 9672 breast cancer patients treated with chemotherapy (314). The results showed that, compared to the 9% of people in the healthy weight group, 11% of the overweight group, 20% of the obese group, and 37% of the severely obese group, were administered dose reductions during their first chemotherapy cycle. This reduction in the dose has been associated with poorer outcomes (315), and could partially explain why adiposity relates to worse prognosis. The rationale for dosing chemotherapy based on body surface area, rather than absolute body mass, is to avoid toxicity, however evidence shows that this strategy could lead to poor clinical outcomes and that toxicity is unlikely. For example, a study examined data from 1,435 stage II breast cancer patients undergoing adjuvant chemotherapy to determine if dosing based on actual body mass increased risk of toxicity (316). Analyses during the first chemotherapy cycle showed that patients with a BMI ≥ 27.3 kg/m^2 who were dosed according to actual body mass did not exhibit excess toxicity (% of women with toxicity: 47% of overweight women vs. 51% of lean women, P = 0.51). Indeed, compared to overweight women who received a dose reduction due to body surface area dosing, overweight women who received their dose based on actual body mass, had an adjusted risk ratio of treatment failure of 0.73 95% CI: 0.53–1.00, indicating that dose reduction can lead to poor clinical outcomes. However, guidelines now advocate dosing chemotherapy for obese patients based on absolute body mass (317, 318). Thus, understanding why obesity is associated with poor treatment outcomes, requires further investigation.

Adiposity Influences Cancer Biomarker Profiles

Obesity is associated with particular molecular subtypes of breast cancer. For example, a study evaluated the link between BMI and breast cancer subtypes (319). In a retrospective analysis of 848 patients with primary operable breast cancer, groups were formed on the basis of BMI: normal weight (BMI = 18–24.9 kg/m^2), overweight (BMI = 25–29.9 kg/m^2) and obese (BMI > 30 kg/m^2). The results showed that triple negative breast cancer was more common among overweight and obese women, whereas HER2-positive tumors were more frequent among women of normal weight.

Body composition can also affect the properties of tumors, as well as the levels and characteristics of some cancer biomarkers. Evidence in support comes from randomized and controlled trials implementing behavioral or lifestyle interventions to bring about changes to physiology. For example, one study randomized 32 overweight or obese stage 0-II breast cancer patients into an intervention and control group as part of a 30 day pre-surgery "weight loss" study (320). The intervention group received counseling on caloric restriction and aerobic exercise to promote a change in body mass of 0.68–0.92 kg/week. The control group received nutritional counseling and upper body resistance exercise which was assumed to elicit a smaller energy expenditure than aerobic exercise. Circulating cytokines and metabolic measurements implicated in cancer progression but also tumor characteristics were assessed. The intervention group exhibited a greater change in body mass than the control group (−3.62 vs. −0.52 kg) and exhibited greater changes in metabolic measurements, including serum leptin and fasting insulin, and inflammatory markers such as TNF-alpha. Most importantly, a greater change to body mass and accelerometer-measured physical activity was positively associated with an infiltration of the CD56+dim cytotoxic sub-population of natural killer cells into tumors. Indeed, tumors from the intervention group were characterized by a greater expression of key genes associated with immune cell recruitment (e.g., CX3CL1, CXCL1, and CXCL12), and higher TNF-alpha, but there were no differences in Ki-67 between groups.

Other evidence for body composition affecting cancer biomarkers comes from cross-sectional studies. For example, one study investigated the association between BMI in 535 post-menopausal women with operable breast cancer and the expression of HER2. The results showed that, with increasing BMI, there was a significant decrease in HER2 overexpression (321). The circulating form of HER2 has also been shown to be positively associated with BMI in a healthy population of males and females aged 45–65 years (322). Other cross-sectional studies have examined the influence of BMI on results from molecular profiling tests. For example, 865 postmenopausal women with breast cancer were divided into groups on the basis of BMI (<25 kg/m^2, 25–30 kg/m^2 or ≥30 kg/m^2). It was shown that IHC4 and Oncotype RS had the most prognostic value for distant recurrences in the group with the lowest BMI and there was no prognostic value in the group with a BMI ≥30 kg/m^2. In the case of Prosigna, the score was most prognostic in patients with a BMI 25–30 kg/m^2. Other cross-sectional studies have examined TILs in the context of body composition. For example, functional tumor infiltrating CD8+TILs were assessed in two groups of breast cancer patients who were classified as either lean (BMI < 25 kg/m^2) or obese (BMI > 32.5 kg/m^2). It was shown that CD8+ TILs from obese patients had a significantly lower expression of Granzyme B (323). Furthermore, there was a significantly lower number of these cells in the lymph nodes draining the tumor in the obese group.

Other studies have examined soluble cancer biomarkers in a variety of body fluids. For example, a study of 128 women with breast cancer (89 post-menopausal) and 254 without breast cancer (125 post-menopausal) measured prostate specific antigen (PSA) in serum and nipple aspirate fluid (324). Among women with breast cancer, PSA measured in nipple aspirates

from pre-menopausal women negatively correlated with BMI ($r = -0.53$, $P = 0.049$), whereas PSA correlated positively with BMI in samples from post-menopausal women ($r = 0.37$, $P = 0.017$). Among women without breast cancer, serum PSA was negatively correlated with BMI in both pre- ($r = -0.56$, $P = 0.001$) and post-menopausal women ($r = -0.37$, $P = 0.017$), but this association was lost when controlling for plasma volume (324). Indeed, obesity is associated with an expansion of blood and plasma volume (325–327) and it is often not considered that the concentration of cancer biomarkers reported in cross-sectional studies could be affected. For example, a study investigated the effect of plasma hemodilution on the concentration of several tumor markers in 6917 healthy women and found that BMI was significantly positively associated with a greater plasma volume, as well as with higher serum concentrations of CEA and α-fetoprotein and lower concentrations of CA 125 and CA 19.9 (328). Even in investigations examining changes over time with serial measurements, results might be affected by shifts in plasma volume. Bouts of exercise that could have been undertaken by study participants and patients in the hours before blood sampling, which is sometimes not controlled for, can decrease plasma volume by up to about −10%, artificially increasing the concentration of some measurements (329, 330). Although these potential inaccuracies in reported values are probably only a minor consideration, they could shift a measurement above or below a cut-off or threshold that influences treatment decisions, or with serial measurements, could give falsely influence estimates of disease progression.

Adiposity Can Affect Tumors Directly and Indirectly

The mechanisms underlying links between obesity and breast cancer treatment have not been determined. Some mechanisms could be indirect and systemic due to the impact that overweight and obesity has on metabolic health, inflammation, and immune competency, whereas other mechanisms could be more direct, or at least related to the characteristics of local tissue surrounding breast tumors. Adipose tissue could in principle contribute to local tumorigenesis, but perhaps counter-intuitively, women with a high percentage of breast adipose tissue, are at a lower risk of disease (331). Indeed, high mammographic density, characterized by radiologically dense breasts consisting of epithelial or stromal tissue which appears light on a mammogram, compared to adipose tissue which appears dark, is a strong predictor of breast cancer risk (332, 333). Although BMI and physical activity should be considered when interpreting mammographic density data (334, 335) it is important to emphasise that the characteristics of breast adipose tissue, such as the phenotype, and the secretory profile, are probably the most important factors that could influence breast tumors.

In vitro and *in vivo* animal studies have examined whether interactions between breast cancer cells and different cell types within surrounding adipose tissue, such as mature and immature adipocytes, and normal and cancer associated fibroblasts, influence tumor progression (336, 337). Using cell co-cultures and mouse models, it was shown that cancer cells triggered phenotypical changes in the surrounding adipocytes, such as increased production of proteases and pro-inflammatory mediators including IL-6, IL-8, CCL2 and CCL5 (336). Indeed, this cross-talk between so-called cancer-associated adipocytes, contributed toward cancer progression and invasion (336). Cytokine production was enhanced further when cancer cells interacted with immature adipocytes stimulating mammosphere formation, resulting in higher invasion and metastatic potential. Indeed, when the cancer cells were injected into mice after co-culture with immature adipocytes for 7 days, the number of tumor initiating cells increased 3-fold, and the volume of metastases in the lungs increased as did the number of circulating tumor cells (337). Further experiments showed that immature adipocytes and the release of cytokines upregulated embryonic stem cell transcription factors c-MYC, SOX2, and NANOG, through Src activation, promoting the expansion of cancer stem cells (337).

Other animal studies have shown that adipocytes from human and mouse breast tissue recruit and activate macrophages (338). For example, one study has used a human-in-mouse breast cancer model whereby human breast adipose stromal cells, modified to model an inflammatory environment of obese breast, are injected into the mouse mammary fat. In this work, mice were randomized to eat either a normal diet (ND) or to eat a diet with increased calories from fat (HFD). It was shown that in mammary glands of HFD mice, total numbers of macrophages were significantly increased ($4.4 \times 10^5 \pm 0.5 \times 10^5$; macrophages/gland) compared with ND mice (2.5×10^5 tumor$\pm$ 0.5×10^5; $P = 0.05$). It was also shown that the recruitment and activation of these macrophages was through the CCL2/IL-1b/CXCL12 signaling pathway. These findings provide a mechanistic role for adipocytes leading to adipose tissue dysfunction in breast tissue, which could precede tumor development (338). A study in mice evaluating obesity-promoted breast tumor growth showed that increased oxidation of fatty acids and reduced glycolysis, both enhanced by the leptin-PD-1-STAT3 axis in CD8+ TILs, promoted obesity-related breast tumorigenesis and contributed to resistance to immunotherapy (323). Inhibiting STAT3 or fatty acid oxidation restored CD8+ T cell effector functions and inhibited tumor development in obese mice. Other murine studies have provided further evidence that obesity can impair cancer immune surveillance. For example, showing that obesity promotes hyperactivation of CD8+ TILs, and an accumulation of granulocytic myeloid-derived suppressor cells (G-MDCSs), which induced Fas/FasL mediated apoptosis of CD8+ T (339).

Research in humans has also examined links between breast cancer and dysfunctional adipose tissue. For example, one study compared two groups of individuals without a breast cancer diagnosis (lean $n = 37$, obese $n = 19$) to patients with breast cancer ($n = 12$) (340). Using RT-PCR to examine expression levels of genes in circulating leukocytes, it was shown that TNF-alpha, IL-6, leptin and ErbB2, were

significantly higher in obese individuals without a cancer diagnosis and among breast cancer patients compared to the lean group. Assuming leukocyte gene expression of ErbB2 is representative of gene expression in breast tissue, then obesity-associated over-expression could have important implications for tumorigenesis and treatment, given its role in metastatic disease. A possible mechanism underlying interactions between disease progression and adipose tissue surrounding breast tumors could be the adoption of an adipose derived secretory phenotype that attracts different populations of immune cells. Adipose tissue dysfunction is characterised by changes to the tissue microenvironment at cellular and structural levels, which results in abnormal secretions derived from adipocytes and local immune cells (197). Changes include adipocyte hypertrophy, hypoperfusion, hypoxia and impaired insulin signaling, leading to an enlargement of adipose tissue, low-grade systemic inflammation due to the release of inflammatory cytokines (341, 342) and possibly exacerbated immunosenescence (343). These changes lead to immune cell accumulation within adipose tissue, most prominently consisting of macrophages with a pro-inflammatory phenotype and effector-memory CD8+ T cells (195, 197). The implications of attracting highly inflammatory populations of immune cells to areas surrounding breast tumors are unknown, but could conceivably have both negative and positive effects, depending on the cell type recruited, perhaps in part providing one explanation for the "obesity paradox". For example, a study investigated 334 breast tumors from patients with long-term follow-up and showed that high frequencies of tumor infiltrating CD8+ T cells were associated with higher cumulative breast cancer specific survival (344). On the other hand, a metanalysis of sixteen studies and a total of 4,541 breast cancer patients showed that overall survival and disease free survival correlated with high frequencies of tumor associated macrophages (overall survival: HR = 1.50, 95% CI: 1.20–1.88 vs. disease free survival: HR 2.23, 95% CI: 1.72–2.90) (345).

Although in obesity, there is often a large accumulation of abdominal adipose tissue, deposition occurs elsewhere, including the breast, and a question that remains is whether regional depots of adipose tissue interact differently with tumors. To further improve our understanding of this question, a study isolated breast tissue-derived and abdominal tissue-derived mesenchymal stem cells (MSCs) from healthy adults undergoing cosmetic surgery (346). MSCs, with the capacity to differentiate into adipocytes, were co-cultured with MCF7 or MDA-MB-231 breast cancer cell lines and compared to co-culture with human macrophages. MSCs from both regions stimulated proliferation of the breast cancer cell lines similarly, and abdominal MSCs had a higher expression of IL-1-beta compared to breast MSCs. Co-culturing MSCs with macrophages led to higher levels of VEGF-A, VEGF-C, SERPINE1, FGF2, IL-1-beta and IL-6 gene expression in macrophages. Thus, MSCs, and perhaps adipocytes from both breast and abdominal depots, interact with macrophages, which could lead to the development of dysfunctional adipose tissue.

In summary, further studies are required to understand mechanistic interactions between adipose tissue—including adipocytes and adipose-associated immune cells—with breast cancer cells. Indeed, if the dysfunction of adipose tissue surrounding breast tumors influences the accumulation of local immune cells, tumor infiltrating lymphocytes, and other tumor characteristics, then this process could have an impact on the expression of tumor biomarkers and cancer progression. Moreover, systemic adipose tissue dysfunction could lead to metabolic, inflammatory and immunological profiles that have been associated with poor clinical outcomes. Encouragingly, if adipose tissue dysfunction and adipose derived secretions contribute to tumorigenesis, then lifestyle interventions could in principle limit disease progression and facilitate treatment. For example, regular exercise, triggers a reduction in fat mass and limits the release of adipokines, resulting in anti-inflammatory adaptations (42, 299, 347).

CONCLUSIONS

Managing heterogeneity in the clinical response exhibited by patients remains a challenge. The first part of this article summarized biomarkers that are available to address this problem, by informing therapeutic options, assessing pathological response and predicting clinical outcomes. The second part of this article summarized factors such as aging, physical activity, and body composition, that might influence the sensitivity and specificity of these biomarkers, by modulating the cellular composition and function of tissues. This article has highlighted that the characteristics of patients, including their age, physical activity level and adiposity, could interact with disease progression and influence treatment effectiveness due to a combination of direct and indirect mechanisms (**Figure 1**). Indeed, processes and profiles associated with lifestyle, including metabolic health, inflammaging and immunosenescence, are gaining increasing recognition as being important factors that can influence cancer and its treatment. The positive outlook is that some of these processes might be reversible, or at least, their development might be slowed or limited, by for example, encouraging patients to lead a physically active lifestyle, at almost any stage of disease. In summary, the measurement of cancer biomarkers in blood or in tumors could be influenced by patient characteristics and their lifestyle, because these factors affect the composition and function of cells and tissues across the body and across the life-course. These factors are not commonly considered clinically or in research, either for practical reasons or because the supporting evidence base is developing. Thus, a broader perspective within cancer care is required which integrates objective measurements of aging, lifestyle and other patient characteristics, using a combination of established biomarkers measured in tissues and in blood, but also broader whole-body measurements of physical functioning and frailty (216, 219, 220). Given the literature presented herein, we hope that this article encourages an interdisciplinary phenomic approach in oncology research and clinical management.

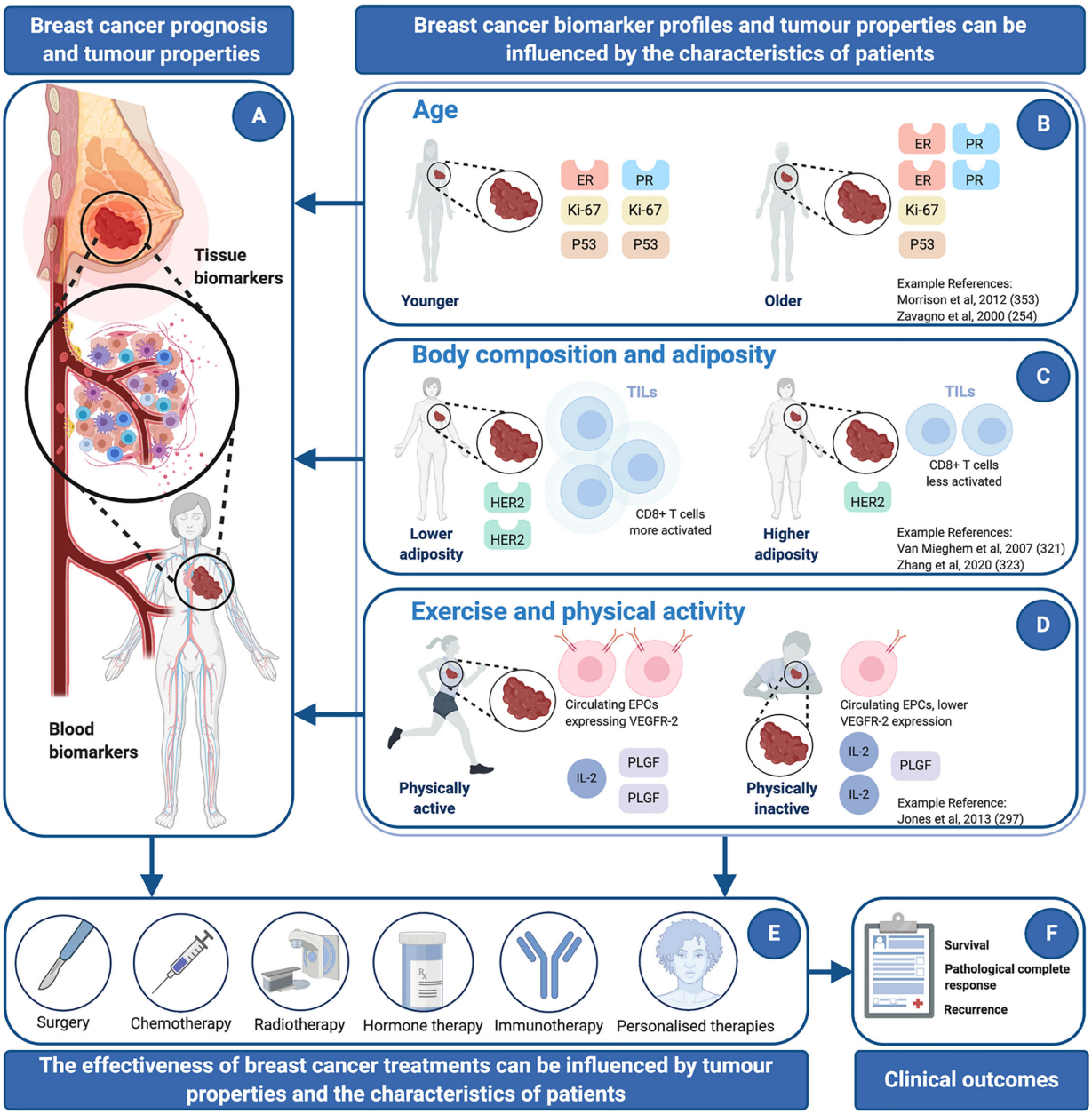

FIGURE 1 | Breast cancer prognosis, tumor properties, and clinical outcomes can be influenced by the characteristics of patients, including: age, body composition and adiposity, or exercise and physical activity. References are considered to be representative examples of robust human studies with breast cancer patients. **(A)** Cancer biomarkers can be assessed in tumor tissue or in blood and can provide information about prognosis and the clinical response to different treatments. **(B)** Some studies have shown that older age is associated with lower expression of tumor proliferative markers (e.g., Ki-67) and proteins implicated in tumor progression (e.g., P53), and higher expression of certain hormone receptors (e.g., ER, PR). **(C)** Higher adiposity has been associated with a lower expression of HER2, a lower magnitude of tumor immune cell infiltration and lower activation status of tumor-resident CD8+ T cells. **(D)** Bouts of exercise and physical activity have been shown to decrease some inflammatory markers (e.g., IL-2) and increase pro-angiogenic factors (e.g., PLGF and EPCs expressing VEGFR-2). Higher tumor vascularity could facilitate the delivery of drugs to a tumor. **(E)** The effectiveness of breast cancer treatments can be influenced by tumor properties [shown in panel **A**] and the characteristics of patients [shown in **(B–D)**]. **(F)** In turn, interaction between tumor properties, the characteristics of patients, and the effectiveness of breast cancer treatments can influence clinical outcomes. EPCs: Epithelial Progenitor cells, ER: Estrogen Receptor, HER2: Human Epidermal Growth Factor Receptor-2, IL-2: Interleukin 2, IL-6: Interleukin 6, KI-67: nuclear protein Ki-67, PGLF: Placenta Growth Factor, PR: Progesterone Receptor, P53: tumor protein 53, TILs: tumor Infiltrating Lymphocytes, VEGFR-2: Vascular Endothelial Growth Factor Receptor-2. Figure created with BioRender.com. Adapted from "tumor Microenvironment 2" and "Types of Cancer Treatment", by BioRender.com (2020). Retrieved from https://app.biorender.com/biorender-templates.

AUTHOR CONTRIBUTIONS

JT and AAE conceived the idea, drafted the manuscript and critically appraised evidence. MB, JC, RJ, RB, KG, PB, and DT undertook a critical review of the manuscript, edited, and contributed toward writing. All authors contributed to the article and approved the submitted version.

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Decreased Peripheral Naïve T Cell Number and Its Role in Predicting Cardiovascular and Infection Events in Hemodialysis Patients

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Patients with end-stage renal disease (ESRD) are at high risk of morbidity and mortality from cardiovascular and infectious diseases, which have been found to be associated with a disturbed immune response. Accelerated T-cell senescence is prevalent in these patients and considered a significant factor contributing to increased risk of various morbidities. Nevertheless, few studies have explicated the relevance of T-cell senescence to these fatal morbidities in ESRD patients. In this study, we designed a longitudinal prospective study to evaluate the influence of T-cell senescence on cardiovascular events (CVEs) and infections in hemodialysis (HD) patients. Clinical outcomes of 404 patients who had been on HD treatment for at least 6 months were evaluated with respect to T-cell senescence determined using flow cytometry. We found that T-cell senescence was associated with systemic inflammation. High-sensitivity C-reactive protein was positively associated with decreased naïve T cell levels. Elevated tumor necrosis factor-α and interleukin 6 levels were significantly associated with lower central memory T cell and higher T effector memory CD45RA cell levels. Decreased $CD4^+$ naïve T cell count was independently associated with CVEs, whereas decreased $CD8^+$ naïve T cell count was independently associated with infection episodes in HD patients. In conclusion, HD patients exhibited accelerated T-cell senescence, which was positively related to inflammation. A reduction of naïve T cell could be a strong predictor of CVEs and infection episodes in HD patients.

Keywords: hemodialysis, T-cell senescence, naïve T cells, cardiovascular event, infection

INTRODUCTION

End-stage renal disease (ESRD), considered as a public health concern, affects more than 1.5 million people worldwide (1). Patients with ESRD usually have a high risk of life-threatening comorbidities, especially cardiovascular and infectious diseases. According to the U.S. Renal Data System, ESRD patients have a 25% annual mortality rate, and almost 50% patient deaths are attributed to

cardiovascular complications (2). Infection is the second leading cause of death, accounting for 35% of all-cause mortality (3). It has been proposed that chronic kidney disease may be a model of premature aging, since uremia could induce premature senescence and many aging-related complications are prevalent in ESRD patients, including those with cardiovascular diseases (CVDs) and infections (4). Recent evidence suggests that uremia can induce T-cell senescence, indicated by a lower thymic output of naïve T cells, a decline in T-cell telomere length, and an increase in differentiation toward the terminal differentiated memory phenotype; T-cell senescence is more pronounced in patients undergoing hemodialysis (HD) therapy (5, 6). Compared with physiological aging, ESRD seems to have the ability to increase the immunological age of T cells by 20–30 years (7). In terms of function, T cells in ESRD patients are pre-activated by secreting more inflammatory cytokines in the resting state, leading to persistent inflammation and providing a breeding ground for CVD (8, 9). On the contrary, T cells in ESRD patients have diminished reaction toward pathogen stimulation, with susceptibility to apoptotic death after activation (9), reduced humoral response to vaccination (10), and impaired maintenance of specific T cell memory (11), resulting in a high incidence of infection. Hence, interventions targeting T cell function could improve morbidity and mortality in such patients.

While it is well-recognized that ESRD-related T cell dysfunction is prominent, few studies have explicated the relevance of T-cell senescence to the fatal morbidity resulting from ESRD, and existing results are based on different markers of immune senescence. It has been reported that telomere length shortening is associated with a higher risk of death, reduced thymic output is associated with severe infection episodes, and terminally differentiated $CD8^+$T cell expansion is closely linked to accelerated atherosclerosis in ESRD patients (5). $CD4^+CD28^-$ T cells, as a terminal differentiated memory phenotype, were independently associated with the presence of atherosclerotic disease in ESRD patients (12). Cytomegalovirus (CMV) infection is considered to act as a critical factor for accelerated T-cell senescence in ESRD patients by exacerbating the selective depletion of naïve T cells and clonal expansion of memory T cells (13). However, since most patients with ESRD are CMV-seropositive (14, 15), it is difficult to distinguish the CMV-independent effects of T-cell aging in ESRD. The question that then arises is whether it would be possible to find one consistent marker for evaluating overall immunological age, assessing the risk of multiple complications, and aiding early intervention in ESRD patients.

Depletion of naïve T cells, the most significant and consistent change reported during aging, is also prevalent in ESRD (8, 15). Our previous study findings revealed that a decrease in the number of naïve T cells is significantly associated with increased mortality in HD patients (16), supporting the idea that selective reduction of naïve T cell is a critical feature in this population and may impact clinical outcomes profoundly. In the present study, we prospectively analyzed whether T-cell senescence is associated with cardiovascular events (CVEs) and infectious episodes in HD patients and aimed to find valuable markers for clinically evaluating immunological aging and predicting risk of ESRD.

MATERIALS AND METHODS

Study Population

This current study included patients who had been on HD treatment for at least 6 months in the Blood Purification Center, Department of Nephrology, Zhongshan Hospital, Fudan University. Patients were enrolled from August to September, 2016 and followed weekly. Individuals who experienced CVE or infection within 3 months were excluded. Those with evidence of hematological diseases, rheumatic diseases, active malignancies, and history of human immunodeficiency virus infection or using immunosuppressants were also excluded. Follow-up lasted for 2 years and ended in October 2018. During follow-up, CVEs and infection episodes were documented. CVEs were defined as coronary artery disease, congestive heart failure, stroke, and peripheral arterial occlusive disease. Infection episodes were defined as infectious diseases requiring regular intravenous antibiotics in hospital or emergency department.

We obtained blood samples from the arterial site of vascular access before the start of the HD session in the middle of the week. Anti-CMV-IgM and IgG antibodies were detected using the Roche Elecsys assay. All procedures were performed at the Department of Clinical Chemistry, Zhongshan Hospital, Fudan University using standard methods. Written informed consent was obtained from all patients that met the inclusion criteria. This study was approved by the Ethics committee of Zhongshan Hospital, Fudan University.

Cell Preparation and Flow Cytometry Analysis

On the day of blood drawing, blood samples mixed with heparin anticoagulant were lysed with red blood cell lysis solution and 0.1 mM EDTA and prepared for flow cytometry analysis with the following fluorescein-conjugated monoclonal antibodies: CD3-PE (Bio- Legend, San Diego, CA, USA), CD4-APC (eBioscience, San Diego, CA, USA), CD8a-Percp/Cy5.5 (eBioscience), CD45RO-FITC (Miltenyi Biotec, Bergisch Gladbach, Germany), and CCR7-APC/Cy7 (BioLegend). The relative expression of CD45RO and CCR7 was used to identify naïve T cell ($T_{Naïve}$, $CD45RO^-$ $CCR7^+$), central memory T cell (T_{CM}, $CD45RO^+$ $CCR7^+$), effector memory T cell (T_{EM}, $CD45RO^+$ $CCR7^-$), and T effector memory CD45RA cell (T_{EMRA}, $CD45RO^-$ $CCR7^-$) subsets of $CD4^+$ or $CD8^+$ T cells. These markers were selected according to previous studies (7, 17). The immunophenotyping methods and gating strategy have been elaborated in the supplementary materials (**Figure S1**).

Statistical Analysis

All data are expressed as mean ± standard deviation or median (interquartile range), as appropriate. Correlations between T cell parameters and laboratory variables were tested using a non-

parametric Spearman rank analysis. Free survival of CVEs and infection episodes were estimated using the Kaplan–Meier curve, and differences between groups were examined using the log-rank test. Univariate Cox regression analysis was used to identify predictors of CVE and infection. Significant predictors were subsequently added to the multivariable model, and backward stepwise Cox regression identified the most parsimonious model. The probability used for the stepwise regression was set at 0.05 for entry of variables and 0.1 for removal of variables. The results of the Cox proportional hazards analysis are presented as the hazard ratio (HR) and 95% confidence interval (95% CI). Statistical significance was considered at $P < 0.05$. All statistical analyses were performed using SPSS version 20.0.

RESULTS

Demographic and Clinical Characteristics of Patients

A total of 404 patients (248 men and 156 women) were enrolled in this study. The average age of patients was 59.4 ± 14.6 years. The median time in HD was 53 (26, 80) months. Of the 404 patients, 94 (23.3%) had diabetes mellitus and 324 (80.2%) had hypertension. The overall frequency of CVD in this cohort was 30.7%; stroke and congestive heart failure were the most prevalent complications, followed by coronary artery disease and peripheral arterial occlusive disease. The underlying kidney diseases included chronic glomerulonephritis (46.8%), diabetic nephropathy (16.8%), polycystic kidney disease (9.4%), hypertension renal disease (3.5%), others (10.9%), and unknown (12.6%). Only one patient (0.2%) was seropositive for CMV-IgM, and 401 patients (99.3%) were seropositive for CMV-IgG. The median level of CMV-IgG was 468 U/ml, and 189 patients (46.8%) had CMV-IgG titers exceeding the upper limit of 500 U/ml. **Table 1** presents the baseline characteristics of the study population.

TABLE 1 | Demographic data of the study population.

Variable	mean ± SD/median (interquartile range)
Age, years	59.4 ± 14.6
Time on HD, months	53 (26,80)
Male (%)	248 (61.4%)
Diabetes mellitus (%)	94 (23.3%)
CVD history (%)	124 (30.7%)
Hypertension (%)	324 (80.2%)
CMV seropositive (%)	401 (99.3%)
BMI (kg/m^2)	21.5 ± 3.2
Kt/Vurea	1.31 ± 0.57
Hemoglobin, g/L	112.4 ± 15.9
White blood cell, ×10^9/L	6.56 ± 2.02
Lymphocytes, ×10^9/L	1.3 ± 0.5
Albumin, g/L	39.0 ± 3.2
Prealbumin, g/L	0.32 ± 0.13
Creatinine, μmol/L	1,000.3 ± 277.3
Uric acid, mmol/L	441.6 ± 88.8
Calcium, mmol/L	2.32 ± 0.24
Phosphorus, mmol/L	2.17 ± 0.65
Total cholesterol, mmol/L	4.11 ± 1.07
Triglyceride, mmol/L	1.45 (1.03, 2.23)
LDL-C, mmol/L	2.27 ± 0.87
HDL-C, mmol/L	1.06 ± 0.59
Homocysteine, μmol/L	34.7 (26.4, 46.6)
NT-proBNP, pg/ml	3,882.0 (1,782.3, 10,324.2)
iPTH, pg/ml	260.7 (150.3, 407.2)
Ferritin, pg/ml	296.9 (139.3, 495.5)
hsCRP, mg/L	4.0 (1.4, 10.2)
TNF-α, pg/ml	33.4 (22.8, 58.8)
IL-6, pg/ml	9.6 (4.2, 36.2)

CVD, cardiovascular disease; CMV, cytomegalovirus; BMI, Body mass index; LDL-C, low density lipoprotein–cholesterol; HDL-C, high density lipoprotein–cholesterol; NT-proBNP, N-terminal pro-brain natriuretic peptide; iPTH, intact parathyroid hormone; hsCRP, high-sensitivity C-reactive protein; TNF-α, tumor necrosis factor-α; IL-6, interleukin 6.

T-Cell Senescence Is Associated With Systemic Inflammation in HD Patients

We examined the association between T cell subsets and circulating inflammatory markers at enrollment. As shown in **Table 2**, high-sensitivity C-reactive protein (hsCRP) was positively associated with decreased $T_{Naïve}$ cell count in both $CD4^+$ and $CD8^+$ T cell compartments ($p < 0.05$). Meanwhile, elevated tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) levels were significantly associated with lower $CD4^+$ T_{CM} and higher $CD4^+$ T_{EMRA} levels ($p < 0.001$).

Decreased $CD4^+$ $T_{Naïve}$ Cell Count as a Predictor of CVEs in HD Patients

During the 650 ± 176 days of follow-up, 86 patients (21.3%) experienced at least one CVE and a total of 99 CVEs were recorded. The incidence of CVE was 13.4% per year. A total of 42 patients died of CVEs, accounting for 56.8% of all-cause mortality. Furthermore, 32 patients had stroke and 14 died of it; 24 patients developed acute coronary syndrome and 12 died of it; 22 patients experienced at least one event of heart failure and 8 died of it; 12 patients developed lower extremity atherosclerotic occlusive disease and 4 died of it; and 4 patients died of sudden cardiac death. The median value of each T cell parameter was used in analyzing the correlation between CVEs. A lower absolute number/percentage of $CD4^+$ $T_{Naïve}$ as well as a higher percentage of $CD4^+$ T_{EM} and $CD8^+$ T_{EM} could significantly predict CVEs (**Figure S2**). When taking age into consideration, only $CD4^+$ $T_{Naïve}$ cells were shown to significantly predict CVEs. In the pairwise comparison, patients with a lower $CD4^+$ $T_{Naïve}$ count had a significantly higher CVE incidence in both the middle-aged [36 < age (years) ≤ 65, $p = 0.014$] and old (age > 65 years old, $p = 0.003$) groups. There was no difference in CVE incidence between middle-aged patients with a lower $CD4^+$ $T_{Naïve}$ count and old patients with a higher $CD4^+$ $T_{Naïve}$ count (**Figure 1**). In the univariate Cox proportional hazard model, other CVE predictors included older age, history of CVD and diabetes mellitus, usage of central venous catheter, lower serum levels of albumin, prealbumin, creatinine, and uric acid, and increased levels of white blood cell count, hsCRP, and N-terminal pro-brain natriuretic peptide (NT-proBNP) (**Table 3**). In the multivariate Cox hazard model, a decreased count of $CD4^+$ $T_{Naïve}$ cells along with older age, history of diabetes, history of CVD, as well as elevated white blood cell count and

TABLE 2 | Correlations between T cell subsets and inflammatory markers in hemodialysis patients.

	TNF-α		IL-6		hsCRP	
	Correlation coefficient	***p***	**Correlation coefficient**	***p***	**Correlation coefficient**	***p***
Cell subset percentage						
$CD4^+$T cells %	−0.198**	<0.001	−0.093	0.062	0.005	NS
$CD4^+$ $T_{Naïve}$%	0.015	NS	0.026	NS	−0.097	0.052
$CD4^+T_{CM}$%	−.225**	<0.001	−.248**	<0.001	0.071	NS
$CD4^+T_{EM}$%	−0.032	NS	−0.016	NS	0.096	0.054
$CD4^+T_{EMRA}$%	0.321**	<0.001	0.312**	<0.001	−0.034	NS
$CD8^+$T cells%	0.180**	<0.001	0.05	NS	−0.002	NS
$CD8^+$ $T_{Naïve}$%	−0.016	NS	−0.023	NS	−0.146*	0.003
$CD8^+$ T_{CM}%	−0.083	0.095	−0.046	NS	0.052	NS
CD8 $^+T_{EM}$%	−0.121*	0.015	−0.061	NS	0.100*	0.045
$CD8^+T_{EMRA}$%	0.104*	0.037	0.073	NS	0.073	NS
Absolute cell number						
$CD4^+$T cells (cells/μl)	−0.085	0.089	−0.129*	001	−0.062	NS
$CD4^+$ $T_{Naïve}$ (cells/μl)	−0.058	NS	−0.085	0.087	−0.108*	0.03
$CD4^+T_{CM}$ (cells/μl)	−0.215*	0.001	−0.260*	0.001	0.012	NS
$CD4^+T_{EM}$ (cells/μl)	−0.021	NS	−0.075	NS	0.043	NS
$CD4^+T_{EMRA}$ (cells/μl)	0.242*	0.001	0.205*	0.001	−0.063	NS
$CD8^+$T cells (cells/μl)	0.075	NS	−0.059	NS	−0.035	NS
$CD8^+$ $T_{Naïve}$ (cells/μl)	0.033	0.051	−0.062	NS	−0.148*	0.003
$CD8^+$ T_{CM} (cells/μl)	−0.07	NS	−0.084	0.093	0.013	NS
CD8 $^+T_{EM}$ (cells/μl)	−0.033	NS	−0.103*	0.039	0.044	NS
$CD8^+T_{EMRA}$ (cells/μl)	−0.016	NS	0.044	NS	0.046	NS

*Spearman rank analysis was applied to investigate the relationship between inflammatory markers and T cell subset level, including the percentages as well as absolute cell counts of naïve ($T_{Naïve}$), central memory (T_{CM}), effector memory (T_{EM}), and effector memory CD45RA (T_{EMRA}) subsets. *p value < 0.05. **p value < 0.001. NS, non-significant, p value > 0.1.*

NT-proBNP was independently associated with CVEs (HR 0.430, 95% CI 0.253–0.731, $p = 0.002$).

Decreased $CD8^+$ $T_{Naïve}$ Cell Count as a Predictor of Infection Episodes in HD Patients

A total of 90 patients (22.3%) experienced at least one infectious episode and 16 died of it, which accounted for 21.6% of all-cause mortality. The incidence of infection was 15.6% per year. A total of 97 infectious events were recorded. The following infections were reported: pulmonary infections [n = 55 (56.7%)], dialysis access-related infections [n = 14 (14.4%)], skin or joint infections [n = 9 (9.3%)], urinary or abdominal infections [n = 10 (10.3%)], septic shock [n = 3 (3.1%)], and infections at other sites or undocumented sites [n = 6 (6.2%)]. The median value of each T cell parameter was used for analyzing the correlation between

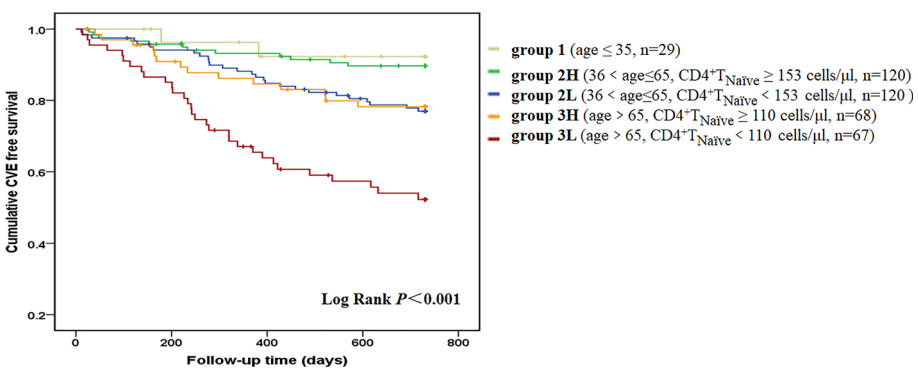

FIGURE 1 | CVE-free survival curves according to age-$CD4^+$ $T_{Naïve}$ group. We divided the patients into five groups according to age and $CD4^+$ $T_{Naïve}$ cell count. Group 1 included young patients (age ≤35 years old, n = 29). Group 2L included middle-aged patients with a lower $CD4^+$ $T_{Naïve}$ cell count [36 < age (years) ≤ 65, $CD4^+$ $T_{Naïve}$ < 153 cells/μl, n = 120]. Group 2H included middle-aged patients with a higher $CD4^+$ $T_{Naïve}$ cell count [36 < age (years) ≤ 65, $CD4^+T_{Naïve}$ ≥ 153 cells/μl, n = 120]. Group 3L included old patients with a lower $CD4^+$ $T_{Naïve}$ cell count (age > 65 years old, $CD4^+T_{Naïve}$ < 110 cells/μl, n = 67). Group 3H included old patients with a higher $CD4^+$ $T_{Naïve}$ cell count (age > 65 years old, $CD4^+$ $T_{Naïve}$ ≥ 110 cells/μl, n = 68). Kaplan-Meier analysis revealed that survival rate was significantly different among the five age-$CD4^+$ $T_{Naïve}$ groups ($p < 0.001$). In pairwise comparison, patients with a lower $CD4^+$ $T_{Naïve}$ count had a significantly higher CVE incidence in both the middle-aged ($p = 0.014$) and old groups ($p = 0.003$). There was no difference between middle-aged patients with a lower $CD4^+$ $T_{Naïve}$ count and old patients with a higher $CD4^+$ $T_{Naïve}$ count.

TABLE 3 | Cox hazard model for CVEs in hemodialysis patients.

Variables	Univariate Cox hazard model		Multivariate Cox hazard model[1]	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age (≥65 years old = 1)	2.967 (1.929, 4.564)	<0.001	1.747 (1.068, 2.857)	0.026
Sex (male = 1)	1.263 (0.810, 1.968)	0.303		
Diabetes mellitus (yes = 1)	2.767 (1.802, 4.249)	<0.001	1.687 (1.060, 2.683)	0.027
CVD (yes = 1)	5.169 (3.323, 8.034)	<0.001	3.118 (1.839, 5.286)	<0.001
Central venous catheter (yes = 1)	2.137(1.383, 3.302)	<0.001		
BMI (kg/m^2)	0.961 (0.902, 1.024)	0.275		
Kt/Vurea	0.556 (0.288, 1.074)	0.081		
Time on HD (month)	0.996 (0.991, 1.001)	0.108		
CMV IgG (U/ml)[2]	1.003 (1.000,1.007)	0.084		
Hemoglobin (g/L)	0.989 (0.976, 1.003)	0.116		
White blood cell (×10^9/L)	1.111 (1.011, 1.220)	0.029	1.155 (1.040, 1.283)	0.007
Albumin (g/L)	0.859 (0.804, 0.916)	<0.001		
Prealbumin (g/L)	0.023 (0.003, 0.199)	0.001		
Creatinine(μmol/L)	0.998 (0.998, 0.999)	<0.001		
Uric acid (mmol/L)	0.995 (0.993, 0.998)	<0.001		
Triglyceride (mmol/L)	0.804 (0.649, 0.996)	0.045		
LDL-C (mmol/L)	0.974 (0.758, 1.251)	0.835		
Phosphorus (mmol/L)	0.928 (0.664, 1.295)	0.660		
Calcium (mmol/L)	0.723 (0.297, 1.759)	0.475		
Log-iPTH (pg/ml)	1.107 (0.616, 1.989)	0.735		
β2-Microglobulin (mg/L)	1.012 (0.986, 1.040)	0.371		
Homocysteine (μmol/L)	1.001 (0.995, 1.007)	0.705		
Log-hsCRP (mg/L)	2.058 (1.439, 2.943)	<0.001		
Log-NT-proBNP (pg/ml)	4.407 (2.777, 6.994)	<0.001	2.388 (1.409, 4.048)	0.001
CD4$^+$ $T_{Naïve}$ count (≥137 cells/μl = 1)	0.352 (0.219, 0.553)	<0.001	0.430 (0.253, 0.731)	0.002
CD4$^+$ $T_{Naïve}$ % (≥36.7 = 1)	0.505 (0.325, 0.784)	0.002		
CD4$^+$$T_{EM}$% (≥33.2 = 1)	1.987 (1.279, 3.087)	0.002		
CD8$^+$$T_{EM}$% (≥22.0 = 1)	1.770 (1.143, 2.741)	0.011		

CVD, cardiovascular disease; BMI, Body mass index; HD, hemodialysis; CMV IgG, cytomegalovirus immunoglobulin G; LDL-C, low density lipoprotein–cholesterol; Log-iPTH, log transformed intact parathyroid hormone; Log-hsCRP, log transformed high-sensitivity C-reactive protein; Log-NT-proBNP, log transformed N-terminal pro-brain natriuretic peptide. $T_{Naïve}$, naïve T cell; T_{EM}, effector memory T cell.

[1]Backward conditional method was used. Model included each T cell parameters and was adjusted for age, gender, history of CVD, history of diabetes, types of vascular access, Kt/Vurea, CMV IgG, albumin, prealbumin, white blood cell, creatinine, uric acid, triglyceride, LDL-C, NT-proBNP, and hsCRP.

[2]For those with CMV-IgG titers exceeding the upper limit of 500 U/ml, the numbers were regarded as 500 U/ml.

infections. Decreased absolute count/percentage of CD8$^+$ $T_{Naïve}$ and increased percentage of CD8$^+$ T_{EMRA} cells were significant predictors of infection (**Figure S3**). Although aging contributes to both infection and depletion of CD8$^+$ $T_{Naïve}$ cells, patients with a lower CD8$^+$$T_{Naïve}$ count in the middle-aged group [36 < age (years) ≤ 65] had a significantly higher infection incidence than those with a higher CD8$^+$ $T_{Naïve}$ count in the same age group (p = 0.04) (**Figure 2**). Other infection event predictors included a history of CVD, usage of central venous catheter, decreased levels of hemoglobin, albumin, prealbumin, creatinine, and uric acid, and increased serum levels of hsCRP, NT-proBNP, ferritin, and globulin (**Table 4**). In the multivariate Cox hazard model, a decreased count of CD8$^+$ $T_{Naïve}$ cells was independently associated with infection episodes in HD patients (HR 0.460, 95% CI 0.279–0.758, p = 0.002).

DISCUSSION

In the current study, CVEs and infections were the major complications accounting for more than 70% of all-cause mortality. Our study finding indicates that a decreased level of CD4$^+$ naïve T cells is a strong predictor of CVEs, while a decreased level of CD8$^+$ naïve T cells is a strong predictor of infectious episodes in HD patients. Loss of naïve T cells might be a hallmark of immune disturbance, leading to a more intense immune incompetence with profound clinical outcomes.

In the original model of the T cell system, naïve T cells are activated in the presence of infection, which then proliferate and generate heterogeneous classes of effector and memory cells with distinctive surface phenotypes, cytokine production abilities, and homing potentials (18). The T cell system has unique mechanisms of replenishment. Thymic T cell generation is the only way to add novel naïve T cells and enrich diversity; however, thymic function rapidly declines during adolescence and early adulthood and is quantitatively irrelevant throughout adult life (19). Instead, homeostatic proliferation is responsible for maintaining the size of the naïve T cell compartment and sustaining the richness of the T cell receptor repertoire (20). Generally, homeostatic proliferation in humans is efficient in maintaining a sizable CD4$^+$ naïve T cell pool (21). CD8$^+$ naïve T cells, on the contrary, are progressively lost with age, which induces a higher homeostatic proliferation of aged-CD8$^+$ naïve T cells than that of aged-CD4$^+$ naïve T cells (20).

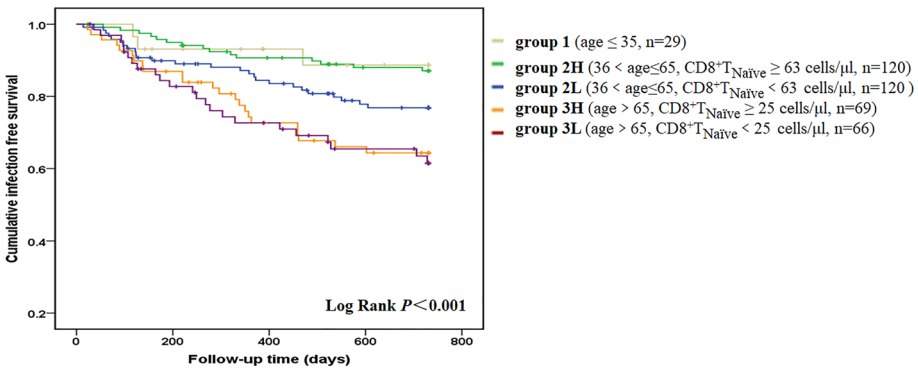

FIGURE 2 | Infection-free survival curves according to age-$CD8^+$ $T_{Naïve}$ group. We divided the patients into five groups according to age and $CD8^+$ $T_{Naïve}$ cell count. Group 1 included young patients (age ≤35 years old, n = 29). Group 2L included middle-aged patients with a lower $CD8^+$ $T_{Naïve}$ cell count [36 < age (years) ≤ 65, $CD8^+$ $T_{Naïve}$ < 63 cells/μl, n = 120]. Group 2H included middle-aged patients with a higher $CD8^+$ $T_{Naïve}$ cell count [36 < age (years) ≤ 65, $CD8^+$ $T_{Naïve}$ ≥ 63 cells/μl, n = 120]. Group 3L included old patients with a lower $CD8^+$ $T_{Naïve}$ cell count (age > 65 years old, $CD8^+$ $T_{Naïve}$ < 25 cells/μl, n = 66]. Group 3H included old patients with a higher $CD8^+$ $T_{Naïve}$ cell count (age > 65 years old, $CD8^+$ $T_{Naïve}$ ≥ 25 cells/μl, n = 69). Kaplan-Meier analysis revealed that survival rate was significantly different among the five age-$CD8^+$ $T_{Naïve}$ groups ($p < 0.001$). In pairwise comparison, old patients had a significantly higher infection incidence, regardless of the $CD8^+$ $T_{Naïve}$ count. Patients with a lower $CD8^+$ $T_{Naïve}$ count in the middle-aged group had a significantly higher infection incidence than those with a higher $CD8^+$ $T_{Naïve}$ count in the same age group ($p = 0.04$).

To the best of our knowledge, this is the first study to identify a decrease in $CD4^+$ naïve T cells as a novel CVE risk factor and a decrease in $CD8^+$ naïve T cells as a novel infection risk factor in patients with ESRD. Notably, compelling data suggest profound lymphopenia of naïve T cells in both the $CD4^+$ and $CD8^+$ compartments in ESRD (15, 22), although the underlying mechanism is not sufficiently understood. It is evident from the literature that there is a reduced thymic output in ESRD (15, 22); however, the more important reason seems to be the failure to maintain quiescence in these cell compartments. Maintenance of quiescence is vital for naïve T cells to retain their self-renewal potential and differentiation plasticity throughout life. In circumstances of inflammation, T cells can leave their usual quiescent state and accumulate as partially differentiated cells, even in the absence of antigen stimulation (23, 24). In ESRD, inflammation is significantly enhanced with uremia (25), and dialysis treatment certainly exposes these patients to microbial products and other antigenic stimulations, which can lead to accelerated activation and turnover of naïve T cells. Thus, chronic inflammation could be responsible for the decreased naïve T cells in ESRD patients, which is supported by our finding that decreased levels of naïve T cells were correlated with elevated levels of the inflammation marker hsCPR in both $CD4^+$ and $CD8^+$ compartments. In earlier studies on aging, the decline in naïve T cells and relative expansion of memory and effector T cell populations were entirely due to chronic CMV stimulation (26). In this context, chronic immune stimulation could be the reason for accelerated T cell aging in ESRD patients, including at least the prevalent CMV infection, renal damage, uremia toxin retention, and increased reactive oxygen species generation. Any attempts to maintain the naïve T cell pool eventually lead to its further depletion and extinction, as such attempts result in the partial loss of stemness and incomplete differentiation and activation of negative regulatory programs (20, 27). In this context, decreased naïve T cells could represent their maladaptive behavior in ageing and even trigger a vicious cycle of aggravated immunosenescence. This is more so in case of $CD4^+$ naïve T cells, as their shrinkage is not common during normal aging. Besides chronic kidney diseases, rheumatoid arthritis is another pathological condition wherein there are several lines of evidence of premature aging of T cells, indicating a defective DNA repair mechanism in $CD4^+$ naïve T cells (28, 29). T cell senescence should be included in the assertion that cellular senescence is an emerging cardiovascular risk factor along with senescence of the endothelial and vascular smooth muscle cells (30, 31). We have reported that the absolute numbers of $CD8^+$ naïve T cells decreased significantly with age in a nearly parallel pattern in HD patients aged 20–89 years (16). In the current study, we found that the levels of $CD8^+$ naïve T cells dropped to an extremely low level in HD patients older than 65 years, which could explain why we did not find a significant correlation between $CD8^+$ naïve T cells and infection in these patients. In the middle-aged patients, a decreased $CD8^+$ naïve T cell count was significantly related to a higher risk of infection episodes. This could be attributed to a decreased T cell receptor diversity in naïve T cells, which are not only vital for a primary T cell response but continue to be a resource for T cell responses to antigens previously encountered. On the contrary, chronic immune stimulation, such as that by CMV infection, can also lead to the clonal expansion of the T cell population, which can severely compromise repertoire diversity. Recent studies indicated that ESRD patients present reduced T cell receptor diversity with clonal expansion (32, 33), leading to a high incidence of infection in these patients.

Generated from naïve T cells, T_{CM} cells home to lymph nodes, lack potent effector functions, and mount rapid

TABLE 4 | Cox hazard model for infection incident in hemodialysis patients.

Variables	Univariate Cox hazard model		Multivariate Cox hazard model[1]	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age (≥65 years old = 1)	2.514 (1.658, 3.813)	<0.001		
Sex (male = 1)	1.207 (0.784, 1.858)	0.394		
Diabetes mellitus (yes = 1)	1.213 (0.755, 1.949)	0.425		
CVD (yes = 1)	1.724 (1.130, 2.630)	0.011		
Central venous catheter (yes = 1)	2.653(1.744, 4.036)	<0.001	2.225 (1.416, 3.497)	0.001
BMI (kg/m^2)	0.985 (0.925, 1.048)	0.626		
Kt/Vurea	0.777 (0.481, 1.255)	0.303		
Time on HD (month)	0.999 (0.995, 1.003)	0.700		
Hemoglobin (g/L)	0.978 (0.967, 0.990)	<0.001	0.983 (0.970, 0.997)	0.014
White blood cell (×10^9/L)	1.013 (0.915, 1.122)	0.800		
Albumin (g/L)	0.864 (0.812, 0.920)	<0.001		
Globulin (g/L)	1.048 (1.003, 1.095)	0.036	1.039 (0.996, 1.084)	0.074
Prealbumin (g/L)	0.079 (0.011, 0.540)	0.010		
Creatinine(μmol/L)	0.998 (0.998, 0.999)	<0.001		
Uric acid (mmol/L)	0.997 (0.994, 0.999)	0.007		
Phosphorus (mmol/L)	0.794 (0.571, 1.104)	0.171		
Calcium (mmol/L)	1.136 (0.475, 2.716)	0.775		
Log-iPTH (pg/ml)	0.902 (0.517, 1.573)	0.717		
Log-hsCRP (mg/L)	1.780 (1.268, 2.498)	<0.001		
Log-NT-proBNP (pg/ml)	2.180 (1.403, 3.388)	0.001	1.559 (0.977, 2.488)	0.062
Log-ferritin (pg/ml)	1.729 (1.002, 2.983)	0.049		
CD8$^+$ $T_{Naïve}$ count (≥46 cells/μl = 1)	0.469 (0.304, 0.725)	<0.001	0.460 (0.279, 0.758)	0.002
CD8$^+$ $T_{Naïve}$ % (≥19.7 = 1)	0.420 (0.270, 0.654)	<0.001		
CD8$^+$$T_{EMRA}$% (≥50.3 = 1)	1.902 (1.239, 2.920)	0.003	1.549 (0.978, 2.453)	0.062

CVD, cardiovascular disease; BMI, Body mass index; HD, hemodialysis; Log-iPTH, log transformed intact parathyroid hormone; Log-hsCRP, log transformed high-sensitivity C-reactive protein; Log-NT-proBNP, log transformed N-terminal pro-brain natriuretic peptide; $T_{Naïve}$, naïve T cell; T_{EMRA}, T effector memory CD45RA cells.

[1]Backward conditional method was used. Model included each T cell parameters and was adjusted for age, history of CVD, history of diabetes, types of vascular access, hemoglobin, albumin, prealbumin, creatinine, uric acid, globulin, ferritin, NT-proBNP, and hsCRP.

secondary responses upon re-exposure to antigens. T_{EM} cells migrate to peripheral tissues and display immediate effector function at the sites of inflammation. T_{EMRA} cells are usually considered to be at an advanced stage of differentiation and are promoted by homeostatic cytokines or low load but protracted antigen exposure (34, 35). T_{EMRA} cells share the same characteristics as senescent cells, such as possessing short telomeres, DNA damage foci, and a secretome of senescence-associated secretory phenotype (36). Consistent with the concept that senescent cells exert systemic detrimental effects, T_{EMRA} cells have been implicated in several chronic disease states, such as rheumatoid arthritis, acute coronary syndromes, as well as poor vaccine responses (37–39). In the current study, T_{EMRA} cells were correlated with proinflammatory cytokines, such as TNF-α and IL-6. It is hard to distinguish causality between inflammation and expanded T_{EMRA} cells. In the present study, a higher percentage of T_{EM} cells was associated with CVEs, and a higher percentage of CD8$^+$ T_{EMRA} cells was associated with infection. However, after including naïve T cells in the model, the association between these cells and clinical events diminished, indicating that an increase in differentiated T cells might partly be due to the decrease in naïve T cells; this is partly explained by some epigenetic studies (40, 41).

Overall, T-cell senescence in HD patients is markedly evident, and the contraction of the naïve T cell pool may act as a major player in developing CVEs and infections in these patients. Mechanistic studies on T cell homeostasis are needed in these patients. The central theme emerging from our finding is to alleviate chronic inflammation and promote cellular quiescence. Modifying HD therapy seems to be a feasible way to ameliorate T-cell inflammation and improve immunity against pathogens using antioxidant electrolyzed-reduced water (42) and introducing hemodiafiltration (43). To the best of our knowledge, only one study has investigated these T cell parameters in healthy individuals for each decade, with T cell subsets defined by co-expression of CD95 and CD62L, and reported that an increased absolute number of CD8$^+$ memory T cells (CD95$^+$CD62L$^-$) correlated with increased mortality (44). Few other studies have reported the relevance of T-cell senescence to morbidity in the aged population. One study conducted in 1,072 elderly individuals from a nursing home indicated that a decreased percentage of CD4$^+$ naïve T cells and CD8$^+$ T_{EM} cells was correlated with frailty (45). In a case-control study conducted in 122 women aged 65 and above, no significant correlation was observed in naïve nor memory T cells between cases and controls (46). However, these studies did not take absolute count of these T cell parameters into consideration, which could miss the vital date of T-cell senescence in aged individuals. Thus, studying T-cell senescence in patients with ESRD can help to shed light onto the alteration of immune function in the general aged population.

Our study had several limitations. First, it remains unclear whether inflammation is the cause or the consequence of T-cell senescence. Second, T-cell senescence can be assessed by several other markers, such as telomere length, recent thymic emigrants, CD57, and CD28. This study cannot exclude the impact of these

unmeasured parameters. Of particular interest is the fact that CMV infection has a substantial impact on T-cell senescence. In the current study, nearly all patients were seropositive for CMV-IgG, and half of them had an extremely high CMV-IgG titer, which could lead to underestimation of the relevance of CMV infection and T-cell senescence in HD patients. Finally, this was a single-center study, which might potentially limit the statistical power and its external validity. Hence, further studies are needed in this area to gain a deeper understanding.

In conclusion, HD patients exhibited accelerated immunosenescence in the T lymphocyte compartment, and these changes were positively related to inflammation. A reduction of naïve T cells was shown to be a strong predictor of CVEs and infection episodes in these patients. Monitoring naïve T cells could be useful for the early identification of patients at a high risk of profound complications.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee, Zhongshan Hospital, Fudan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FX analyzed the data and drafted the manuscript. BS and JZ made the diagnosis and designed the experiments. XD, XHC, and ZZ revised the manuscript. FX and XSC collected the data. All authors contributed to the article and approved the submitted version.

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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.2021.644627/full#supplementary-material

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