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HIGHLIGHTS

REVIEW

Aging of the immune system: Focus on inflammation and vaccination

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Major advances in preventing, delaying, or curing individual pathologies are responsible for an increasingly long life span in the developed parts of our planet, and indeed reaching eight to nine decades of life is nowadays extremely frequent. However, medical and sanitary advances have not prevented or delayed the underlying cause of the disparate pathologies occurring in the elderly: aging itself. The identification of the basis of the aging processes that drives the multiple pathologies and loss of function typical of older individuals is a major challenge in current aging research. Among the possible causes, an impairment of the immune system plays a major role, and indeed numerous studies have described immunological changes which occur with age. Far from the intention of being exhaustive, this review will focus on recent advances and views on the role that modifications of cell signalling and remodelling of the immune response play during human aging and longevity, paying particular attention to phenomena which are linked to the so called inflammaging process, such as dysregulation of innate immunity, altered T-cell or B-cell maturation and differentiation, as well as to the implications of immune aging for vaccination strategies in the elderly.

Keywords: Aging · B lymphocytes · Longevity · NK cells · Signaling · T lymphocytes · Vaccine

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Introduction

A long human life span—exceeding eight decades or more, for example—is becoming increasingly attainable in the developed countries. Globally, the number of persons aged >60 is expected to more than double by 2050, increasing from 901 million in 2015 to 2.1 billion in 2050, and that of persons aged >80 is projected to increase from 125 million in 2015 to 434 million in 2050 (United Nations: World Population Prospects; http://esa.un.org/unpd/wpp/). Unfortunately, an equally long health span—years of healthy life, devoid of disease and diminished vigor—lags seriously behind the gains in lifespan [1]. Why is the case? In large measure, the remarkable increase in lifespan enjoyed by modern humans is due to major advances in preventing, delaying or curing individual pathologies such as infections, hypertension, type 2 diabetes, and even some forms of cancer [2].

While modern biomedical interventions are successful at, for example, reducing cardiovascular disease due to hypertension or high blood cholesterol levels, basic aging processes continue to impair, for example, the host response to lung function, cognition, muscle strength, and bone integrity. A major challenge in current aging research, then, is to identify the basic aging processes driving the multiple pathologies and loss of function that afflict older individuals, with the long-term goal of developing effective interventions which ameliorate their effects.

What are these basic aging processes? For humans, this question remains formally unanswered. Nevertheless, a very large body of experimental evidence from a wide variety of organisms ranging from yeast to primates strongly suggests there are at least nine evolutionarily conserved hallmarks of aging that almost certainly derive from a small handful of basic aging processes [3]. These hallmarks of aging include stem cell exhaustion, altered intercellular communication, genomic instability and telomere attrition, epigenetic alterations, loss of protein homeostasis (proteostasis), altered nutrient and growth factor sensing, mitochondrial dysfunction, and cellular senescence [3]. There are still many open questions regarding the prime causes and ultimate effects of these hallmarks. However, emerging studies are beginning to identify commonalities among the causes and effects of at least some of these hallmarks. One of these commonalities has been linked to the immune system: low levels of chronic inflammation, otherwise known as inflammaging or inflamm-aging [4].

Inflammaging is a hallmark of virtually every major age-related disease and phenotype and has been shown to be a defining pathological characteristic of aging tissues across multiple species [5]. Inflammaging is characterized by the low level persistent infiltration of immune cells, primarily but not exclusively cells of the innate immune system, and elevated levels of several pro-inflammatory cytokines and chemokines [6], both within the tissue microenvironment and the systemic milieu. Because cells of both the adaptive and innate immune systems change with age, as discussed more extensively later in this review, the phenotypes of these infiltrating immune cells remain to be thoroughly characterized.

While a general consensus on a specific biomarker of inflammaging has never been reached, increased levels of circulating inflammatory mediators such as pro-inflammatory cytokines and acute phase proteins, e.g. interleukin-6 (IL-6) and C-reactive protein (CRP) are commonly used as indicators of inflammaging. In particular, analysis on thousands of elderly subjects show that IL-6 and CRP levels systematically increase in an age-dependent manner, even in subjects never diagnosed with diseases commonly associated with age, such as cardiovascular disease, myocardial infarction, stroke, type 2 diabetes, or cancer [7].

The origin(s) of the cytokines and chemokines that attract immune cells during inflammaging are incompletely understood. One possible hypothesis--based upon evidence observed in disease characterized by an accelerated aging of the immune system, such as HIV infection [8]—is that microbial products translocated from the gut might find their way into the circulation and ultimately into tissues more easily in elderly people, because of an age-related increases in gut and/or vascular permeability [9]. The microbial composition and diversity of the gut ecosystem changes with aging [10], as *Bifidobacteria* have been reported to decrease [11], while facultative anaerobes, including *Streptococci, Staphylococci, Enterococci, and Enterobacteria*, increase with age [12]; this alteration is associated with increased serum levels of IL-6 and IL-8 [13].

Another origin might be the age-related accumulation of senescent cells—cells that have entered a state of irreversibly arrested cell proliferation (growth) and altered function as a consequence of many of the stresses that are known to increase with age [14]. These stresses include genome and epigenomic damage, activation of oncogenes, metabolic imbalances and mitochondrial dysfunction, among others. The senescence growth arrest almost certainly evolved to suppress the development of cancer [15]. However, another hallmark of senescent cells is the acquisition of a senescence-associated secretory phenotype (SASP), which entails the chronic transcriptional induction and secretion of numerous pro-inflammatory cytokines, chemokines, growth factors and proteases [16]. As senescent cells chronically release chemokines, they may promote leukocyte recruitment, a well-known function of chemokines and, as shown in an in vivo model with senescent tumor cells, innate immune cells can migrate into the vicinity of the senescent tumor area [17, 18]. The pro-inflammatory nature of the SASP is generally considered deleterious [16, 19]. However, using a mouse model in which senescent cells can be detected in living animals, SASP was also recently shown to promote wound healing and optimize the formation of certain embryonic structures. In this model, senescent fibroblasts appear at wound sites a few days after skin injury, and these wound-associated senescent cells promote optimal wound healing by secreting PDGF-A, a SASP factor, which in turn promotes myofibroblast differentiation. [20]. Finally, SASP can optimize the formation of certain embryonic structures [20, 21]. Thus, although the SASP can promote tissue repair and remodelling, which also requires a controlled inflammatory response, but it can also become maladaptive and promote aging phenotypes and pathologies when chronically present.

The age-related accumulation of senescent cells was recently shown to shorten both lifespan and health span in mice [22]. Senescent cells secrete a distinct suite of inflammatory cytokines and chemokines depending upon whether senescence is induced by genotoxic stress, [23] or mitochondrial dysfunction, as in the form of deficiencies in mitochondrial sirtuins or damage to mitochondrial DNA [24], each of which are considered distinct hallmarks of aging [3]. Thus, activation of the immune system by senescent cells might account for more than one hallmark of aging. Likewise, activation of immune function by other hallmarks of aging – loss of proteostasis, for example–might also account for multiple aging phenotypes and pathologies.

Adipose tissue has been shown to play a major role in the regulation of inflammatory status. Adiponectin, which is associated with lean states and insulin sensitivity, has been hailed as an anti-inflammatory force in adipose tissue by regulating the production of anti-inflammatory cytokines, and polarizing macrophages toward anti-inflammatory M2 phenotype [25, 26]. Leptin, on the other hand, is produced in states of abundant adipose tissue and systemic inflammatory distress, and has been shown to induce the production of TNF-α, IL-6, and IL-12 in both human and murine monocytes [27, 28]. The normal aging process often leads to increased levels of visceral and subcutaneous adipose tissue. Higher numbers of resident macrophages and T cells from adipose tissue have been found in aged mice, and their number is correlated with higher inflammation. Concomitant with greater body fat percentage, aged mice also have more adipose tissue T cells (ATTs) than young mice, which can contribute to create a proinflammatory environment in visceral fat [29].

It has been suggested that this age-associated accumulation of adipose tissue is the cause of elevated inflammatory cytokines observed in obese individuals [30, 31], and indeed up to 30% of circulating IL-6 could derive from adipose tissue in human healthy subjects [32]. The contribution of adipose tissue to inflammaging is further supported by studies showing that elderly subjects who exercise regularly and are leaner have fewer senescent T cells and lower circulating pro-inflammatory cytokines [33], and that healthy centenarians with low adipose mass and high insulin sensitivity do not show elements of the proinflammatory profile [34].

Innate immunity: still a complex question

Likely because of the clear and easily detectable effects of thymic involution on naïve and memory peripheral blood T lymphocytes [35], studies on immunosenescence have been focused on adaptive immunity for decades. For a long time, innate immunity was considered basically unaffected by aging but, especially after the birth of the concept of inflammaging, several studies have demonstrated that crucial components of the innate immune system also undergo profound changes, which are related to an increased risk of infections and higher infection-related mortality. Moreover, the importance of molecules containing damage-associated molecular patterns (DAMPs), such as mitochondrial DNA, in activating innate immune cells and maintaining the status of inflammaging is emerging [36].

The number of neutrophils does not change with age, but profound functional alterations have been observed in this cell type [37, 38]. In particular, neutrophils from elderly subjects are characterized by a reduced capability to migrate towards a chemotactic signal, probably because of a constitutive activation of the lipid kinase phosphoinositide 3-kinase [PI3 K] [39]; the same phenomenon has been observed in mice, and has been attributed to reduced expression of ICAM-1 [38]. It must be noted that such reduced chemotactic capacity could also lead to a diminished egress of neutrophils from inflamed tissue, thereby contributing to higher local inflammation, as observed in aged mice after burn-associated lung injury [40]. Concerning phagocytosis and killing of ingested microorganisms, neutrophils from elderly subjects show a well-preserved capability to ingest non-opsonized particles [41], but a reduced capability to uptake opsonized particles, or pathogens such as *Escherichia coli* [37, 42]; in mice, neutrophils from aged animals also display a reduced capability to form neutrophil extracellular traps (NETs) in a model of severe skin infection by *Staphylococcus aureus* [43]. This reduction could be partially due to the lower expression of CD16 in neutrophils from elderly subjects [37]. Interestingly, centenarians, the best example of successful aging, show well-preserved neutrophil functions, such as bacterial phagocytosis, chemotaxis and superoxide production, which are comparable to those of young subjects [42].

A crucial mechanism for activation of innate immune response is the engagement of pattern recognition receptors by specific agonists. Peripheral blood mononuclear cells (PBMCs) from old individuals (≥ 65 years) have been shown to have a delayed and altered transcriptional response to stimulation with TLR4, TLR7/8, and RIG-I agonists; this altered response is accompanied by a decreased production of the pro-inflammatory and antiviral cytokines TNF-α, IL-6, IL-1β, IFN-α, and IFN-γ, and of the chemokines CCL2 and CCL7 [44].

Monocytes can be schematically divided in three main subsets on the basis of their phenotype: classical ($CD14^{++}CD16^{-}$), non-classical ($CD14^{+}CD16^{++}$) and intermediate ($CD14^{++}CD16^{+}$) [45]. Aging has not been shown to significantly alter the absolute number and the frequency of overall monocytes in humans [44], but does determines significant changes in the relative distribution of their subsets, with a marked reduction of the classical subset and an increase in the number of intermediate and non-classical monocytes [46]. As to functionality, significant age-related reduction of reactive oxygen species (ROS) production and phagocytosis capability have been described [46, 47], along with profound dysregulation in the release of different cytokines after the activation of monocytes through Toll-like receptors (TLR). The synthesis of TNF-α and IL-6 after TLR1/2 engagement, for example, is severely reduced in human monocytes, while release of TNF-α upon TLR4 stimulation is increased [48]. Furthermore, monocytes from aged donors have been shown to release higher levels of IL-8 after stimulation of TLR1/2, TLR2/6, TLR4, or TLR5 [49]. Such dysregulation appears to be caused by both alteration in surface TLR expression and impairment of downstream signaling: while TLR2

expression is constant, TLR1 expression declines with age, and activation of MAPK and ERK1/1 pathways after TLR1/2 triggering is severely reduced in cells from elderly subjects [49]. In contrast, signaling downstream of TLR5 has been shown to increase with age [49]. It has to be underlined, however, that most of these data concerning humans have been obtained in isolated monocytes treated in vitro, and some of the contrasting results observed could be due to enhanced responsiveness from cells with progressive differentiation in vitro [50]. Similarly, some in vivo data have been obtained on rodent models, and are often contrasting, probably because of different strains and experimental condition used. In humans, the functional consequences of similar, possible alterations are less known. However, it has been shown recently that there are no age-related differences in the capacity of the synthetic TLR4 agonist glucopyranosyl lipid A to induce expression of co-stimulatory molecules or production of cytokines by human antigen-presenting cells [51].

With regards to dendritic cells (DCs), age-related changes in the frequency and absolute number of plasmacytoid DCs (pDC) and myeloid DCs (mDC) were discordantly reported [44, 52–55]. Conversely, it is well established that Langerhans cells (LCs) markedly diminish with age [56, 57], and that such a reduction could contribute to the higher risk of skin infection in elderly subjects [58]. Concerning the capability to secrete cytokines upon stimulation, contrasting data exist for mDCs: while some studies have indicated an increased secretion of pro-inflammatory cytokines in elderly subjects, others showed no change or a decreased production [59, 60]. pDCs are characterized by a marked impairment of pro-inflammatory cytokine release with aging: pDCs display a reduction in intracellular levels of TNF-α, IL-6 and IL-12, as well as IFN-α, IFN-β, and IFN-γ upon viral or TLR stimulation [52, 53]; however, phagocytosis appears well preserved [41]. As the expression of TLRs in pDCs is constant over the life [61], it is likely that this impairment is caused by defects in signal transduction, as discussed below.

Data obtained in mice indicate that DC recruitment to lymphoid organs is also impaired with aging, probably because a combination of both direct alterations in DC capacity to respond to cytokine and chemokine stimulation, and indirect effects such as age-related defects of lymphoid organ microenvironment. In humans, a reduced mobilization of LCs from the skin to lymphoid organs upon TNF-alpha stimulation have been shown in aged subjects [56], while an age-associated increase in TLR4- and TLR8-dependent cytokine production has been observed in human MDDCs [59]. The lower capacity of pDCs from elderly subjects to release IFNs and pro-inflammatory cytokines has been associated with a reduced response to influenza vaccine [62]. However, basal production of pro-inflammatory cytokines in the absence of TLR engagement has been found to be higher in cells from older compared with young individuals, suggesting a dysregulation of cytokine production that may limit further activation by TLR engagement [44].

Finally, minimal attention has been paid to the effects of age on mast cells, basophils and eosinophils. Concerning mast cells, mouse models have given inconsistent results, and data show no age-related changes, or a reduced activity [63, 64]; degranulation was found increased in mast cells from aged mice, probably because of a lower expression of FcγRIIB/III, a negative regulator of their function [65]. No recent studies are available on basophils, while eosinophils have been studied in young and elderly asthmatic patients, and indicate no age-related changed in either number or functions of these cells [66].

The general picture that emerges is that of a profound dysregulation of innate immune functions, with some functions down-regulated, and others up-regulated or even enhanced. In particular, an increase in the basal production of proinflammatory cytokines, observed in different cell types, could be a major contributor to the age-related increase of the levels of such molecules observed in several cohorts of elderly subjects [19, 67, 68].

T lymphocytes in old humans: a matter of quantity and quality

T cells are generated in the thymus, a primary lymphoid organ that undergoes gradual decay with age. This process is referred to as thymic involution, and is characterized by the progressive deterioration and disappearance of functional thymic compartments (cortex and medulla) and accumulation of adipose tissue, so that only traces of functional thymic tissues are found at age 70 or more [69]. The exact causes of thymic involution are however not fully understood. Age-related changes in the levels of thymostimulatory growth hormones (e.g., decreasing levels of GH and IGF-1), or steroid hormones (increased during puberty), potentially thymosuppressive, and inflammatory cytokines (e.g., increasing levels of IL-6), as well as from oxidative stress-induced damages may play a role in this process [69, 70]. However, the early-in-life initiation of thymus decay in most vertebrates suggests a potential evolutionary role of this process, although this remains to be understood [71]. Aging is also associated with apparent quantitative changes of naïve and effector memory $CD4^+$ or $CD8^+$ αβ T-cell subsets. The reduction in the frequency of naïve T cells, together with the increasing proportion of terminally differentiated T lymphocytes and the decrease in cells expressing T-cell receptor rearrangement excision circles (the DNA molecules deriving from somatic recombination of TCR alpha chain, which are present only in T cells recently egressed from thymus) have long been hallmarks of immune aging [72]. These cellular changes are commonly reported in the circulation (i.e., the blood) [73], but can also be extended to lymphoid tissues and organs (e.g., spleen, lymph nodes, lung, and gut) of old subjects [74].

Studies of young subjects having undergone thymectomy during childhood have provided clear mechanistic insights into the decline of the naïve T-cell compartment in elderly humans [75, 76]. These studies showed that, independently from age, reduced thymic function and lower production of naïve T cells, together with the consumption of these cells (e.g., upon activation and differentiation during a viral infection), lead to reduced numbers of naïve T cells [75, 76]. This setting of partial

lymphopenia results in increasing naive T-cell homeostatic proliferation, although this is not sufficient to maintain a constant number of these cells [77, 78]. Reduced number of naïve T cells is associated with a less diverse TCR repertoire of the total T lymphocyte compartment overtime [79]. Nonetheless, despite their lower frequency, the repertoire richness of the naïve T cells declines only modestly in healthy elderly adults, i.e., —two- to fivefold if compared with that of young adults [80]. Of note, the elderly naïve T-cell repertoire was characterized by large T-cell clones (distinct from memory clones), suggesting that an uneven homeostatic proliferation occurs in the naïve T-cell compartment.

In addition to quantitative changes, naïve T cells display functional defects with advanced age. Altered TCR signaling and TCR-induced ERK phosphorylation, resulting in blunted activation, have been reported in $CD4^+$ and $CD8^+$ naïve T cells from old people [81, 82]. In naïve $CD4^+$ T cells, the desensitization of the TCR cascade was even associated with increased activity of dual-specific phosphatase 6 [DUSP6], a negative regulator of the ERK pathway [81]. Of note, the increased expression in T cells of another dual specificity phosphatase, DUSP4, was also found to cause defective TCR responses and aging markers reminiscent of T-cell senescence [83]. Both these quantitative and qualitative defects of naïve T cells result in a lower capacity of aged individuals to induce de novo antigen-specific T-cell responses. Using an in vitro model of T-cell priming, it was indeed shown that $CD8^+$ T cells from elderly individuals consistently mounted impaired responses specific to a model neoantigen, such as Melan-A/MART-1 [82]. Studies in old mice also revealed a decreased trafficking capacity and reduced motility of naïve $CD4^+$ T cells in lymph nodes prior to antigen encounter, thus indicative of delayed immune cell recruitment and antigen recognition [84]. Reduced T-cell priming capacity (i.e., diminished $CD4^+$ and $CD8^+$ T-cell responses specific for neoantigens) with advanced age was recently demonstrated in vivo, during a primary virus infection experimentally induced in old humans by immunization with live-attenuated yellow fever vaccine [85]. Overall, these deficits likely compromise priming of early adaptive immune responses in old individuals, thus contributing to their susceptibility to infection or cancer.

The increased proportion of terminally differentiated oligoclonal effector memory T-cell populations in the elderly has been shown to be the consequence of recurrent or chronic immune activation [86]. The latter usually results from the numerous and recurrent challenges that the immune system faces over time, in particular related to infection with persistent viruses, such as cytomegalovirus (CMV). Terminally differentiated ($CD28^-CD57^+$) T lymphocytes are characterized by strong effector functions such as expression of cytotoxins (e.g., Perforin and Granzyme B) and cytolytic capacity, and a marked inflammatory profile by way of increase proinflammatory cytokine (e.g., IFN-γ, TNF-α, MIP-1β) secretion [86]. As a consequence, the accumulation of these cells likely participates in the establishment of the hyperinflammatory status, characteristic of the old person. However, terminally differentiated T cells are constrained by limited proliferative capabilities in tandem with short telomeres, and to a large extent, have been defined as approaching senescence [86, 87]. Of note, the intensity of the immune response upon activation can be regulated through the action of various co-inhibitory receptors or immune check points (e.g., PD-1), which can, for instance, lower the degree of expansion and differentiation of activated cells. On the one hand, this type of regulation enables the immune system to adapt to the levels of antigenic stimulation, which could be beneficial in terms of limiting the immune alterations eventually associated with aging. On the other hand, it may also potentially limit the efficacy of the cellular immune response in the setting of infectious or malignant diseases. Moreover, a less efficient response to infections with increasing age may be related to a narrowing of the repertoire, ensuing recurrent antigen exposure, and resulting in either holes in the repertoire or even loss of effective T cells. Detailed analyses of the human TCR repertoires of influenza A virus-specific $CD8^+$ T cells, for instance, revealed a direct correlation between increasing age and narrowing of the TCR repertoire [88]. This may be associated with an age-associated increase in the proportion of low-avidity virus-specific $CD8^+$ T cells, as shown in the case of CMV infection for instance [89]. Recent studies have also provided new insights into the molecular defects of terminally differentiated "senescent" cells, reporting defective mitochondrial function, and elevated levels of ROS as well as p38 MAPK, associated with alterations of energetic metabolism as well as autophagy, a major cellular lysosomal degradation pathway [90, 91]. Of note, blocking the p38 signaling pathway has been shown to enhance the proliferation of such terminally differentiated T cells without compromising their capacity for cytokine secretion [92]. Moreover, the use of spermidine, which enhances autophagy, could help rejuvenate $CD8^+$ T-cell responses in old mice infected with influenza or murine CMV [93]. These studies therefore identify potential intervention targets for restoring responsiveness for these T cells and improving aged immunity.

Similarly to the changes observed on αβ T cells, the γδ T-cell compartment is also significantly affected by age. It is characterized by a decline in total γδ T-cell frequency along with phenotypic (accumulation of highly differentiated cells) and TCR repertoire changes (inflation of Vδ2-γδ T cells), phenomena which are accentuated by CMV infection [94, 95]. Functional analyses of γδ T cells from old individuals are nonetheless required to uncover potential qualitative defects with aging. Altogether, these findings provide further insights into the quantitative and qualitative cellular immune alterations and insufficiencies that accompany human aging.

Natural killer cells: changes in their subsets

Based on the expression of the surface markers CD56 and CD16, three NK-cell subsets have been characterized in humans. $CD56^{dim}CD16^+$ cells represent approximately 90% of circulating NK cells and are considered as mature mainly cytotoxic subset; $CD56^{bright}CD16^{neg/dim}$ cells constitute approximately 10% of the NK-cell population, and are considered immature with a

cytokine-mediated immune-modulatory role [96]. Furthermore, a scarce subset of NK cells, devoid of CD56 expression and displaying a reduced functional capacity, has been identified in healthy controls and in chronic viral infections such HIV and hepatitis C virus (HCV) [97].

Reports of changes in NK-cell phenotype and function with old age have been inconsistent. The proportion of $CD56^{bright}$ NK cells appears to be reduced in the elderly, which is likely the consequence of an impaired production of new NK cells with advanced age, due to a lower output from the bone marrow [98]. Instead, percentages and absolute numbers of $CD56^{dim}CD16^{+}$ NK cells have been described to be either maintained, increased, or decreased in the elderly population [99, 100]. NK cells from elderly subjects exhibit generally normal IFN-γ production capacity, but a defective capacity to secrete chemokines post-stimulation [101], accompanied by a reduced cytotoxic potential against MHC class I molecule negative target cell lines [99].

The impact of aging on the expression of NK-cell receptors has been recently reviewed [102] revealing that CD16 expression and function, which is a key receptor for antibody-dependent cellular cytotoxicity (ADCC), is not altered in the elderly, whereas the expression of the activating natural cytotoxicity receptors (NCRs), NKp30 and NKp46 and DNAM-1 are diminished [103]. There seem to be limited age-related changes in KIR and NKG2 repertoires of NK cells. A decrease in NKG2A expression occurs from young to elderly adults [104, 105]. In contrast, an increased frequency of KIR expression was observed in NK cells from cord blood to adults without any further increases in the elderly [98].

Similarly to T cells, CD57 can be a marker of replicative senescence for NK cells which have high expression of KIR, low expression of NKG2A, decreased sensitivity to cytokines, reduced replicative potential and high cytotoxicity properties [106]. Down-regulation of NKG2A, acquisition and high expression of KIR and expression of CD57 have been shown to correlate independently with terminal differentiation, as shown by reduction in proliferation capacity, homing molecules, response to cytokines, and expression of activation markers [107].

With a half-life estimated about 12 days in healthy young individuals [100], NK cells have been classically considered short-lived effector cells. However, the analysis of NK-cell homeostasis in old donors, showing a decreased de novo production of NK cells despite a relatively well preserved number of peripheral NK cells, suggests the persistence of a high proportion of long-lived NK cells in the elderly [108]. Little is known on the factors involved in the generation of long-lived NK cells in humans. Recent evidence has demonstrated that CMV infection could be a parameter associated with this expansion of long-lived 'memory-like' NK cells, which are characterized by the expression of CD94/NKG2C and CD57 [105, 109]. Moreover, this chronic viral infection leads to imprints in the human KIR repertoires [110, 111]. Lately, this unusual clonal expansion of $NKG2C^{+}CD57^{+}$ NK cells has been found in elderly CMV-seronegative donors, indicating that parameters related to aging other than CMV could influence the peripheral repertoire [105]. Mechanisms involved could include common factors between CMV infection and immune aging such as immune senescence, pro-inflammatory environment and increased homeostatic turnover.

In conclusion, aging is associated with a gradual loss of the $CD56^{bright}$ NK-cell subset, probably due to limited production of its precursors, and with the expansion of highly differentiated mature $CD57^{+}CD56^{dim}CD16^{+}$ and dysfunctional $CD56^{-}CD16^{+}$ NK cells. Even if the majority of elderly people exhibit a normal NK-cell compartment, a minority of individuals show a breakdown of NK-cell repertoire diversity which might influence immune surveillance. This is particularly relevant in the context of cancer development, which is negatively associated with the level of NK-cell-mediated cytotoxicity [112]. Both the failure to replenish the naïve NK-cell pool, due to either inefficient NK-cell differentiation or a highly skewed NK-cell repertoire caused by the selective expansion of virus-specific NK cells, might impair NK-cell function in the elderly.

Aging of B-cell function

The number of circulating B cells has been shown to significantly decrease with age, and changes in the relative frequencies of the different B-cell subsets have been reported. The evaluation of specific subsets is complicated not only by variations between individuals but also by the use of different phenotyping protocols. There is general consensus, however, that by using anti-CD19, -CD27 and -IgD antibodies, it is possible to identify four major circulating B-cell subsets: naïve [$IgD^{+}CD27^{-}$], IgM memory [$IgD^{+}CD27^{+}$], switched memory [$IgD^{-}CD27^{+}$], and late/exhausted memory [$IgD^{-}CD27^{-}$] {reviewed in [113]}. Using these markers, it has been shown that the percentage of switched memory B cells, the predictors of optimal antibody responses [114], decreases with age [115, 116]. Conversely, the percentage of late/exhausted memory B cells, the antigen-experienced and pro-inflammatory B-cell subset, increases with age [117, 118]. The term "exhausted" indicates terminally differentiated, senescent cells expressing the cell cycle regulator $p16^{INK4}$, which decelerates cell progression from G1 to S and induces cell cycle arrest. Late/exhausted memory B cells also have shorter telomeres [117, 118]. In addition, they secrete pro-inflammatory cytokines before stimulation and for this reason they are pre-activated and "refractory" to undergo in vitro class switch when stimulated with antigens and mitogens, as explained below. Moreover, it has been shown that bone marrow from old patients contains a low numebr of plasma cells [119].

Aging decreases antibody responses to exogenous antigens and vaccines, leading to greater susceptibility of elderly individuals to infectious diseases such as influenza. Functional alterations in T cells have been considered the most significant contributors to immunosenescence and sufficient per se to explain the age-related decrease in antibody responses of elderly individuals. However, some studies have managed to analyze defects in a variety of components of the innate and adaptive immune systems which occur with age. For example, in the case of influenza vaccination, the following defects have been characterized: decreased T-cell function and loss of CD28 expression, reduced specificity and class of antibody produced and decreased memory B cells, reduced

natural killer cell cytotoxicity on a per cell basis, and reduced number and/or function of circulating dendritic cells (reviewed in [120]).

Age-related intrinsic B-cell defects, responsible for sub-optimal antibody responses in elderly individuals to infections and vaccines have been identified [95, 121–123], and include decreases in expression and function of the key transcription factor E47 (see below), along with a reduction in activation-induced cytidine deaminase (AID), the enzyme of class switch recombination and somatic hypermutation. AID is a measure of optimal B-cell responses and its decreased expression in B cells from elderly individuals has been shown to lead to a reduced ability to generate higher affinity protective antibodies [124]. For example, the serum antibody response to both seasonal and pandemic influenza vaccines, as well as the in vitro B-cell response after vaccination, are both decreased with increasing age and are significantly correlated [114, 124, 125]. AID expression is also significantly correlated with antibody affinity maturation for the HA1 globular domain of the pandemic (p)H1N1 HA, as measured by antibody-antigen complex dissociation rates and Surface Plasmon Resonance [125].

AID is transcriptionally regulated by E47, a class I basic helix-loop-helix protein encoded by the E2A gene. E47 mRNA expression has also been shown to be decreased in B cells from elderly individuals [115]. The reduced E47 and AID mRNA expression levels in B cells from elderly individuals are due to reduced mRNA stability, which is in turn due to the higher expression of the inflammatory micro-RNAs (miRs) 16 and 155, which bind to the 3'-untranslated region of E47 and AID mRNA, respectively, inducing mRNA degradation [115]. Inflammaging not only induces higher expression of inflammatory miRs, but has also been shown to drive TNF-α expression in B cells from elderly individuals, and these levels are positively correlated with serum TNF-α and negatively correlated with the response of the same B cells after in vitro stimulation, which is measured by AID [121].

The higher levels of serum and B-cell-intrinsic TNF-α observed in elderly individuals have been associated with the age-related increase in CMV seropositivity. TNF-α activates the immediate-early promoter/enhancer of CMV, creating a "vicious cycle" in which the production of pro-inflammatory cytokines is increased. CMV may down-regulate B-cell responses either directly through TNF-α [118] or indirectly through the induction of terminally differentiated T cells and senescent T cells [126], and reduced generation of memory T cells [127].

Elderly individuals have a significant reduction in B-cell repertoire diversity and this correlates with their health status [128, 129]. Influenza and pneumococcal vaccine-induced expansion of B cells with short and hydrophilic IgH CDR3 regions is lower in older individuals, and the impaired anti-pneumococcal IgM and IgA responses correlates with the spectratypes for their IgM- and IgA-expressing B cells [130]. Moreover, elderly individuals have decreased numbers of B-cell lineages but increased pre-vaccination mutation load in their repertoire, resulting in a less efficient response, and the diversity of the lineages is thus greatly reduced as compared with that in young individuals [131].

Altered signaling modifies cell functions

In almost all immune cells, one of the most prominent changes occurring with aging regards signaling, that is integrating all the molecular events converging from the surface receptors to an adequate cellular response [132]. The modifications in the numbers of the most common immune receptors, i.e., TLR, Fcγ, and chemokine receptors, are controversial, even if, with some exceptions, the number itself is not changing [102]. Studies show that the signaling pathways are generally altered either in their proximal events (such as those including MyD88, PI3K, Lyn) or at the distal events (such as those including NFkB) [133]. JAK, Erk and PI3K represent the three most important pathways which are strictly interconnected. Their dysregulation can lead to altered chemotaxis, free radical production, killing in neutrophils and monocytes/macrophages, and reduced chemotaxis and antigen presentation in DCs [reviewed in [102]). For example, it has been recently demonstrated that the inhibition of PI3K, whose signaling in resting human neutrophils is constitutively increased in elderly subjects, significantly improves their functions by restoring neutrophil migratory accuracy [39]. The causes of such alterations are numerous, ranging from the hostile inflammatory milieu, leading to an increased basal level of cell activation, to intrinsic reasons, such as membrane alterations, or the disequilibrium between feed forward pathways and the inhibitory feedback loops [134].

In order to achieve an optimal T-cell response, the coordinated action of surface receptors and various signaling pathways, including the metabolic ones, is required. Several studies found age-related alterations in T-cell signaling pathways [135], including impairment of PTK phosphorylation, decreased $Ca2^{+}$ mobilization, and lowered PKC, PI3K and MAPK activation [136]. TCR density remains unchanged, but CD28 decreases by 20%-30% during aging, likely due to increased plasma levels of TNF-α. These alterations lead to decreased activity of the transcription factors NF-κB and NF-AT [137].

PTK Lck is obligatory for initiation of TCR signaling. Its activity is finely tuned by a multiple component module, comprising PTPase CD45 and PTK Csk bound to scaffold protein PAG (CBP). Lck activity cycles between primed, active and inactive states. Dysregulation of the Csk/PAG/CD45 loop in aged T cells favors the inactive form of Lck [138], providing a molecular clue to altered T-cell responses in aging. Negative feedback inhibitory events are also compromised during aging. For instance, SHP-1 activity has been shown to be higher in healthy elderly subjects than in young individuals, an observation consistent with the decreased T-cell response. Importantly, pharmacological inhibition of SHP-1 resulted in recovery of TCR/CD28-dependent lymphocyte proliferation and IL-2 production, suggesting the possibility of improving T-cell responses in the healthy elderly [138]. Furthermore, Li et al. [81] identified an age-associated defect in T-cell receptor (TCR)-induced ERK phosphorylation in naive $CD4^{+}$ T cells. The defective ERK signaling was caused by the dual specific phosphatase 6 (DUSP6), whose protein expression increased with age due to a decline in repression by miR-181a. Reconstitution of miR-181a lowered DUSP6 expression in naive

$CD4^+$ T cells in elderly individuals [81]. DUSP6 repression using miR-181a or specific siRNA and DUSP6 improved $CD4^+$ T-cell responses, such as increased expression of activation markers, improved proliferation and supported preferential T helper type 1 cell differentiation. Intrinsic alterations have been demonstrated at the level the T-cell membrane, as the cholesterol content in the membrane was found to be increased, interfering with the coalescence of the lipid rafts that are necessary for adequate signaling [139].

The redox state of the cell also strongly influences T-cell signaling. Activation of CD28 has been shown to result in decreased levels of reduced glutathione (GSH) and increased levels in cytosolic ROS [140]. In T cells from aged individuals, ROS remain high [141]. High ROS levels in T cells can inhibit TCR signaling through lowered expression of TCR/CD3, diminished phosphorylation of ZAP70 and altered $Ca2^+$ mobilization [136]. The persistence of low amounts of pro-inflammatory cytokines, concomitant with increased production of ROS, both of which are features of inflammaging, converge to diminish T-cell function in older persons, in the form of reduced IL-2 production and clonal expansion/proliferation [142].

Alterations in T-cell activation in the healthy elderly may also result from accumulation of memory T cells [143, 144]. Recently progresses have been made in linking of the development of the memory phenotype signaling, and the concomitant cellular metabolism orchestrated by the mTOR pathways [145]. It is now accepted that the memory phenotype is emerging because of persistent activation of the MAPK p38. The fundamental metabolic requirements of senescent primary human $CD8^+$ T cells were elucidated in a recent study, where it was shown that p38 MAPK blockade could reverse $CD8^+$ T-cell senescence via a mTOR-independent pathway, i.e., via the autophagy pathway [92]. Inhibition of mTOR has been shown to increase the general immune response to vaccination in the elderly [145], and might be relevant in designing new therapeutic strategies. Finally, T-cell metabolism also drives the differentiation of TH1 cells to various other subsets [146], but data are lacking on the behavior of these cells from aged individuals.

Immune aging and vaccinating the elderly

The fact that vaccines are the most effective measure to prevent infectious disease is widely accepted in the pediatric setting, and tremendous progress has been achieved in developing novel and improved vaccines for children over the last years. There is still a great need for vaccines tailored to optimally stimulate the aged immune system, as the elderly suffer more frequently from severe infections and experience poorer outcomes from these infections as compared to younger adults [147]. However, vaccine recommendations for the elderly vary from country to country and include vaccination against influenza, *Streptococcus pneumoniae* and *Herpes zoster* as well as booster vaccinations against tetanus/diphtheria, and in some cases pertussis and polio (Table 1).

Vaccine-induced immune responses are frequently lower in the elderly compared to younger adults. In most studies, antibody concentrations are measured to determine immunogenicity of vaccines, but lower antibody responses cannot be attributed solely to defects in B-cell function. Age-related changes in antigen uptake, processing and presentation, as well as functional defects of T cells, also lead to reduced antibody responses [148, 149]. In addition to cell-intrinsic defects, inflammaging can also contribute to impaired vaccine responses, as measured by antibody production.

The immunogenicity of subunit and split influenza vaccines is usually measured by the hemagglutination inhibition assay (HAI), and has been shown to generate lower results in the elderly compared with responses from younger adults. A meta-analysis of more than 30 studies demonstrated unadjusted odds ratios (OR) of 0.48 (95% CI: 0.41-0.55 for H1N1 Ag); 0.63 (0.55-0.73; H3N2 Ag) and 0.38 (0.33-0.44; B Ag) for seroconversion (HAI titer increase $\geq$ 4-fold) and 0.47 (0.40-0.55; H1N1 Ag), 0.53 (0.45-0.63; H2N3 Ag), and 0.58 (0.50-0.67; B Ag) for seroprotection (HAI $\geq$40) in a comparison of elderly versus young adults [150].

In elderly patients, frailty, a multifactorial syndrome characterized by reduced stress resistance and physiological reserve [151, 152], and associated with increased serum levels of IL-6 [153], has been shown to impact susceptibility to influenza and responsiveness to influenza vaccine [154]. Expression profiles predicting vaccination responses have been investigated prior to and in the early phase after influenza vaccination [155]. Pre-vaccination expression of genes associated with T-cell and B-cell function were positively correlated with influenza-specific antibody responses, while monocyte- and inflammation-related genes were negatively correlated with influenza-specific antibody responses, supporting the concept that inflammatory responses at baseline might be detrimental to vaccine-induced antibody responses [155]. It has been shown in mice that the inflammatory condition associated with obesity limits the antibody response to, and efficacy of, influenza vaccination [156]. Decreased influenza-specific antibody levels and B-cell function have also been described for obese humans [157]. It has been suggested that elevated baseline inflammation may aggravate or cause intrinsic defects in T cells and B cells, hampering their responses to antigenic stimulation (summarized in [158]). Cell-mediated immunity, namely the cytolytic activity of $CD8^+$ T cells after vaccination, is also lower in the elderly [159]. Various strategies have been pursued in order to improve vaccine-elicited antibody responses in the elderly, leading to the licensing of several novel vaccines against influenza. These include an intradermal vaccine [160], a high-dose vaccine [161], and a vaccine adjuvanted with the oil-in-water emulsion MF59, containing a synthetic muramyl peptide which has been shown to possess low toxicity and significant immunostimulatory activity in humans [162]. All of the above formulations show slightly higher immunogenicity in the elderly compared to the standard trivalent inactivated vaccine. The MF59-adjuvanted vaccine also induces more antibodies against heterologous viral strains compared to the standard influenza vaccine [163].

Table 1. Recommendations for vaccination of adults and older adults in selected countries for 2015

Country	USA	Germany	Austria	UK	Italy
Guideline	[a]	[b]	[c]	[d]	[e]
Influenza	Annually	Annually >60	Annually, particularly >50	Annually >65	Annually for all adults, particularly >65
S. pneumoniae[a)]	Once >50, PCV13, after 1 year PPV23	Once >60 PPV23	Once >50 PCV13, after 1 year PPV23	Once >65 PPV23	Once >65 PCV13, followed by PPV23
Herpes zoster	Once >60	–	Once >50	once >70	once >60
Diphtheria[b)]	Every 10 years	Every 10 years	Every 10 years, >60 every 5 years	–	every 10 years
Tetanus[b)]	Every 10 years	Every 10 years	Every 10 years, >60 every 5 years	–	every 10 years
Pertussis (acellular)[b)]	Once during adulthood	Once during adulthood	Every 10 years, >60 every 5 years	–	every 10 years
Polio (inactivated)[b)]	–	–	Every 10 years, >60 every 5 years	–	

[a)] for persons without prior vaccination with PCV13 or PPV23.
[b)] for persons with adequate primary vaccination earlier in life.
a. Recommended Adult Immunization Schedule United States. 2015 http://www.cdc.gov/vaccines/schedules/downloads/adult/adult-schedule.pdf. Accessed 15-1-2016
b. Empfehlungen der Ständigen Impfkommision (STIKO) am Robert-Koch-Institut. 2015 http://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2015/Ausgaben/34_15.pdf?__blob = publicationFile. Accessed 15-1-2016
c. Impfplan Österreich. 2015 http://bmg.gv.at/cms/home/attachments/8/9/4/CH1100/CMS1389365860013/impfplan.pdf. Accessed 15-1-2016
d. Complete Immunisation Schedule UK. 2015 https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/473570/9406_PHE_2015_Complete_Immunisation_Schedule_A4_21.pdf. Accessed 15-1-2016
e. Piano Nazionale Prevenzione Vaccinale. 2015 http://www.quotidianosanita.it/allegati/allegato1955037.pdf. Accessed 15-1-2016
General recommendations for age groups are shown, while additional recommendations for specific risk groups, e.g. persons with underlying diseases, are not included here and can be found in the cited documents.

Studies analyzing clinical efficacy or effectiveness against influenza are difficult to compare as their outcome depends heavily on the study population, on read-out parameters, and on epidemiological parameters (such as prevalence and virulence of the virus, the degree of mismatch between the vaccine strains and circulating virus strains, among others). Meta-analyses have estimated clinical efficacy and/or effectiveness of influenza vaccine and it can be concluded that protection is lower in the elderly than in young adults [164, 165]. Substantial research has been performed in order to develop a universal influenza vaccine which would be able to protect from all strains of influenza by inducing broad, long-lasting immune responses, and which could solve the issue of annual re-vaccination. Several viral proteins have been suggested as candidate antigens and a variety of delivery platforms, such as viral vectors, adjuvants or DNA vaccines have been tested [166].

A 23-valent polysaccharide vaccine against *Streptococcus pneumoniae* has been used for many years in the older population. Meta-analyses reported efficacy against invasive disease, but the efficacy against pneumonia is frequently in doubt, as results from clinical studies are inconclusive [167]. Recently, a 13-valent conjugate vaccine has been licensed for adults and its clinical efficacy was 45.6% (95.2% CI: 21.8–62.5; $p < 0.001$) for confirmed vaccine-type community-acquired pneumonia and 75.0% (95% CI: 41.4–90.8; $p < 0.001$) for vaccine-type invasive disease in a large randomized placebo-controlled study enrolling more than 84 000 elderly persons [168]. Data from this study were used to analyze the effect of age on vaccine efficacy using a statistical model, and a decrease of vaccine efficacy for vaccine-type community acquired pneumonia and invasive disease from 65% (95% CI: 38–81) in 65-year-old subjects, to 40% (95% CI: 17–56) in 75-year-old subjects was determined [169].

The incidence of herpes zoster, caused by reactivation of the varicella zoster virus, increases with age. A live-attenuated vaccine is available, which has been shown to reduce the incidence of herpes zoster by 51.3% (95% CI: 44.2–57.6) and of post-herpetic neuralgia, a severe complication occurring frequently in the elderly, by 66.5% (95% CI: 44.5–79.2) in the vaccinated population compared to placebo [170]. The protective effect against post-herpetic neuralgia was independent of age, whereas clinical efficacy against herpes zoster declined to only 27.6% in persons older than 69 years. The current live-attenuated vaccine cannot

be used to vaccinate immunocompromised persons, who are at great risk of herpes zoster also at a younger age. A novel, inactivated vaccine containing the viral glycoprotein E, adjuvanted with the liposome-based $AS01_B$ system (MPL and QS21), has recently been developed [171]. In a phase III randomized placebo-controlled trial clinical efficacy against herpes zoster was 97.2% (95% CI: 93.7–99.0; $p < 0.001$) for persons over the age of 50 and did not decrease for older age groups (>70 years) [171]. The $AS01_B$ adjuvant system efficiently induces IgE-specific $CD4^+$ T cells [172] and cell-mediated immunity; antibody responses are also higher compared with those induced by the live-attenuated vaccine [173]. Experiments in mice showed that adjuvants containing MPL and QS21 rapidly induce chemokines and cytokines at the intramuscular injection site, attracting monocytes and granulocytes [174]. Increased numbers of neutrophils, monocytes, and DCs were also observed in the draining lymph node and it has been hypothesized that the adjuvant-mediated recruitment and activation of APCs, particularly of $MHCII^{high}$ DCs, is responsible for the efficient stimulation of adaptive immune responses. However, these results have been obtained in young mice, and it is still not known if this mechanism is preserved in aged animals [174].

Regular booster vaccination against tetanus and diphtheria throughout life is recommended in many countries. However, the levels of tetanus- and to an even greater extent diphtheria-specific antibodies in the elderly are frequently below that considered to be protective [175]. Single booster shots late in life were shown not to elicit long-lasting antibody responses against diphtheria in a substantial proportion of the elderly [176]. Appropriate vaccination documentation is crucial to timely deliver booster vaccinations, but is often poorly documented in the elderly. Epidemiological data show that pertussis is relevant for older age groups and does not solely affect infants [177]. Some countries recommend regular booster vaccination against pertussis in combination with the tetanus/diphtheria vaccine or at least one booster shot during adulthood. Immunogenicity of the vaccine is lower in the elderly compared with that in younger adults [177].

Substantial effort is put into the development of vaccines against several pathogens that are of relevance for the elderly. Particularly, persons with underlying diseases and frail elderly have a risk of severe disease caused by respiratory syncytial virus (RSV). Estimations in the United Kingdom reported up to 18 000 hospitalizations and 8400 deaths caused by RSV per season with 79% of hospitalizations and 93% of deaths in persons older than 65 years [178]. Several vaccine candidates against RSV are currently in early clinical development [179], and these vaccine candidates should also be tested in adults and the elderly. Vaccines against nosocomial pathogens such as *Staphylococcus aureus*, *Clostridium difficile*, *Escherichia coli*, *Klebsiella pneumoniae,* and *Candida ssp*. could provide great benefit for the elderly, as this age group has a high risk of hospitalization and nosocomial infections. Clinical development is ongoing for several vaccine candidates [180], and successful vaccination against nosocomial pathogens has the potential to save many lives and to substantially reduce healthcare costs.

Conclusion

The increase in human lifespan poses several new questions and complex challenges to the medical and scientific community, including for immunologists. Today, the immune system has to defend the organism for several decades, and thus has to work effectively for a substantial number of years; this is a reality that was not considered when Jenner developed the smallpox vaccine. Moreover, every day immune cells have to cope with external insults (such as oxygen, UV light, chronic infection), personal and social behaviors (nutrition, obesity, psychological stress, lack of exercise, hyper-training, pollution, smoking, economic status) and unavoidable internal changes (cell metabolism, turnover and production of DAMPs). Our community is well aware of this challenge, and indeed an unprecedented attention is now paid to aging and longevity, that includes the search for new strategies for an optimal maintenance of immunological performances in the long, last part of our life.

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