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## Introduction

The life expectancy in industrialized countries has consistently increased every decade for the last 150 years. From the early 1900s to 2006, the United States alone experienced an increase in life expectancy from 49.2 to 77.7 years [1]. Although most of these increases have occurred in industrialized countries, the world as a whole has also seen improvements. It is projected that, by the year 2025, there will be more than one billion people over the age of 60 worldwide (see World Health Organization website). Increases in life expectancy reflect advances in both public health policy and improved treatments for infectious diseases. However, the increases in average human lifespan also present growing challenges to a nation's economic, medical, social fabric, and public health programs. Older age is associated with a significantly higher incidence of various chronic conditions, such as cardiovascular disease, cancer, diabetes, neuropathologies, and

immune system dysfunction. Persons with these conditions constitute a large burden on the health care system, and in many cases, the lack of adequate treatments have negative effects on quality of life and independent living. Thus, improved understanding of the fundamental molecular basis of aging is critical in order to adequately address the health of an increasingly large proportion of the population.

Our understanding of the aging process has greatly increased in the last two decades, but many more questions on the molecular and cellular mechanisms of aging and on the optimal biomarker for “biological age” remains to be addressed. Mechanistically, some of the more thoroughly tested aging theories relate to studies on telomeres, reactive oxygen species, DNA mutation accumulation, and specific aging regulatory proteins such as p53 and sirtuins [2–5]. All these proposed aging mechanisms have links to metabolism regulation, genomic integrity, and physiological stress, and each has a different degree of impact, depending on the cell type and organ system being studied. Telomeres, terminal chromosomal regions that help maintain DNA integrity, provide an example of cell and organ-specific aging changes. When cells undergo division, the telomeres shorten, due to incomplete DNA replication. Once a critically short telomere length is reached, regulatory proteins, such as p53, become activated and this activation blocks the cell cycle and further cell division, a stage known as replicative (or cellular) senescence [6].

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Telomere-based aging mechanisms would appear to primarily affect rapidly proliferating cells, since, as the hypothesis suggests, telomere shortening occurs with cell division. Increased proportions of lymphocytes and epithelial cells with short telomeres and additional features of replicative senescence are present in older humans [7–9]. However, telomere shortening of non-dividing cells has also been observed, suggesting that additional mechanisms, such as oxidative stress or DNA damaging agents, can cause a cell to reach replicative senescence *in vivo* [10, 11]. The abundance of proposed molecular pathways demonstrates our growing understanding of the underlying mechanisms of aging; however, the numerous putative pathways also point to challenges in the field of gerontology. There are multiple factors that can affect the aging of an individual cell, underscoring the complexity of gaining a comprehensive understanding of how these pathways contribute to aging on the organism level.

Another issue in studying human aging is the need to establish metrics for evaluating the rate of aging and the biological age of a particular individual. The various factors that affect individual aging undoubtedly contribute to the different rates of aging among humans. An extreme example is provided by studies of monozygotic twins, who, though genetically identical at birth, do not die on the same day [12–15]. In fact, by age 80, the lifespans of identical twins are no more closely correlated with each other than with the lifespans of unrelated persons within their community. These observations buttress the multiple studies demonstrating that extrinsic parameters, such as the environment and stochastic factors, can also account for intra-species age differences in aging rates. Because of this, aging parameters in elderly cohorts show much greater variability than measurements done in young adults. Regardless of the heterogeneity in the aging rate, all humans are subject to the aging process, whether it is driven primarily by intrinsic (genotype) or extrinsic (environment) factors.

To account for aging rate differences, current research is focused on identifying biomarkers that define the biological – in contrast to the

chronological – age of an individual. The dilemma arises in documenting suitable biomarkers that reflect aging, rather than disease. According to a National Institute of Health group, a biomarker is defined as a characteristic that objectively measures a normal biological process, pathogenic process, or a pharmacological response to a therapeutic intervention [16, 17]. Using this definition, biomarkers have been utilized for a number of different clinical applications, such as depicting stages of cancer, or validating drug efficacy [16, 18]. In the context of aging, biomarkers can be utilized as indicators of good health and predictors of longevity. Because of the ease of performing longitudinal studies, lymphocytes have been the main source of biomarker research in humans. Further, lymphocytes, due to their continuous circulation, may reflect fundamental aging changes occurring at the organism level. Finally, many of the pathologies associated with aging, such as osteoporosis, atherosclerosis, and cancer, have immune components [19].

When referring to living systems, homeostasis is defined as the ability of the organism to maintain internal equilibrium by adjusting physiological processes. Conceptually, the lack of homeostasis can explain both the etiology of diseases and also many facets of the aging process. The inability of organisms to repair and regenerate their cells eventually results in a decline in the functional capacity. These functional declines accumulate within various organ systems, causing further progressive dysfunction, leading to certain age-related diseases. The immune system provides an excellent example of this phenomenon. The age-related failure of the immune system to maintain homeostasis, for example, by the decreased capacity to produce functional lymphocytes and the accumulation of senescent memory cells, leads to many age-related pathologies. This conceptual model helps us understand how the various signals/factors that affect lymphocyte homeostasis can also impact the aging process of an individual lymphocyte.

Homeostatic immune tissue imbalances may be caused at multiple levels and by numerous mechanistic pathways. We can classify the factors that influence homeostasis as intrinsic

(genetic), extrinsic (epigenetic), and stochastic (random biological and nonbiological factors) [12]. How these factors, in turn, affect our immune response as we age is modulated by the timing within the lifespan at which they first act on the organism. Adaptive immunity plays a key role in activating both the learned and innate immune response to pathogens and cancer. Malfunctions in the adaptive immune system during aging increase and, as a direct consequence, so do infection rate, cancer incidence, allergy, and autoimmunity. To understand how these aging malfunctions (age-related pathologies) occur, one must look no further than our lymphocytes as the primary culprits, particularly our T and B cells, with natural killer (NK) cells playing a lesser role. The interaction of lymphocytes with their environment, either in primary or peripheral lymphoid tissues, can explain many observations of the immune aging process. In this regard, changes in development of hematopoietic stem cells, primary lymphoid tissue in the bone marrow (BM) and thymus, and finally phenotypic changes in the lymphoid cell itself can all cause homeostatic imbalances to the immune response that ultimately contribute to age-related pathologies. This chapter addresses these and other topics to explain age-related changes in human lymphocytes, and how these relate to the emergence of age-related pathologies and chronic diseases.

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### **Age Changes in the Developmental Environment and in Hematopoietic Stem Cells**

Our bodies are estimated to be composed of 50–100 trillion cells [20]. The sheer number of cells is evidence of the remarkable ability of our cells to proliferate and maintain tissue integrity. The capacity for cell renewal that maintains these tissues declines with age, and is the basis for many age-related pathologies. In no other organ is the decline in cellular expansion and resultant age-related disease more apparent than in the immune system. Lymphocytes, strategic cells involved with implementing both innate and adaptive

immune responses, undergo multiple types of age-related changes. Many of the quantitative and qualitative aging lymphocyte alterations can be traced to the age-related remodeling of the BM tissue, which is the site of hematopoiesis; these alterations can subsequently impact various stages of lymphocyte differentiation. Within BM tissue resides a population of hematopoietic stem cells (HSC), the precursors that give rise to all lymphoid and myeloid cell lineage. Developmentally, the BM is the primary lymphoid tissue that houses the HSC, where lymphoid cells develop into B cells and which also supplies the thymus with the T cell precursors that will differentiate into the T cell lineage. Therefore, changes in the stromal microenvironment of the BM and thymus, as well as within the HSC themselves, not only correlate, but are at least partially responsible for a decline in number and function of naïve lymphocytes with age [21].

Structurally, the composition of the BM stroma is dramatically altered during aging, due to its replacement with increasing amounts of adipose tissue. The underlying mechanism for this structural change is not completely understood, but the adipose tissue increase is likely related to the reduced production of growth hormones by the anterior pituitary gland. Growth hormone administration to rodents was shown to reverse adipose tissue growth and promote lymphoid development [21, 22], but questions regarding the target cells and signaling pathways involved in the effect have not yet been addressed. Part of the issue relates to the fact that the term “stromal cells” is actually a generic term used to describe a heterogeneous population of large cells in the BM that can include reticular cells, endothelial cells and macrophages. Thus, the mechanism of how all or some of these cells are replaced by adipose tissue and how this process becomes ablated by growth hormones requires further investigation [22].

A second important age-related change within the BM relates to the decreased capacity of HSC to replicate and generate lymphoid progeny. The two important features of HSC that link these cells to the aging process are self-renewal and differentiation. Differentiation refers to the ability to

generate both common lymphoid progenitor (CLP) and common myeloid progenitor (CMP) cells [23]. As both the BM tissue and the HSC cells undergo age-related changes, the balance of lymphoid and myeloid progeny, which is equivalent during youth, shifts to greater proportions of myeloid lineage cells in old age [24].

To conceptualize the aging process in the BM and/or HSC, it is useful to categorize the changes as either intrinsic or extrinsic in nature. Intrinsic to aging is the genotype of the BM and HSC, which can determine hematopoietic turnover [25, 26]. For example, screens for genetic polymorphisms in rodents have linked regions within chromosome 17 to higher levels of HSC self-renewal, and chromosome 1 to higher levels of hematopoietic progenitor cells [25]. These observations indicate that different genetic regions, and therefore different genetic controls, affect the various downstream steps in T and B lymphocyte development. Future studies will require identification of specific genes within those chromosomal regions, and defining their role in HSC function. It is also unclear whether genetic regions are involved in DNA repair, metabolism, or stress responses, and whether defects in these pathways are involved in HSC self-renewal. Alternatively, these chromosomal regions might primarily affect the interaction of HSC with the BM stroma or may even exert direct effects on HSC. Other experiments support the significance of intrinsic genetic factors within HSC themselves, as competition studies comparing old versus young donor HSC repopulation in a common recipient demonstrates the superior ability of young HSC to outgrow and repopulate the bone marrow [27, 28]. Furthermore, there are numerous experiments that also established the importance of the extrinsic phenotype of the stroma on HSC [29–32].

The internal milieu of the BM is unique, since it must accommodate distinct microenvironments needed for HSC as well as for the different blood cell lineages. It has been proposed that HSC have specific stromal cell requirements, although phenotypic differences in stromal cells have not been clearly identified in the BM [22]. This presents challenges in the analysis of B lymphocyte precursors. Indeed, although the multiple stages of

B cell differentiation have been well-characterized, the developmental microenvironment niche still remains to be defined. Some groups believe the niche to be a localized region near the endosteum, but others believe it to be scattered throughout the BM. Until the niche is correctly identified, it will not be possible to accurately measure age-related stromal changes and to develop methods to reconstitute the appropriate microenvironment [22, 32–34].

Studies in mice that are connected through parabiosis have been used as a model system to differentiate between extrinsic and intrinsic changes on HSC function due to age. This experimental system has provided evidence for how the local microenvironment affects HSC self-renewal and, therefore, possible lineage commitment [30]. In these experiments, rodents of different age combinations (e.g., young/young, old/young) enabled young niche effects on old HSC descendants to be measured. It was observed that when an old mouse was physically connected to a young mouse, both HSC levels and lymphoid to myeloid ratios were restored. Ultimately, be it by intrinsic or extrinsic factors on the BM, aging has a net effect of decreasing HSC replication and development [35, 36]. As a consequence of this decrease in HSC, the CLP numbers and functions are also reduced [36].

The thymus is the second organ where lymphocytes undergo development and maturation, and which, like the BM, undergoes age-related changes. Precursor T cells migrate from the BM to the thymus, where the T cell receptor (TCR) undergoes *V(D)J* rearrangement by Rag1/Rag2 proteins as the cells travel through different thymic compartments. In the cortex of the thymus, the so-called double-positive (i.e., CD4+CD8+) thymocytes undergo positive selection, and the newly generated TCR is tested, based on its ability to bind a composite of self-peptide/self-major histocompatibility complex (MHC) presented by cortical epithelial cells. The surviving thymocytes then migrate to the central medullary region, where they undergo negative selection, based on their TCR ability to recognize self-peptides expressed by dendritic cells and medullary thymic epithelial cells [37, 38].

The aging changes to the thymic microenvironment further affect the aging of T lymphocytes as a result of the continued differentiation of the thymus tissue as we age.

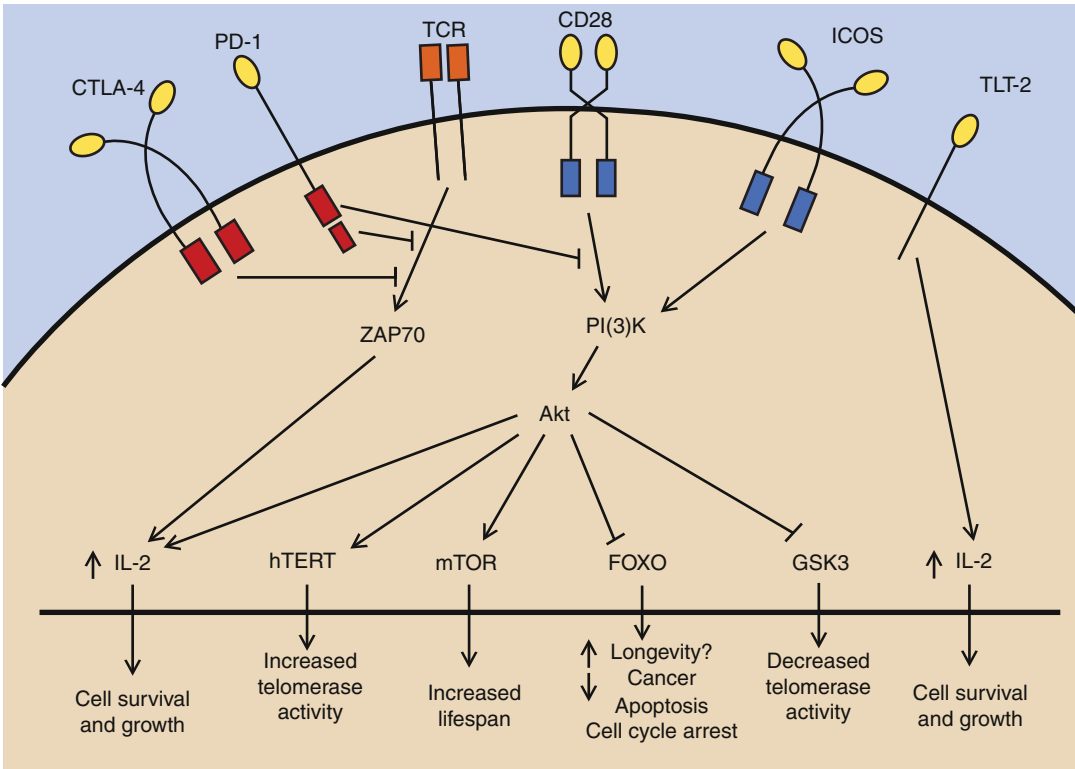
Involution is the most obvious aging change that occurs in this primary lymphoid tissue. Although the process of thymic involution begins soon after birth, the effects of structural changes within the thymus become most evident during old age [39]. Thymic atrophy, which involves both a reduction in thymic mass as well as the increased abundance of adipose tissue, correlates with a decrease of naïve T cells in the periphery. Mechanistically, it is known that several different hormones [sex steroids, keratinocyte growth factor (KGF), and growth hormone (GH)] affect thymic architecture and peripheral T cell numbers. Experiments using sex steroid ablation of androgens by either physical or chemical castration documented a deceleration of thymic involution and an increase of CLP production [40, 41]. GH receptors expressed by many blood cells of hematopoietic origin can act directly or indirectly on thymocytes or peripheral T cells to promote lymphopoiesis [42, 43]. GH also has indirect effects, mediated by insulin growth factor-1 (IGF-1) and interleukin (IL) IL-7. IGF-1 can enhance CLP and CMP reconstitution by modulating homing receptors and/or inducing IL-7 [44]. IL-7, in turn, supports thymocyte development and peripheral T cell survival and proliferation. It has been reported that during aging, thymic involution causes a decrease in naïve cells in the periphery due to a decrease in stromal-derived IL-7 [45]. Further research showed that sustained production of IL-7 in the thymic stroma (via gene transduction) leads to a maintenance of CD25 expression (the  $\alpha$  chain of the IL-2 receptor) in thymocytes, although thymic involution was nevertheless not prevented [45]. The role of IL-7 in thymopoiesis has also been tested in clinical studies, in which administration of recombinant IL-7 resulted in expansions of CD4 and CD8 T cells in patients being treated for cancer [46], an observation that may provide a therapeutic approach for reconstituting thymic function during aging.

## Age-Associated Quantitative and Qualitative Lymphocyte Changes

The major immune system changes that occur during aging are within the T cell compartment. T cells are the cells primarily responsible for activating and maximizing immune responses. As such, these cells are a major focus of immunosenescence research. The importance of B cells, and their role in generating high affinity protective antibodies, is also an area of active research, since vaccinations remain among the best methods for prevention of infectious diseases and potentially cancer. Nevertheless, although there are specific B cell–intrinsic effects of aging, it is assumed that many of the antibody defects observed in the elderly are secondary to T cell alterations.

Following maturation in the thymus, T cells migrate to the periphery, where some naïve T cells circulate in the blood stream, while others occupy specific tissues awaiting antigen encounter. During the period prior to antigen encounter, naïve lymphocytes are stimulated by self-antigens to proliferate for the purpose of self-renewal and survival, homeostatic functions that maintain cell numbers [47]. This apparently innocuous activity is, nevertheless, important in the elderly, as discussed below. During an immune response to a pathogen, antigen presenting cells (APC) express on their surface antigen-derived peptides that are attached to MHC molecules and other receptors. Antigen-specific T cells that recognize this complex, bind to the APC and become activated, using a dual signal system. The TCR provides the primary signal for T cell activation, and equally important it also determines antigen specificity. The second signal, referred to as the costimulatory signal, is a modulatory signal that can be initiated by a member of a large receptor family (Fig. 2.1), each of which elicits a distinct outcome (e.g., activation, differentiation, anergy).

The principal costimulatory protein used for T cell activation is the CD28 receptor. Once both the TCR and CD28 have been triggered, T cells will become activated and will secrete cytokines and carry out cell-specific effector functions.



**Fig. 2.1** CD28/CTLA-4 receptor family induces pathways that are crucial in regulating T-cell activation, tolerance, and immunosenescence. This family of receptors may have overlapping cooperative or antagonistic functions that can be downregulated (i.e., CD28) or upregu-

lated (i.e., CTLA-4) as T cells age, particularly within the CD8+ T cell subset. As illustrated here, the coreceptors are critical in regulating PI(3)K/Akt signaling pathways that in turn affect many age-related mechanisms. Ligands to the CD28 family can be reviewed elsewhere [48]

However, other co-receptors, such as ICOS and TLT-2 and a multitude of cytokines (i.e., (IL)-2, IL-4, Interferon (IFN)- $\gamma$  [gamma], IL-7 and IL-15), are required for antigen-specific naïve T cells to further differentiate and proliferate into their corresponding effector cells, with a small proportion of these cells becoming memory cells. The main effector subsets of activated T cells are CD8+ T cells, which become cytotoxic to target cells and are characterized by their upregulation of FasL and secretion of granzymes and perforins into target cells, and CD4+ T cells, which differentiate into Th1/Th2 and Th17 helper T cells, which activate and maximize both the adaptive and innate branches of the immune system. In addition, CD4+ T cells can also differentiate into regulatory T cells ( $T_{reg}$ ), which downregulate the immune response

and prevent autoimmunity. It should be noted that these differentiated effector lineages may not be terminal; indeed, CD4+ T cells may retain a degree of plasticity. For example, there is evidence that, under the correct cytokine (IL-6) signal,  $T_{reg}$  cells can be induced to become Th17 cells, a newly identified class of T cells that is involved in clearing pathogens during host defense reactions and in inducing tissue inflammation in autoimmune disease [49]. In summary, T cells have three common features that are necessary for adequate immune protection: (1) antigen recognition and activation, (2) T cell clonal expansion (proliferation), (3) differentiation of naïve T cells into memory T cells (central memory vs. effector memory) and effector cells. Age-related changes in these three features have been investigated as possible



underlying causes of immune deterioration, as discussed below.

The first T cell feature that undergoes age-related change is antigen recognition/activation. As noted above, the TCR recognizes antigens presented by APC and this recognition causes T cells to rearrange their membrane proteins into the so-called immunological synapse, which enhances receptor cross-linking and activation of the T cell. It has been observed that the TCR undergoes age-dependent defects that lead to defective cytoskeleton modification required for an immune synapse [50]. These changes lead to inadequate T cell activation during aging, which, in turn, causes decreases in both IL-2 and effector cells of the Th1 and Th2 lineages [51]. Moreover, the TCR is a complex of proteins, and, with increasing age, at least one of these proteins (CD3- $\gamma$  [gamma]) appears to increase in expression [52].

Another age-related change that affects T cell activation is the decreased TCR repertoire, i.e., the degree of diversity within the different antigen-recognition units. The greater the TCR diversity, the more capable is the immune system to protect against a wide variety of pathogens. Most of the documented decreases in the TCR repertoire occur within the CD8+ T cell subset [53, 54]. Multiple theories to explain these phenomena have been proposed, including continuous stimulation and clonal expansion of T cells induced by latent viral infections, such as cytomegalovirus (CMV) and Epstein-Barr Virus (EBV), as well as by an increase in the number of peripheral cells by self-renewal homeostatic mechanisms. Irrespective of the cause, the result of the constricted TCR repertoire is a reduction in the range of pathogens to which the immune system can respond. This phenomenon may explain the increased incidence of infection in older individuals, as discussed below [55].

Among the most dramatic age-associated phenotypic changes related to T cell activation is the significant increase in the proportion of CD8+ T cells lacking expression of the CD28 costimulatory receptor. This signature change may exert far-reaching effects, due to the critical role of CD28 in modulating activation and immune

responses. Indeed, it has been clearly demonstrated that activation of T cells in the absence of a CD28 signal can lead to anergy or apoptosis [56]. However, during aging, the absence of CD28 signaling is not due to defective stimulation, but rather to the complete suppression of CD28 gene expression. This loss of CD28 gene and protein expression is associated with multiple changes suggestive of replicative senescence, including critically short telomeres [57]. Studies in humans, comparing T cells in young adults (20–39 year olds) versus seniors (60–90 year olds) demonstrate a reduction of CD28 expression that occurs mostly within the CD8+ T cell subset, and is a rare event in CD4+ T cells [58].

Because CD28 is the primary costimulatory protein for T cell activation, since reduction in its expression correlates with age, efforts to understand the underlying mechanism have been extensive and informative. The molecular pathways modulating CD28 expression have been elucidated at the transcription level, where two motifs  $\alpha$  [Alpha] and  $\beta$  [Beta], located within the minimum CD28 promoter, are required for CD28 expression and where the  $\beta$  [Beta] motif is primarily downregulated. Interestingly, the  $\beta$  [Beta] motif for CD4+ and CD8+ T cells has different binding profiles, which may help explain the age-associated CD28 expression differences between CD8+ and CD4+ T cells [59]. Other efforts to understand the role of CD28 in aging have focused on reducing exposure of T cells to TNF $\alpha$  [Alpha], a cytokine secreted by chronically stimulated T cells, which has been shown to suppress CD28 expression [60], and genetically-induced sustained expression of CD28 in CD8+ T cells [61]. This genetic manipulation resulted in an average  $2 \times 10^{20}$  fold increase in proliferative potential compared to the control cells, and a delay in the appearance of multiple senescence-specific changes, such as loss of telomerase activity, increased production of pro-inflammatory cytokines, and upregulation of CTLA-4 expression.

The above results also highlight the intricacies and interactions between various costimulatory proteins that may be critical in the cellular aging of T lymphocytes. For example, ICOS and TLT-2 (the latter found only in murine cells) are other

mammalian receptors in T cells that stimulate proliferation, cytokine production, and, in the case of ICOS, survival. ICOS and TLT, therefore, have functions that overlap with CD28 and may, under certain conditions, substitute in for CD28 functions, a scenario likely to take place during T cell activation, since CD28 expression is transiently up- and downregulated after activation [59]. Alternatively, T cells also express other CD28 family receptors that are inhibitory to TCR-dependent activation and cause decreases in proliferation, cytokines, and cell survival, in addition to promoting inhibitory functions of T<sub>reg</sub> cells. Key CD28 antagonist surface proteins are CTLA-4, BTLA, and PD-1, but other CD28 non-family member proteins have been discovered and investigated (CD85j and CD158b/j) [58, 62]. The primary mechanism for CD28 modulation involves blocking and competing with CD28, and antagonizing the signaling pathways of activation (Fig. 2.1).

Since the loss of CD28 is closely associated with an age-based terminal differentiation of T cells (replicative senescence), many studies have compared gene expression profiles for CD28+ and CD28- cells within both the CD4+ and CD8+ subsets. These studies have shown several changes in gene expression in T cells lacking CD28 with decreased expression of CD27, adenosine deaminase (ADA), CCR2, CCR6, and CCR7 and increased expression of CD57, CD26, HLA-DR (MHC class II), and Killer immunoglobulin receptor (Kir) genes: KIRDL2, KIRDL3, and KIRDL4 (summarized in Tables 2.1 and 2.2, respectively) [52, 58]. The significance in the change in surface receptor gene expression again highlights potential aging mechanisms critical for immune function. For example, studies on chemokine receptors explain why these changes are likely to control the outcome of diseases in the elderly [63].

Aging is also associated with alterations in cell proliferation and in the relative proportions of certain classes of T cells in the periphery. Most of the CD28 functions described earlier have an overall net effect on lymphocyte numbers in the periphery, but other factors also influence cell numbers. The generation of T lymphocytes depends on two

main mechanisms: migration of T cells from the thymus and self-renewal of post-thymic T cells through homeostatic proliferation.

A number of techniques have allowed determination of human T cell quantitative parameters affected by thymopoiesis and homeostatic proliferation [64]. Quantitatively, the adult human contains  $3 \times 10^{11}$  T cells with a  $10^8$  T cell repertoire, which means that, on average, humans have 1,000 naïve clonal T cells per antigen target [65]. T cell turnover measurements have indicated that naïve CD4+ and CD8+ T cells have a half-life of 100–200 and 100 days, respectively [65], longer than the half-life of memory T cells, which ranges between 10 and 50 days. Thus, naïve T cells turn over once a year during the human lifespan and can survive up to ten times longer than memory cells [65]. Studies in mice have shown that naïve cells from old mice function less well than naïve cells from young mice, and are unable to provide memory to the same degree as the young, a scenario that may be true in humans as well [66]. In addition, with increasing age, naïve and memory T cell numbers and functions decline, which may, at least in part, be a consequence of developmental changes in the stromal microenvironment (BM and thymus) and HSC that ultimately yield fewer CLP, as well as to possible additional intrinsic functional changes in homeostatic control.

Puberty in humans, which begins at approximately age 11, marks the accelerated involution of the thymus by sex hormones. However, a decline in absolute numbers of naïve cells is not observed until the seventh decade of life [65]. Thus, despite the steady decline with age in naïve T cells being produced by the thymus, the absolute number of lymphocytes seems to be maintained by homeostatic mechanisms. Evidence supporting this notion emerges from studies on thymectomized children, who nevertheless are able to maintain normal T cell numbers in the periphery [67]. The consequences of increasing homeostatic proliferation to maintain T cell numbers have several negative outcomes by old age: a decrease in the overall TCR repertoire, increased clonotype frequency, and T cell exhaustion. Although T cell numbers in the periphery can be



**Table 2.1** Summary of gene expression patterns in CD28null CD8 T cells [52]

Genes expression	Description
<b>Activation receptors</b>	
↑ CD28	Stimulator of T cells; ↑: proliferation, cytokines, survival, and glucose metabolism
≈ CTLA-4	Inhibitor of TCR activation; ↓: proliferation and cytokines
≈ ICOS	Stimulator of T cells (weaker signal than CD28); ↑: proliferation, cytokines, survival, and germinal center formation
≈ BTLA	Inhibitor of TCR activation; ↓: proliferation and cytokines
≈ PD-1	Inhibitor of TCR activation; ↓: proliferation and cytokines and survival
↑ 4-1BB (CD137)	TNFR family member, role in cell division, ↑: survival and effector functions
↓ CD40L	Costimulatory receptor required for effective B cell activation
↑ KLRD1, KLRF1, KLRG1, CNCR1, and CD16	Stimulatory and inhibitory receptors also expressed in NK cells. Some shown to bind polymorphic MHC class I. The ligands for some of these receptors are unknown
↓ KLRB1	Receptor linked to CD8 T cells specific to chronic hepatitis C virus
<b>Cytolytic molecules</b>	
↓ GZMK	Granzyme induces apoptosis, only produced by CD8 T cells
↑ GZMM, GZMA, GZMB, GZMH	Granzymes produced by CD and NK cells involved with inducing apoptosis
↓ PRF1	Creates pore structure on target membranes facilitating granzyme entry
↓ FASL	FAS ligand induces apoptosis
<b>Cytokine/chemokine receptors</b>	
↓ IL-3, IL12A, and IL-13	Cytokines induce: 1 – myeloid differentiation, 2 – Th1 differentiation; 3 – survival, differentiation, and proliferation respectively
↓ CCR2, CCR7, and CCR6	Chemokines induce: 1 and 2 – migration; 3 – aids in B-lineage maturation and differentiation respectively
↑ CCL4, XCL1, XCL2, and CX3CR1	Regulate adhesion and migration of T cells into sight of infection

Gene expression is indicated as increase (↑), decrease (↓), or no change (≈)

maintained by homeostatic mechanisms, the TCR repertoire can only be increased by thymic output. The naïve T cell repertoire can be maintained for a few decades after the dwindling of the thymic output, but nevertheless during the seventh decade the overall repertoire in humans decreases from  $10^8$  to  $10^6$  different TCRs [65].

Although the underlying cause for the decrease in TCR repertoire in older humans remains to be established, one possible cause is the presence of large populations of clonally expanded T cells, most of which occur within the CD8+ subset [55, 68]. It remains to be determined if the constriction of the T cell repertoire is the cause, or the effect, of the large populations of clonally expanded CD8+ T cells. Evidence for the latter seems more likely, since chronic infections with CMV and EBV are associated with high proportions of

virus-specific memory clonotypes [68, 69]. The final and, arguably, most deleterious outcome of both chronic antigenic stimulation and extensive homeostatic proliferation is the generation of large populations of T cells which have reached the end stage of replicative senescence, particularly within the memory CD8+ T cell subset.

Replicative senescence involves a variety of features that may negatively impact the immune system of older individuals. Senescent T cells are unable to divide, and have shortened telomeres, inability to upregulate telomerase, altered cytokine profiles, resistance to apoptosis, reduced cytotoxic function, complete suppression of CD28 gene transcription [70–75], and reduced responses to vaccines [76, 77]. CD8+ T cells with similar features have been documented in vivo in

**Table 2.2** Summary of gene expression patterns in CD28null CD4 T cells [58]

Genes expression	Description
<b>Receptors</b>	
↓ CD28	Stimulator of T cells; ↑: proliferation, cytokines, survival, and glucose metabolism
↑ HLA-DRA, HLA-DPB1, HLA-DPB5, HLA-DPB4, HLA-DPB1, HLA-F, and HLA-DPA1	Genes encode for the human class I major histocompatibility complex used for antigen recognition
↑ CD26	Functions in cell adhesion
↑ CD3E	Component of the TCR-CD3 receptor complex required for T cell activation
↓ CD27	TNF family member, role in long term memory, ↑: survival and effector functions
≈ CD40L	Costimulatory receptor required for effective B cell activation
↑ CD58	Cell adhesion molecule
↑ KIR2DL2, KIR2DL3, and KIR2DL2	Stimulatory and inhibitory receptors normally expressed in NK and CD8 T cells. Unknown function in CD4 T cells
≈ KLRD1, KLRG1, and KLRK1	Unknown function on CD4 T cells
<b>Cytokine/chemokine receptors</b>	
↑ IL-17RB	Mediates the activation of NF-kappaBeta
↓ CCR2, CCR7, and CCR6	Chemokines induce: 1 and 2 – migration; 3 – aids in B-lineage maturation and differentiation, respectively
↑ CCR4, CCR8	Aid in Th2 memory T cells functions and ↑ migration to sight of infection

Gene expression is indicated as increase (↑), decrease (↓), or no change (≈)

the context of several forms of human cancers, consistent with the notion that chronic long-term exposure to tumor-associated antigens may drive them to the end stage of senescence. Finally, CD8+ T cells with features of replicative senescence have been shown to exert suppressive influences on antigen presentation, helper T cell function, T cell proliferation, and cytotoxicity [78, 79]. Memory T cells have shorter telomeres and reduced proliferative potential compared to naïve T cells from the same individual [80]. Further, telomere shortening of peripheral blood lymphocytes has been associated with multiple age-related pathologies, including atherosclerosis, type 2 diabetes, premature cardiac infarction, and Alzheimer’s disease [19].

Whereas the most dramatic age-related changes occur in T cells, there is evidence of altered B cell function during aging as well. B cells contribute to immune responses primarily in three ways: they produce antibodies against pathogens, play a role in antigen presentation, and are the source of regulatory B cells [81, 82]. The effector function of B cells is initiated in the

germinal centers of lymphoid tissues where B cells undergo positive selection based on their antigen affinity, with elimination of most cells by apoptosis, leaving only a few surviving cells to undergo clonal proliferation. During proliferation, the differentiation process continues and includes somatic hypermutation of the variable regions of the immunoglobulin (Ig) genes. The rearranged Ig genes, which encode for variable regions for both heavy and light chains, are needed for affinity maturation, which enhances the efficacy of the antibody. Further differentiation of B cells is aided by T cells and dendritic cells, which provide costimulatory signals through CD86, CD80, and CD40 receptors [83, 84]. During subsequent activation by T cell-dependent (and to a lesser extend T cell-independent) mechanisms, a subset of B cells will undergo Ig isotype switching, from IgM and IgD to IgG, IgA, or IgE expression [85, 86]. After resolution of the infection, a small number of antigen-selected cells become long-lived memory B cells, which, upon antigen re-encounter, can initiate a more rapid humoral response.

During aging, there are several quantitative and qualitative changes that affect antibody production. There is a reduced production of CLP in the BM, leading to a decrease in precursor B cell production [87] and a reduction in the number of B cells migrating to the spleen (likely due to a defect in signals to exit the BM); as a consequence cell turnover is reduced, which affects the B cell repertoire [24, 86, 88]. The total B cell number in the periphery, nevertheless, remains relative constant for most of a human's adult life, due to prolonged survival of most B cells, as well as to homeostatic expansions, but by old age, the number of B cells eventually declines [89, 90].

In the elderly, decreases in peripheral B cells affect the duration of the humoral response; the titer and affinity of the antibodies produced are both also reduced. The observed reduction in somatic hypermutation of Ig genes by antibody secreting plasma cells in the BM [91] and the decline of Ig class switch are critical [92]. Experiments that mechanistically explain the age-related decline of Ig class switching in human B cells have documented the importance of regulating activation-induced cytidine deaminase (AID) [93, 94]. AID initiates Ig class switching by deamination of cytidines, prompting excisions of base pairs and DNA strand breaks [95]. The sequential steps for upregulation of AID have also been determined. It was discovered that p38 MAPK stabilizes the transcription factor E47 mRNA, which then leads to increased E47 and the upregulation of *AID* gene expression [94]. Mice undergo a similar intrinsic B cell change, but in mice *AID* may also affect somatic hypermutation [94, 96]. This key marker of aging B cells may well be the equivalent of T cells losing their CD28 receptor in its far reaching implications for immune activation and function.

Another important phenotypic change to the B cells during aging is the increased proportion of IgM+ B cells in the periphery, which is indicative of a decrease in antibody isotype switching. In addition, specific variable Ig region genes are preferentially upregulated in old organisms, an observation that may explain the low titer and reduced affinity of antibodies in older persons [93, 97]. Besides intrinsic aging factors, B cells

are dependent on CD4+ helper T cells to generate an optimum immune response. Therefore, age-changes to CD4+ helper T cells (e.g., reduced CD28 expression) may also be a factor in the reduction in both B cell activation and somatic hypermutation, causing a decrease in higher-affinity antibody response. Clinical evidence for these observations include the higher incidence of infection and the poor humoral immune responses to vaccines in the elderly [98]. In addition, the specific lymphoid tissue within which a B cell matures can affect the rate of somatic hypermutation; for example, the spleen and tonsils appear not to have any changes whereas Peyer's patches and peripheral B cells show signs of decreased somatic hypermutation [92, 99]. A possible explanation may be the decrease in numbers and sizes of germinal centers necessary for B cell maturation [94, 100]. Collectively, these extrinsic and intrinsic age-related B cell defects provide possible mechanisms that explain the age decline of the humoral immune responses, increased rates and severity of infections, and reduced vaccine responses.

In contrast to B and T cells, which are central to adaptive immunity, NK cells belong to the innate branch of the immune system. They originate from the same CLP that give rise to T cells and B cells. Although research on CLP and adaptive immune changes during aging has been extensive, studies on aging NK effects have been much more limited. Nevertheless, there has been some notable research on the role of NK cells during aging in mammals. Young rodents that are resistant to mousepox, the murine equivalent of smallpox, become less able to control this disease at middle age, a change that has been attributed to NK cell, rather than T cell, defects [101]. Reduced NK function may also affect immunity to influenza during aging. A striking example of the importance of NK function was documented in mice subjected to caloric restriction, which is normally associated with increased maximal lifespan and reduced cancer in mice. However, following pulmonary infection with influenza, the calorically-restricted mice showed significantly increased morbidity, which was linked to dimin-

ished NK cell function [102]. The underlying mechanism for NK decline during aging has not been elucidated, but one line of evidence now points to malfunctioning of toll-like receptors (TLR), which, like the TCR and CD28 on T cells, are required for NK activation [103].

## Aging and Hematological Malignancies

There is little doubt that cancer is an age-related disease. The strongest evidence in support of this conclusion is the fact that cancer incidence increases exponentially at the midpoint of maximum lifespan, with >32 % of males and >21 % of females subsequently developing some type of invasive cancer [104, 105]. Many genome-related causes for cancer development have been described. Among them are the cumulative somatic mutations from a lifetime of exposure to endogenous and exogenous DNA damage and proofreading errors during DNA replication [104]. Exacerbating the situation is the progressive age-related decline in DNA repair capabilities and the increase in mutation rate as we age, although the mutation rate of  $2 \times 10^{-7}$  per gene per cell division does not explain the stochastic events of initiation and progression required for the observed high incidence of cancer during aging [104, 106–108].

Mechanistically, replicative senescence, which strictly limits cell division capacity of normal cells, is a barrier to lymphoma development. As noted above, replicative senescence is triggered by the shortening of telomeres and moderated by the action of p53 [7–9]. However, escape from p53-induced senescence leads to further shortening of telomeres, which can cause chromosomal abnormalities and instability, due to fusion-bridge-breakage cycles; these chromosomal instabilities can be restored in cancer cells with the reactivation of telomerase activity [109–112]. Of interest, however, is the type of cells that develop into cancers as we age. Indeed, it is intriguing to note that, although genetic alteration to DNA is occurring in all cells, epithelial carcinomas are the most prevalent type of cancer in elderly humans (83.6 %), with sarcomas and lymphomas being less com-

mon [104]. There are several observations that can explain this tumor spectrum. One idea is that different cell types may be regulated differently. Evidence to support this notion comes from studies in mice, a species that normally displays a cancer spectrum that is opposite to that of humans (i.e., mostly lymphomas and sarcomas). Interestingly, knockout mice that lack telomerase activity, but are heterozygous for *TP53*, show a dramatic increase (from 2% to 55 %) in epithelial carcinomas, resembling the human tumor spectrum [104, 111], suggesting that telomere shortening may be a more critical mechanism in the development of epithelial carcinomas, as compared to sarcomas and lymphomas.

Whereas epithelial-derived tumors are more common in elderly humans, 3–4% of all cancers in the world are lymphomas [113]. In 2001, the World Health Organization (WHO) created a consensus for classifying hematological and lymphoid malignancies based on genetic abnormalities, clinical features, and immunophenotypes, which helped separate cancers from such broad classifications as Hodgkin lymphomas, non-Hodgkin lymphomas, leukemias, and myelomas [113, 114]. These efforts to classify hematological malignancies have revealed extensive heterogeneity based on age and gender. For example, it was observed that the rates and cancer numbers for both Hodgkin lymphomas and non-Hodgkin lymphomas are higher for males than for females. Epidemiology studies also revealed that non-Hodgkin lymphoma rates increase steadily with age in both males and females. These findings contrast sharply with observations on Hodgkin lymphomas, where two peaks for the cancer incidence for both males (30–34 and 75–79 years) and females (20–24 and 75–79 years) have been observed [113]. These studies suggest that Hodgkin lymphoma may be comprised of several diseases, which may explain the two cancer incidence peaks, and the fact that some of these diseases are more prone to occur later in life.

Data from the Hematological Malignancy Research Network (HMRN) (at HMRN.org) on a UK population using the WHO malignancy classification revealed that diffuse large B cell lymphoma, follicular lymphoma, and marginal zone lymphoma account for 70% of the total

lymphoma subtype frequencies among all age groups. This frequency pattern is shared by North America, Europe, and Oceania. What is highly relevant to the field of aging is that most of the non-Hodgkin lymphomas have a median diagnostic age over 70 years, again linking aging as a the greatest risk factor for cancer incidence. As further evidence for the correlation between age and lymphomas is an HMRN 5-year study on a population of 3–6 million people, where no cases of mantle cell lymphomas were detected prior to the age of 48 years (HMRN.org) [113].

If an age-based immune deregulation plays a role in lymphomagenesis, then it might be predicted that certain pathogens that cause depletions, or altered lymphocyte subset distribution within the immune system would also increase the incidence of lymphomas. A prime example is the human immunodeficiency virus (HIV), which is associated with a 10–300-fold increased risk of several B cell lymphomas [115, 116]. Other viruses that modulate the immune system via mechanisms distinct from that of HIV, such as human T cell leukemia virus (HTLV-1), EBV, and human herpes virus (HHV-8), are also associated with increase in the incidence of lymphomas. It is interesting to note that these viral pathogens manifest themselves with increased severity at older ages. Nevertheless, the relative contribution of genetic versus the virally-induced mechanisms remain to be elucidated.

It has been suggested that the age-related decline of the immune system allows cancer cells to escape immunosurveillance, and may even contribute to the spectrum of tumors that predominate during aging. One line of evidence supporting this view has emerged from murine studies on T cell subsets, which were shown to be predictive of subsequent resistance to lymphomas, mammary adenocarcinomas, and fibrosarcomas [117]. The researchers took into account the aging changes in the proportions of T cell subsets in the periphery (with phenotypes similar to those discussed above), and by using a statistical method of principal components, were able to decipher differences among T cell subset patterns at 18 months of age that predicted lifespan in mice dying of aforementioned cancers. Furthermore, as early as

8 months of age, T cell subset patterns were also significant predictors for these cancers, consistent with the notion that premature immunosenescence may predispose the mice to early death from cancer. Further studies will be required to determine whether similar immune biomarkers present early in life are predictive of certain forms of cancer in humans. In that regard, it should be noted that longitudinal studies in humans have identified a so-called immune risk profile (IRP) that is predictive of all-cause early mortality (including cancer) in a group of octogenarians [118]. Future research is required to determine how early in life these and other immune biomarkers will be informative regarding subsequent cancer development.

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## Summary and Conclusions

The ultimate outcome of the age-related decline in immune function is the increased incidence and severity of infections, cancer, and age-related pathologies. These clinical manifestations of immunosenescence are caused by both inherent cellular malfunctions as well as by certain pathogens. With increasing age, the incidence of and morbidity from certain infections that are easily controlled during youth become more problematic. Certain viral, bacterial, and fungal diseases, such as CMV, *Streptococcus pneumoniae*, and *Aspergillus* infections, become more prevalent as we age, suggesting that some of the immunological declines may be pathogen driven [90, 118–120]. Paradoxically, despite the overall decline in immune control over pathogens, certain aspects of the immune response actually increase with age, as evidenced by increased titers of autoantibodies and increased incidence of some allergies. Most importantly, with increasing age, there is a significant increase in certain types of cancers. Indeed, older age is the greatest risk factor for cancer [104].

In order to develop strategies that result in immune reconstitution, increased understanding of age-related remodeling of the immune system is critically important, since many age-related diseases could be ameliorated/prevented by an “improved lymphocyte.” As the elderly population continues to increase, age health-related issues



will become more urgent to address. However, the multifactorial nature of the aging process suggests that single interventions might be unlikely to succeed. Multimechanistic aging targets may be required to retard the process of immunosenescence and/or to rejuvenate aging lymphocytes. Regardless of the approach taken, one biological hurdle that must be addressed is the need for telomere maintenance. Experiments that restore telomerase activity, by reintroducing exogenous telomerase genes, or by some pharmaceutical treatment that can upregulate telomerase activity in human T cells, is one possible approach for delaying immunosenescence and enhancing immune function [70, 121, 122]. The other biological hurdle involve reversing the age-associated oligoclonal expansion of CD8<sup>+</sup> T cells, which seems to be greatly exacerbated by latent CMV infection. One possible approach is suggested by experiments in mice, in which thymic grafts led to increased thymopoiesis and lymphocyte production [123, 124]. Cultured thymic fragments were transplanted into recipient mice, and, surprisingly, the mice showed a reversal of the oligoclonal to polyclonal ratio within the TCR repertoire [124]. Although this approach would not be practical for the large aging human population, it nevertheless provides clues on a possible strategy for remodeling the aging immune system. Importantly, the above strategy suggests that immune system rejuvenation may actually be possible.

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