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Lymphocyte generation and population homeostasis throughout life

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Abstract

Immune aging is a multi-faceted process that manifests as reduced competence to fight infections and malignant cells as well as diminished tissue repair, unprovoked inflammation and increased autoreactivity. The aging adaptive immune system, with its high complexity in functional cell subpopulations and diversity of B and T cell receptors, has to cope with the challenge of maintaining homeostasis while responding to exogenous stimuli and compensating for reduced generative capacity. With thymic involution, naïve T cells begin to function as quasi-stem cells and maintain the compartment through peripheral homeostatic proliferation that shapes the T cell repertoire through peripheral selection and the activation of differentiation pathways. Similarly, reduced generation of early B cell progenitors alters the composition of the peripheral B cell compartment with the emergence of a unique, auto-inflammatory B cell subset, termed age-associated B cells (ABCs). These changes in T and B cell composition and function are core manifestations of immune aging.

Introduction

Advances in technology and healthcare have tremendously improved the quality of life and life expectancy of the world's population. Infections, heart disease, diabetes and other diseases that were once death sentences have now become manageable chronic diseases. Great progress has been made in healthy aging evident by the dramatic increase in life expectancy from ~50 years of age in the 1900s to more than 70 years of age. This increase in life expectancy has led to a higher average age of the world's population. In turn, a larger population of older individuals represents a challenge for healthcare due to increased age-associated morbidities.

Optimal function of the immune system is central to maintaining a healthy life. As an organism lives longer, it accumulates alterations that affect its functional ability to maintain

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homeostasis and to respond to stress. Unfortunately, the age-dependent decline in function observed in multiple cells, tissues and organs is also evident in the immune system. The age-associated decline in immune function, termed immune aging, is associated with reduced vaccine efficacy, increased incidence of cancer and autoimmune diseases, and increased morbidity and mortality from infectious diseases [1]. The immune system is highly integrated with spatially and temporally regulated signaling cascades and cell-cell communication steps, in which small changes in regulations can have profound negative impact in overall function.

Recently identified effects of aging on the innate immune system include deficiencies in macrophage, NK and granulocyte function; and altered function of major sensing receptors [2]. However, overall generation of innate immune cells is largely intact in older individuals and the immune system of older individuals is characterized by the production of proinflammatory mediators, referred to as inflamm-aging. Age-related changes in the adaptive immune system have been linked to many of the health-related concerns that affect older individuals. [3]. In this review we will focus on the mechanisms to maintain large and diverse T and B cell repertoires into older age and the consequences of declining cell generation and failure in homeostatic mechanisms for T and B cell competencies.

The thymus – essential for building a T cell repertoire but involutes with age

The thymus is the central T cell-generating organ responsible for producing the naïve T cell pool. During naïve T cell generation, lymphoid progenitor cells migrate from the bone marrow to the thymus where they commit to the T cell lineage and differentiate into functionally mature T cells to be exported to the periphery. The structure and function of the thymic stroma determines the thymus' ability to produce T cells by providing a series of micro-environmental niches that promote T cell development and repertoire selection. The thymus develops during the fetal stage, reaches its maximum output in early postnatal life, and declines in size and output during early adulthood through a process of age-related involution and thymic activity is minimal to absent in later adult life.

Thymic involution is characterized by the gradual disorganization of thymic compartments, changes in thymic endothelial cell subset ratios, and reduced T cell production. Aging alters the expression of FoxN1 and keratinocyte growth factor, key differentiation and growth factors for thymic epithelial cells [4]. Histological changes observed in the involuting thymus include decreased volumes in the cortical and medullary regions, disorganization of epithelial cell architecture and of the cortico-medullary junction, and replacement of the stroma by adipose tissue. The origin of the adipose tissue observed in the involuted thymus is not clear. Two current hypotheses are epithelial-to-mesenchymal trans-differentiation or epithelial cell death leading to expansion of adipose tissue [5]. Adipocytes have the potential to produce proinflammatory cytokines that can negatively affect thymopoiesis. Regardless of the adipocytes' origin, their increase has the potential to accelerate the loss of thymic function with aging.

The structural defects observed in the involuted thymus are aggravated by aging hematopoietic cell defects that skew cells toward myeloid differentiation. With aging, there is a diminished differentiation towards lymphoid lineage and an increased propensity towards myeloid differentiation [6]. Aged lymphoid precursors have difficulty in seeding the old thymus. Impaired seeding may be due to intrinsic cell defects or changes in stromal niches. In support of the former, early T cell precursors isolated from old mice showed a tenfold decrease in seeding fetal thymic organ cultures compared to young cells [7].

How much and how long thymic activity contributes to T cell generation in humans has been a question of controversy. During growth periods in childhood and adolescence, the seeding of new thymic emigrants is certainly important in building up the repertoire. Indeed, surgical removal of thymic tissue in early childhood changes the composition of the T cell compartment in young adults, reminiscent to the T cell population changes associated with immune aging [8]. But even under these extreme circumstances, the unmasking of the immune aging features requires concurrent CMV infection. Whether any thymic T cell production occurs under steady state condition after early adolescence is less clear. Small islands of functional thymic tissue can be found in thymic tissue specimens from older individuals that have otherwise completely lost the normal thymic architecture. However, it is unlikely that production from these tissues is quantitatively important.

Similar to thymus tissue size and function, TCR excision circles (TREC) that form during TCR rearrangement and that have been taken as an indicator of recent thymic emigrants, exponentially decline with age and are highly infrequent in older individuals [9]. Since TRECs do not replicate when T cells proliferate, the loss of TRECs is consistent with cumulative homeostatic proliferation in the absence of thymic activity. Thus, the persistence of few TREC⁺ T cells may just reflect cells that have not proliferated or are the original parent cell [10]. Similar considerations apply to phenotypic markers that are preferentially found on recent thymic emigrants, such as CD31 and PTK7. Cells expressing these markers decline with age and are very infrequent in older individuals; if still present in adults, they do not indicate ongoing thymic activity but instead are likely naïve cells that have not replicated. Indirect evidence that thymic T cell generation is irrelevant in adult life comes from studies quantifying naïve T cell turnover. Rates do not increase in older individuals indicating that homeostatic proliferation does not increase to compensate for declining thymic production [11]. Only in the very old, an increase in proliferating cells is seen that may reflect a compensation for increasing peripheral loss.

Even if the thymus does not contribute to T cell production in the healthy adult under steady state condition, it may do so in a lymphopenic environment. Lymphopenic stress conditions have been explored to assess thymic potential capacity under several clinical settings, including the introduction of HAART to patients with HIV infection; lymphocyte repopulation after T cell depletion to treat autoimmune disease; or repopulation after bone marrow transplantation. Antibody-based T cell depletion explored in the 90's in the treatment of rheumatoid arthritis and other autoimmune diseases provided a unique research opportunity because of the absence of confounding factors associated with HIV infection or chemotherapy. Middle-aged female patients with rheumatoid arthritis treated with the very potent depleting antibody Campath1H infrequently generated naïve T cells and most

repopulation derived from oligoclonal proliferation of memory T cells [12]. Hakim et al. studied female patients with breast cancer after bone marrow transplantation for thymic size, T cell subpopulation frequencies and frequencies of TRECs [13]. In this study, about 50% of patients in the age group between 40 and 50 years showed evidence for thymic activity while this was the rare exception in patients older than 50 years. Taken together, these data suggest that thymic function declines over the course of a lifetime, becoming virtually absent in elderly individuals.

Maintaining the T cell pool by peripheral proliferation into older age

Since T cell receptor (TCR) rearrangement is restricted to thymocyte differentiation, addition of T cells with novel receptors is strictly dependent on thymic function. With the dramatic decrease in thymic output after puberty in humans, novel T cells are no longer added in any quantitatively meaningful number throughout adulthood. To maintain the size of the naïve T cell compartment and compensate for T cell loss, peripheral homeostatic mechanism must work properly. Here, it is important to appreciate differences in T cell homeostasis between mice and humans [14]. In mice, the thymus is a prominent contributor to T cell generation throughout life (i.e. with loss in thymic production, naïve CD4 and CD8 compartments shrink). In contrast, homeostatic proliferation in humans is an effective means to maintain the naïve CD4 and to a lesser extent CD8 T cell pool [15]. The size of the naïve CD4 T cell compartment significantly shrinks with age, however, even the very old individual has a sizeable naïve CD4 compartment and a contribution of age is difficult to detect when controlled for confounders such as CMV infection [16]. In contrast, age is clearly associated with a loss of naïve CD8 T cells independent of confounding variables. The reason for this differential susceptibility between naïve CD4 and CD8 T cells is unclear, but likely related to differences in peripheral maintenance rather than thymic production. Peripheral expansion of naïve T cells relies on low-intensity TCR stimulation and homeostatic cytokines, in particular IL-7 [17, 18]. Optimally-tuned tonic TCR and homeostatic cytokine stimulation is required to maintain a balance between providing sufficient signals for survival and replacement without triggering turnover. Lowering TCR and cytokine receptor thresholds or excess of cytokines may even accelerate aging, as proposed by Reynolds et. al., in which increased IL-7 driven proliferation leads to increased cell death, decreasing the cell compartment size several years later [19].

Functional consequences of cumulative homeostatic proliferation with age

For homeostatic proliferation to be an effective mechanism of T cell replacement, cells need to maintain the phenotype and function consistent with their prior differentiation stage. Indeed, the phenotype is largely conserved. However, increase in homeostatic proliferation due to excess of trophic cytokines or to lymphopenia leads to changes in the phenotypic and functional status of the cells, so that naïve cells assume a pseudo-memory phenotype termed virtual memory (VM) cells (Figure 1). VM cells can dominate the central memory compartment in wild-type mice. Studies in old mice have shown that naïve T cells frequently assume VM phenotype and function in the absence of immunization [20, 21]. The mechanisms by which VM cells arise in old mice are different from those that produce VM cells in young adult mice. In young adult mice, VM cells arise as a result of neonatal

lymphopenia and excess IL-7 in an “empty” neonatal peripheral compartment [22]. These VM cells display superior immediate effector function and proliferation when compared to true naïve cells. In contrast, VM cells in old mice exhibit increased TCR avidity and decreased peptide/MHC dissociation rates [21]. Whether virtual memory cells also occur in the human memory compartment with age is likely but has not yet been proven. However, fully differentiated T cells [23] and clonally expanded T cells [24] exist in the naïve compartment of older individuals and these clones may be memory T cells that have regained a naïve phenotype as has been described for memory stem cells after yellow fever vaccination [25]. They may also include the human equivalent of murine virtual memory cells that clonally expanded due to selective homeostatic proliferation but maintained a naïve phenotype.

Cumulative homeostatic proliferation naïve T cells can lead to functional changes without causing a phenotypic switch to memory. This is certainly the case for telomeric erosion that is seen for naïve T cells in relationship to age and reflects their replicative history [26]. Moreover, aged naïve CD4 T cells have a lower expression of miR-181a, which leads to activation of negative regulatory pathways in TCR signaling involving dual-specific phosphatase 6 and other phosphatases [27]. Loss of miR-181a is characteristic for T cell differentiation; with thymocytes having the highest concentration and progressive loss of expression during the transitions to naïve, effector and memory T cells. Thus, the age-associated loss of miR-181a can be interpreted as incomplete differentiation. Similar conclusions can be drawn from chromatin accessibility data where naïve CD8 T cells from old individuals exhibit epigenetic signatures reminiscent of T cell memory differentiation (own unpublished observation).

Maintaining T cell receptor repertoire diversity – the ultimate challenge of T cell aging

Maintaining a diverse TCR repertoire is critical for an immune system to respond to the large plethora of possible antigens. TCR repertoire diversity is generated early in life by stochastic receptor rearrangement in the thymus. Thymic involution is therefore a threat for TCR diversity because homeostatic proliferation can, at best, maintain diversity but not compensate for T cell specificities that are lost. Initial intrathymic and peripheral clonal expansion of different TCR clones in the neonate is relatively equal suggesting similar T cell clonal sizes of 100 to 1000 cells across all T cell clones early in life. For a clone to get extinct, the entire clonal progeny has to be lost. Thus, to maintain diversity, peripheral proliferation needs to be non-selective. Using *in silico* modeling of T cell homeostasis, we have shown that additive or synergistic effects in peripheral selection can lead to a sudden dramatic loss in diversity [28]. Our modeling predicts that this crash in diversity is not prevented by thymic activity or restored by thymic reactivation. Peripheral selective forces certainly exist, ranging from recognition of self-antigen with different affinities to variable responsiveness to cytokine signaling. Therefore, clonal sizes are expected to become more variable with aging, and at the extreme, an entire clonal progeny can be lost leading to repertoire contraction.

There are several studies in mice and humans analyzing the influence of age on TCR diversity, using techniques such as anti-TCRV β Ab staining, TCR CDR3 length analysis and high-throughput next generation sequencing. It is important to note that the experimental strategies can influence results in ways unrelated to T cell repertoire maintenance. T cell activation and mobilization may influence the composition of the peripheral blood T cell repertoire. Even under steady state condition, the circulating T cell pool may not be representative of the entire T cell population, although this is likely less of an issue for naïve T cells in humans. Analyzing unseparated CD8 and CD4 cell pools does not take into account the extensive changes in T cell subpopulation during aging. Moreover, most studies to-date do not analyze the TCR repertoire in sufficient depth; and thus, the age-related changes observed reflect increased frequencies of clonally expanded population that accumulate with age rather than a loss in richness, i.e. the number of receptors with different sequences. These limitations emphasize the need to use high-throughput sequencing techniques of purified subsets of T cells to study changes in TCR diversity with aging. However, even with high-throughput sequencing, richness is difficult to estimate if the global richness is much larger than the sample of cells sequenced. Of note, the entire T cell pool encompasses nearly 10^{12} T cells while sequencing is usually performed on purified subsets of about 10^6 cells. To control for these shortcomings, we analyzed replicates of purified populations of naïve and memory CD4 and CD8 T cells. The TCR repertoire richness of naïve CD4 and CD8 populations were about 3- to 5-fold lower in healthy individuals older than 65 years when compared to young adults [24]. Given the enormous TCR richness found in this study, this repertoire contraction is unlikely to be biologically relevant, however, it should be noted that study participants were selected for being in excellent health [24]. Biologically more relevant may be an increase in clonality observed with age. Such increased clonality was much more prevalent in naïve CD8 compared with CD4 T cells and may represent memory T cells with a naïve phenotype or naïve T cells clonally selected under homeostatic proliferation, discussed above. In either case, these cells could impair immune responses due to competition for space or ultimately cause a crash in repertoire diversity.

In addition to uneven homeostatic proliferation, expansion of T cells in the memory pool recognizing latent viruses influences T cell diversity. Chronic or latent infections such as cytomegalovirus (CMV), which infects 60-70% of humans in the western hemisphere, strongly affect the memory T cell repertoire [29]. CMV induces inflation of effector memory CD8 and CD4 T cells with increasing oligoclonality [30-32]. Whether CMV-induced expansion of the memory T cell pool can have a negative effect on the repertoire of naïve T cells remains debated. However, most of the CMV-specific effector T cells have a different homing receptor profiles than naïve T cells and therefore do not compete for the same space and are unlikely to affect the naïve repertoire.

Vulnerability of B cell generation and peripheral homeostasis to aging

Similar to the aging T cell compartment, the aging B cell compartment has reduced frequencies of naïve cells coinciding with an increase of oligoclonal memory subsets. In addition, the functional capacity of B cells substantially declines with age. Overall, older individuals exhibit a significant deterioration of effective antibody responses, which are

highlighted by weaker neutralizing antibody responses to vaccination and natural infection, as well as increased production of autoreactive antibodies by B cells [33]. Alterations in B cell generation, homeostasis and selection all contribute to age-associated B cell dysfunction (Figure 2).

B cells are derived from hematopoietic stem cells (HSCs) and develop within the bone marrow. Skewing of the HSC compartment towards the myeloid lineage and away from lymphocyte precursor development (lymphopoiesis) is a well-described event during aging. This cellular skewing has been linked to changes in the cytokine milieu within bone marrow stem cell niches as well as with intrinsic epigenetic and transcription dysregulation and mutational changes of genomic DNA in HSCs [34]. Age-associated decline in lymphopoiesis within the bone marrow reduces the generation of early B cell progenitors and alters the composition of the peripheral B cell compartment in older individuals [35]. Plasma cell niches within the bone marrow are also reduced in the older individuals and could directly cause a decrease in longevity of antibody responses observed during B cell aging. The age-associated reduction in cellular output from the bone marrow in combination with maintenance of total peripheral B cell numbers observed with age indicates that there are peripheral compensatory mechanisms to deal with reduced B cell generation. These mechanisms comprise of an increase in mature B cell lifespan and increased homeostatic expansion of antigen-experienced B cells that lead to decreased repertoire diversity in aged B cell populations [33]. Decreased repertoire diversity directly correlates with decreased overall health in elderly individuals.

B cell selection mechanisms with age

Once committed to the B lineage, B cell precursors must undergo immunoglobulin gene rearrangement during the pre- and pro-B cell stages, followed by expression of the B cell receptor (BCR) to become an immature B cell and exiting the bone marrow [36]. In the periphery, immature B cells undergo further selective pressure during transitional stages before completing maturation. During these stages of development, self-reactive B cells are eliminated through negative selection and BCR heavy and light chain rearrangement (central tolerance). Additionally, peripheral B cells undergo negative and positive selection mechanisms (peripheral tolerance) that allow for the maintenance and expansion of antigen-specific B cells and differentiation into antibody-secreting plasma cells. These selection events include competing for cytokines important for survival, in particular, B cell activating factor (BAFF) and the ability to undergo affinity maturation through somatic hypermutation.

Breakdown of selection mechanisms that typically promote immune tolerance can lead to the induction of autoimmunity. The appearance of autoantibodies and increased frequency of autoimmune diseases in older individuals suggests a failure in B cell tolerance mechanisms during the aging process, likely during transitional B cell development. In the search for understanding this selection failure, a new phenotypically distinct B cell subset has been discovered. This subset, termed age-associated B cells (ABCs), accumulates in the peripheral B cell population with age and are defined by high expression of the transcription factor T-bet and surface CD11c [37]. ABCs rapidly respond to innate stimulation through toll-like receptors but, unlike most B cells, need little to any BCR stimulation for activation.

ABCs also have a survival advantage compared with classical mature B cells, in that they do not rely on BAFF as a survival factor, and therefore, can outcompete mature B cells for space within the aging B cell compartment. Moreover, ABCs secrete high levels of autoantibodies and are increased in patients with autoimmunity (e.g. common variable immunodeficiency, Sjogren's syndrome), suggesting these cells bypass normal selection mechanisms. Of note, no difference in the ability of B cells to undergo somatic hypermutation is observed between young and elderly adults, thus differences in affinity maturation within germinal centers unlikely induces the development of ABCs. Thus, age-related differences in B cell selection and homeostatic expansion, in particular the expansion of ABCs, may contribute to increased autoantibodies during age. Further studies of central and peripheral tolerance mechanisms will provide better insight into B cell dysfunction during immune aging.

Conclusion

Age-related changes in T and B cell generation and peripheral maintenance have a significant impact on immune function and the ability to protect against infections and malignant cells. In case of T cells, the lack of thymic production possibly throughout adulthood, but certainly in the later years of life, places strong pressure on homeostatic proliferation mechanisms to maintain a large and diverse T cell pool. Increased homeostatic proliferation due to excess trophic cytokines or lymphopenia has the unintentional consequence of generating VM cells. Furthermore, chronic latent infections, in particular with CMV, leads to increased clonality and expansion of effector populations within the memory compartment with possible, currently debated consequences for the naïve repertoire and immune competence. Similarly for B cells, the increased propensity of the HSC compartment to differentiate into the myeloid lineage rather than lymphocyte precursors, results in reduced generation of early B cell progenitors, altered composition of the peripheral B cell compartment and the emergence of ABC cells. Maintaining peripheral T and B cell homeostasis over lifetime is essential to preserve immune competence in older age and a prerequisite for healthy aging.

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Figure 1. T cell generation and maintenance during aging

The thymus generates novel naïve T cells early in life, providing a diverse T cell receptor (TCR) repertoire. The maintenance of the naïve T cell pool during adult life is entirely dependent on homeostatic proliferation. In old age, aberrant homeostatic proliferation results in contraction of the T cell pool (especially CD8⁺ T cells), decrease in TCR repertoire diversity and generation of virtual memory cells (VM).

Figure 2. B cell generation and maintenance during aging

In older individuals, hematopoietic stem cells (HSC) are skewed towards the myeloid lineage, reducing the number of lymphoid progenitors and subsequently, B cell precursors in the bone marrow. B cell precursors undergo a series of selection events, where initial pre-B cells are positively (+, green bars) selected for functional BCR heavy chain arrangements followed by negative selection (–, red bars) of self-reactive immature B cells, prior to exit from the bone marrow. Once in the periphery, transitional B cells undergo further selection for survival and antigen-specificity. Reduced naïve B cell output from the bone marrow in the elderly leads to memory cell expansion by homeostatic proliferation and contraction of repertoire diversity. In addition, there is accumulation of age-associated B cells (ABCs) and autoreactive antibodies.