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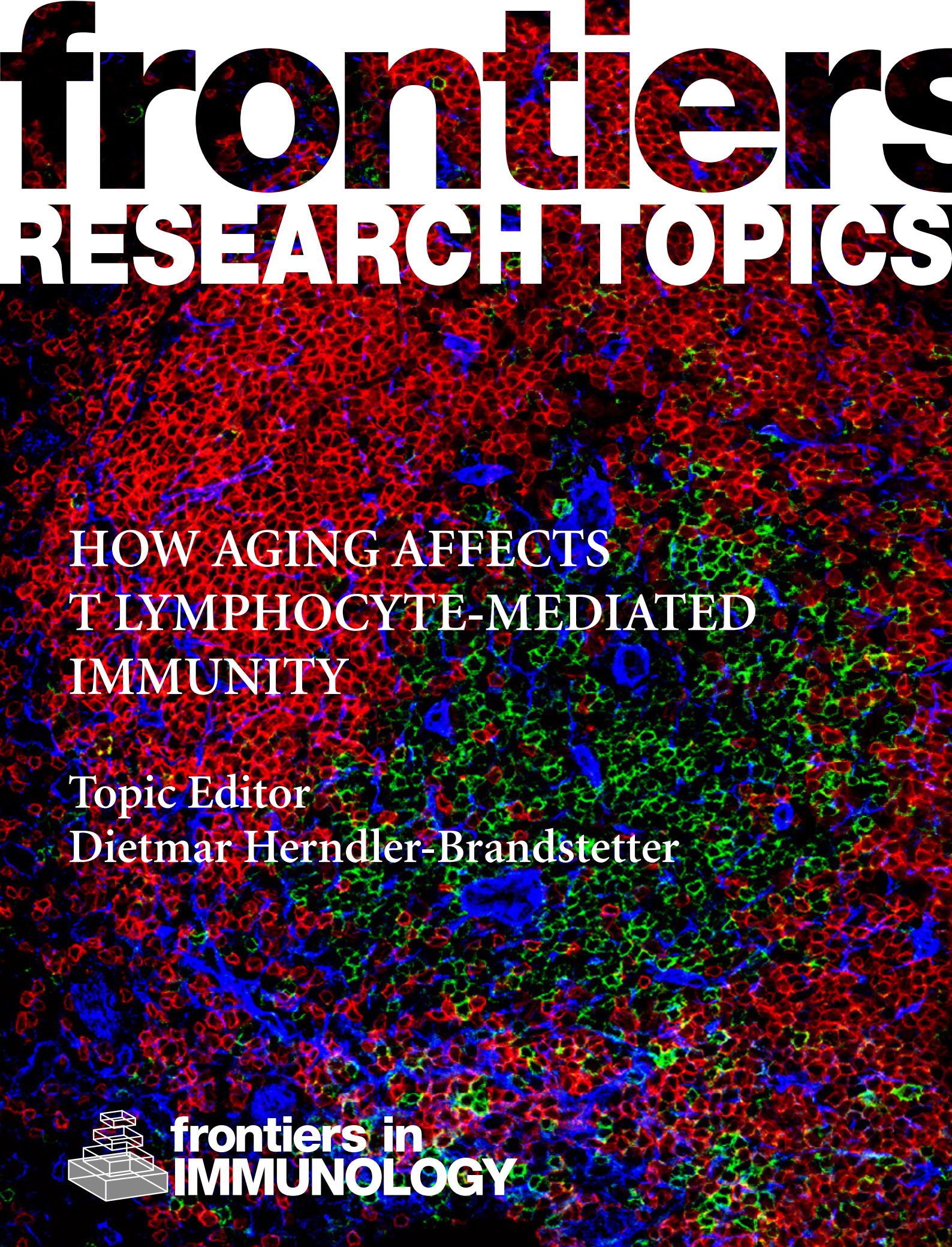

HOW AGING AFFECTS T LYMPHOCYTE-MEDIATED IMMUNITY

Topic Editor
Dietmar Herndler-Brandstetter

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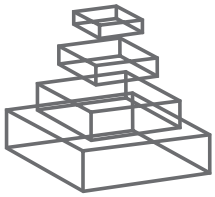

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HOW AGING AFFECTS T LYMPHOCYTE-MEDIATED IMMUNITY

Topic Editor:
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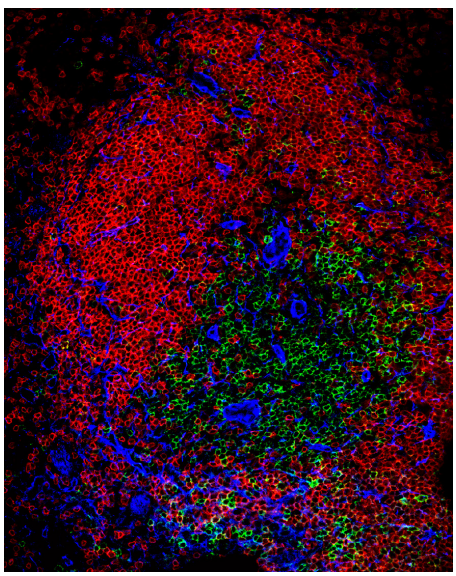

This confocal microscopy picture shows the organization of B cells (red, B220), T cells (green, CD3) and blood vessels (blue, laminin) in the spleen of a C57BL/6 mouse. This structural organization is a prerequisite for the initiation of adaptive immune responses. Copyright: Dietmar Herndler-Brandstetter

Table of Contents

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EDITORIAL
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How aging affects T lymphocyte-mediated immunity

Dietmar Herndler-Brandstetter*

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Keywords: aging, biomarkers, memory T lymphocytes, lifespan, autoimmunity, vaccination, cytomegalovirus

Increasing age has been associated with an insufficient protection following vaccination and an increased incidence and severity of infectious diseases. The predicted acceleration of global population aging will accentuate the need to understand the mechanisms that drive the age-related decline of the immune system and to, eventually, identify strategies to improve immune function in elderly people. One type of immune cell appears to be most dramatically affected by the aging process: T lymphocytes. As T lymphocytes are key players of the adaptive immune system, this research topic summarizes our current understanding on how aging affects the microenvironmental niches and molecular networks that are important for the generation, survival and function of naïve, memory and effector T lymphocytes.

Age-related changes of the bone marrow and the thymus microenvironment lead to a decreased thymic output of functional, naïve T lymphocytes. The article by Palmer (1) suggests that the thymic stroma is a key factor in regulating thymic atrophy and that both, intrinsic and extrinsic factors contribute to the age-related decline in thymopoiesis. Several studies also demonstrate that the kinetics of the involution of the thymus is not uniform throughout life but can be divided into distinct phases, which are probably controlled by different molecular mechanisms.

The article by Chen et al. (2) provides an extensive review about how aging affects genome-wide transcriptional changes in different cell types, such as thymocytes, CD4 and CD8 T cell subsets in mice and humans. Gene networks and signaling pathways that are altered in aged T cells are highlighted and they may prove useful to select robust biomarkers of T cell aging. Moro-Garcia et al. (3) address the impact of aging on the phenotype and function of naïve, memory, and highly differentiated $CD4^+$ T cells. The authors also propose that the homeostatic cytokine interleukin (IL)-15 can rescue functional properties of highly differentiated $CD4^+CD28^-$ T cells that accumulate in elderly people.

The impact of aging on a clinically highly relevant T cell subset, namely regulatory T cells (Treg), is reviewed by Fessler et al. (4). The authors discuss how aging affects Treg cell generation, homeostasis, diversity, and function. The review article also highlights the need to address the gaps in our understanding of Treg cell aging. This will be of relevance when it comes to implementing adoptive Treg cell therapies to treat autoimmune diseases in older individuals. The impact of aging on Treg cells is also addressed by an original article by Raynor et al. (5). The authors show that serum IL-2 levels decline during aging, which is associated with the accumulation of a unique $CD25^{lo}$ Bim^{lo} $FoxP3^+$ Treg population in old mice. Although the mechanism of the age-dependent decline in Bim expression in Treg cells remains to be determined, the accumulation of $CD25^{lo}$ Bim^{lo} Treg cells in old mice seems to be mediated by IL-15.

The article by Goronzy et al. (6) addresses the paradox of an age-dependent decline in immune responses while autoimmune diseases increase in incidence. The authors hypothesize that the same defects that account for the decreased ability to generate protective immune responses also contribute to the increased risk of autoimmunity. The major risk factor for autoimmunity in old age appears to be the reshaping of the peripheral T cell receptor repertoire, which is driven by the age-dependent involution of the thymus. The consequent increase in homeostatic T cell proliferation prolongs peripheral T cell survival and selects for self antigen and cytokine responsiveness, thereby facilitating the generation of autoreactive T cells. Fulop et al. (7) review longitudinal studies that analyzed immune parameters predictive of survival. These studies defined an immune risk profile and showed that persistent viral infections, in particular *Cytomegalovirus* infection, can accelerate human T cell aging.

In an opinion article, Aspinall and Lang (8) focus on the impact of influenza virus infection in the elderly and highlight the current problems of designing a more effective vaccine for people older than 60 years of age. The authors propose a portfolio approach and emphasize that future developments should move toward a more personalized treatment regimen that may even be combined with therapeutic approaches that target immune aging itself. In another opinion article, Flavell and colleagues (9) accentuate the need to define a robust set of markers of T cell aging. The authors summarize our current knowledge about markers of T cell aging and emphasize the lack of data about T cell phenotypes and functions in various human organs and tissues, which would be important to better understand the fate of different T cell types and the role of specific markers. Eventually, the identification of a set of markers for immunosenescence would be a valuable clinical tool and would allow to evaluate potential therapeutic interventions aiming to prevent or reverse immune cell aging, and to personalize vaccination strategies for elderly people at risk, thereby increasing life and health span.

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The effect of age on thymic function

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Age-related regression of the thymus is associated with a decline in naïve T cell output. This is thought to contribute to the reduction in T cell diversity seen in older individuals and linked with increased susceptibility to infection, autoimmune disease, and cancer. Thymic involution is one of the most dramatic and ubiquitous changes seen in the aging immune system, but the mechanisms which underlying this process are poorly understood. However, a picture is emerging, implicating the involvement of both extrinsic and intrinsic factors. In this review we assess the role of the thymic microenvironment as a potential target that regulates thymic involution, question whether thymocyte development in the aged thymus is functionally impaired, and explore the kinetics of thymic involution.

Keywords: thymus, immunosenescence, thymic involution, thymic stroma, thymocyte

THE IMPACT OF THYMIC INVOLUTION ON PERIPHERAL T CELL SENESCENCE

Advance aging correlates with a reduced ability of the immune system to generate antigen specific responses to pathogens and vaccination. This collectively results in a higher incidence of infection, neoplastic, and autoimmune diseases which are preferentially observed in older individuals. These profound changes exhibited by the aging immune system is termed immunosenescence, which affects both innate and adaptive immunity (1–3).

The thymus is responsible for the development of self-restricted, self-tolerant, immunocompetent T cells but has no self-renewal properties relying on the continuous replenishment of new T cell progenitors from the bone marrow. Maturation of these cells occur through a series of proliferation and differentiation stages dependent upon receiving instructions from the specialized thymic microenvironment (4, 5).

One of the most acknowledged changes of the aging immune system is regression, or involution of the thymus (6–8), which seems to occur in almost all vertebrates suggesting that this is an evolutionary ancient and conserved process (9). Age-associated thymic involution involves a decrease in tissue mass and cellularity, together with a loss of tissue organization with the net outcome being a reduction in naïve T cell output [**Figure 1**; (6–8)]. This decline in naïve T cell output is believed to have a major impact on the properties on the peripheral T cell pool such that with increasing age, these cells exhibit alterations in phenotype and function, loss of diversity, and replicative senescence (10, 11). Moreover, it is these age-related changes in peripheral T cells that are believed to contribute significantly toward the features of immunosenescence (12, 13), suggesting that the altered thymic activity is a key trigger toward the decline of immune function in the aged (14).

While animal models show that the maintenance of naïve peripheral T cells in the adult do indeed require the release of cells from the thymus (15, 16). In humans, however the relationship between thymic activity and naïve T cell homeostasis is a matter of debate, with the recent observations that peripheral proliferation and not thymic output contributes to the maintenance of naïve T cells in young adults (17). Nevertheless, using signal-joint T cell receptor (TCR) excision circles (sjTREC) as a measurement of thymic function, numerous studies have shown lower sjTREC levels in elderly individuals are associated with a reduction of naïve T cells (18–20).

Moreover, a direct correlation between thymic function and naïve T cell number comes from studies examining the peripheral immune system of thymectomized individuals (21). In one such study which looked at patients 20+ years after thymectomy, the authors observed a decreased proportion of naïve T cells, reduction in TCR diversity and noted that such changes were more marked in individuals infected with *Cytomegalovirus* (22). Furthermore, thymectomized individuals exhibited a delayed primary response to tick-borne encephalitis vaccination (23). Interestingly, these and other studies seem to suggest that the thymus may play a role in maintaining immune efficacy in the adult (21). Indeed, reports, using mice, have demonstrated the need for the continual production of naïve T cells to mount an effective immune response against bacterial (24), viral (25), and fungal infections (26); with the latter study showing that mice thymectomized at 5 weeks of age exhibited a delayed response to Pneumocystis infection. Furthermore, amongst HIV-infected patients under highly active antiretroviral therapy, those individuals that show enhanced T cell output appear to demonstrate a better prognosis (27, 28). Furthermore, a recent study proposed that thymic function is a key marker in determining mortality in elderly humans (29). Thus, the notion that thymus activity may play an important role in host defense of the adult is interesting and clearly merits further investigation.

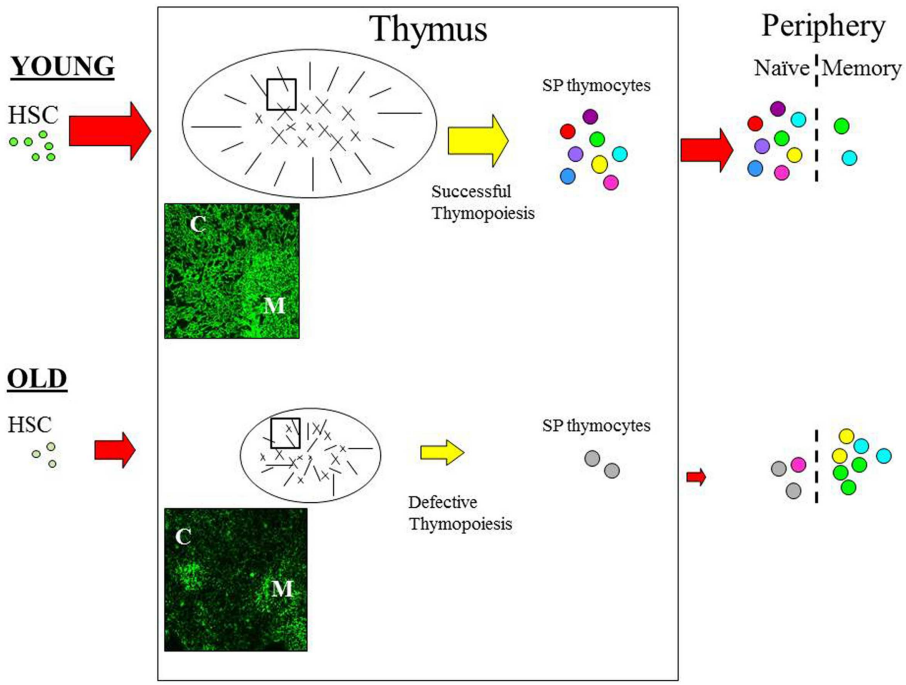

FIGURE 1 | The effect of age on thymic function. Schematic diagram outlining the pathway of T cell development. Aging can impact on variety of pathways during the development of T cells. With increasing age HSC appear to have a reduced lymphoid potential and increased myeloid differential capacity. Age-related involution is associated with reduced thymic mass and altered architecture resulting in reduced thymic output in the aged thymus. In the young, T cell development is functional and the peripheral T cell is pool is diverse; as depicted by the various colors. Furthermore, normal thymopoiesis provides a positive effect on thymic structure; thymic cross-talk. In contrast, T cell output is significantly reduced in the aged thymus resulting in loss of diversity and alteration in the phenotype and function of peripheral T cells; with the majority of cells being in the memory pool. Modifications in the thymic microenvironment are likely to have an impact on thymopoiesis resulting in defective RTE, which in turn disrupt thymic cross-talk leading to further alteration of TEC structure. Immunofluorescence image of young (6 weeks) and old (18 months) murine thymus stained with anti-keratin antibody (55) which detects cortical (C) and medullary (M) TEC. In the young thymus, antibody staining shows the cortical epithelium as a network of long thin processes, while the medullary region reveals a squamous appearance. In contrast, the staining on old thymic section revealed a reduced network of cortical epithelial cells, the medullary region is smaller and more diffuse while the cortical-medullary junction is less distinctive; as also depicted in the schematic. C, cortex; M, medulla. Picture (100× magnification).

CHANGES IN THYMOCYTE DEVELOPMENT WITH AGE

Although the exact mechanisms involved in age-associated thymic involution are not fully understood, a picture is emerging suggesting defects are present within both developing thymocytes and thymic stroma (30). Thymopoiesis involves a series of sequential developmental steps. Briefly, bone marrow progenitors enter into the thymus and are identified by a lack of both CD4 and CD8. Referred to as double negative (DN) thymocytes, these cells differentiate to become double positive (DP), expressing both CD4 and CD8, and subsequently mature into either single positive (SP) CD4 or SP CD8 T cells, through the process of positive and negative selection, and then exit into the periphery (4, 5).

Given that the thymus requires the continual input of bone marrow progenitors, any age-related alterations in hematopoietic stem cells (HSC) function could conceivably contribute toward thymic involution. Studies have demonstrated that aged HSC appear to exhibit an increased bias toward myeloid differentiation together with a reduced capacity toward lymphoid maturation; which has been observed in mice and human (31, 32). Such alterations in HSC function may manifest within early thymocyte progenitor (ETP) activity. Indeed, aged mice have fewer numbers of ETP, which exhibit reduced proliferation and differentiation potential (33, 34). ETP obtained from young mice are able to differentiate into all the stages of T cell development when seeded into fetal thymic organ culture, in contrast aged ETP showed a reduction of T cell differentiation activity (33). Furthermore, ETP from aged mice show an increased frequency of cells undergoing apoptosis together with a reduced number of $Ki67^{+}$ cells (34). ETP are contained within the earliest stages of DN thymocytes and other studies have highlighted further age-related changes within the later stages of DN thymocyte development; with the observation of a decrease in proportion of $CD44^{+}CD25^{+}$ (DN2) and $CD44^{-}CD25^{+}$ (DN3) cells (35–38). Additionally, a population of $CD44^{+}CD24^{-}CD3^{+}$ DN cells has been shown to accumulate in the thymus of older mice (35, 39–41). Interestingly, a similar population has been identified in adult murine bone marrow which appears to be associated with a role in reducing hematopoiesis (42), giving rise to the possibility that the accumulation of such cells in the aging thymus might have a negative impact on thymopoiesis thereby contributing to thymic involution.

Further stages in thymocyte maturation also exhibit phenotypical alterations with age; in particular, studies have demonstrated

an age-associated decline of CD3 expression on DP and SP thymocytes (40, 41, 43). Such changes may result in impaired TCR-dependent stimulation. Indeed, it has been demonstrated that aged thymocytes, in comparison to young cells, showed reduced Concanavalin A-induced proliferation (37, 40, 41, 44), with the observation that aged cells failed to enter into the G_2M phase of the cell cycle (41).

Arguably, these age-related changes in thymopoiesis are likely be acquired by RTE; leading to the possibility that such cells will exhibit reduced immunocompetence. Indeed, several studies have showed that aged RTE undergo phenotypic maturation with delayed kinetics, exhibit decreased proliferative capacity, defective calcium signaling following TCR stimulation, and reduced helper and memory activity (45–47). Furthermore, peripheral T cells from older mice exhibit increased resistance to apoptosis which again may be acquired during thymocyte development as it has been demonstrated that thymocytes from older animals are more resistant to apoptosis (41, 44, 48). It is unlikely that the impairment of aged RTE is acquired in the periphery, but is imprinted during their development in the aged thymus and propose that such flawed cells are also likely to contribute further to peripheral immunosenescence. Moreover, these studies also question, the notion regarding whether T cell development is functionally active in the aged and in light of these studies, this often held view may need to be revised (40).

AGE-ASSOCIATED CHANGES IN THE THYMIC STROMAL ENVIRONMENT

The thymic stroma plays a crucial role in thymopoiesis by providing the signals necessary to promote proliferation and differentiation due primarily to the influence of cortical and medullary epithelial cells (4); thus age-related changes in the thymic niches could potentially promote thymic involution. In fact, we have argued that the extrinsic defects within the aged microenvironment contribute significantly to age-associated thymic involution (1, 14, 49). Several studies have demonstrated that with age, the thymic microenvironment undergoes structural, phenotypical, and architectural changes (50). This include down regulation of various thymic epithelial cell (TEC) markers such keratin, MHC class II together with alterations of cortical and medullary markers (37, 51–55). Furthermore, the structural integrity of the thymic niche is disrupted with age, including disorganization of the cortical and medullary junction; together with increase fibrosis, adipose tissue, and the accumulation of senescent cells in the aged thymus (40, 55–57).

The age-associated changes in thymopoiesis would principally imply intrinsic defects, however, closer examination reveal that perhaps such alterations could be due, in part, to extrinsic defects within the aged thymic stromal niche resulting in impaired T cell development. For instance, studies have revealed that the production of IL-7, which is necessary for thymopoiesis (58), decreases with age (59). This may be due to the observed loss of MHC class II^+ TEC in the aged thymus which has been identified as the cell type responsible for producing IL-7 (54). Moreover, IL-7 administered in older mice (60) and rhesus macaques (61) was shown to increased thymic output. Interestingly, bone marrow from young mice injected into lethally irradiated older mice failed to restore thymic architecture and was still accompanied by a reduction in quantitative thymic function (62). In an elegant study addressing the repopulation potential of thymic progenitors, Zhu and colleagues transplanted fetal thymic lobes under the kidney capsule of 1-month-old and 18-months-old mice and observed that the total number and proportion of developing thymocytes in the grafts were similar in older and younger host mice (56, 63). Similar results were obtained when transplanting RAG deficient thymic lobes in that the ability of wild-type thymic progenitors to develop stromal patterning was not dependent on the age of recipients (63). In contrast, it was observed that intrathymic injection of young ETP fail to develop in older animals but did so in the thymus of young recipients (63). Furthermore, recent studies revealed that age-associated thymic involution results primarily with changes in gene expression profile in thymic stromal cells (64).

Above all, these studies suggest that the thymic stroma is a key factor in regulating thymic involution and perhaps the acquired intrinsic defects in aged thymocytes could be due to the inability of the aged thymic microenvironment to support and maintain thymopoiesis (56). Furthermore, the inter-dependency of both thymocyte and TEC to maintain a functional thymic structure (i.e., thymic cross-talk), is also likely to be a contributing factor toward thymic involution (65). Indeed, disrupting the integrity of TEC in the adult thymus has been shown to mimic thymic involution. The transcription factor Foxn1, which is essential for TEC development (66), has been shown to be important for maintaining TEC activity and reducing *Foxn1* expression in the postnatal thymus mimics features of thymic involution (67, 68). In contrast, over expression of Foxn1 delays age-associated thymic involution (69). Moreover, rejuvenation of the aging thymus has been successful when targeting TEC, with the administration of exogenous keratin growth factor being shown to enhance thymic cellularity, restore thymic architecture, and improve immune function in aged mice (70). Similar results have also been seen when using growth hormone (71), sex steroid ablation (72), ghrelin (73), and IL-22 (74). However, although such treatment have been effective in directly enhancing thymic activity in the aged, in some instances, this may also be due, in part, by promoting hematopoiesis in the bone marrow (71, 75).

In addition to the age-related changes observed in TEC, there is an accumulation of adipose tissue particularly in the human thymus and there is increasing evidence indicating that thymic adiposity may inhibit thymic function (57). In mice, Yang and colleagues demonstrated that inducing obesity in mice accelerated thymic involution (76). In contrast, in another study, the same group observed that caloric restriction resulted in reduced thymic adiposity and delayed thymic involution (77). Although it is unclear how increase thymic adiposity alters thymic function, it has been proposed that this is due to the cytokines produced by adipocytes (57) and while involution occurs before fat deposition, suggesting that it is not initiating thymic involution, it may however exacerbate the impact of age on thymic function.

Studies have also noted an increase in the proportion of fibroblasts in the aging thymus of several species including mice (1, 54), human (52), and fish (78); suggesting that this may be a common feature. Several tissues such as heart (79), kidney (80), and liver (81) also show increased fibrosis with age which is associated

with senescence and impairment of tissue function. Reports have implicated a role for TGFβ (82) and metalloproteinases (80) in the accumulation of fibroblasts in various tissues, which may be activated in response to inflammation as a result of wounding (83). It is currently unknown whether similar events also occur in the thymus, but may exacerbate the aforementioned alterations seen with age.

KINETICS OF AGE-ASSOCIATED THYMIC INVOLUTION

An often held view is that thymic involution is triggered during puberty. This is based on studies showing that sex steroids have a detrimental effect on thymocytes and that chemical or surgical castration in older rodents is able to restore thymic size (34, 38, 64). While sex hormones are likely to contribute to thymic regression, the role of these steroids being responsible for initiating thymic involution is now being questioned (84). Indeed, several studies using a variety of thymic indices (cellularity, epithelial space, number of recent thymic emigrants) have observed that thymic involution occurs early in life, prior to puberty and that the rate of decline is not linear, but appears to be phasic. In mice, thymic cellularity begins to decrease within the first few weeks after birth (37, 45, 53, 85) and a similar picture is evident in human (51, 52, 86), equine (87), and zebrafish (88) thymus.

After this rapid early decline, involution appears to proceed at a steady rate, with studies examining human thymus suggesting a rate of 3% of thymic tissue is lost per year until middle age, followed by a rate of 1% per year (6, 89); which perhaps may cease in later life with studies showing TREC levels being barely detectable in individuals over the age of 85 years (18, 19).

Overall, these studies strongly suggest that the kinetics of age-associated thymic involution is not uniform throughout life, but characterized by distinct phases and perhaps controlled by different mechanisms. Indeed, the onset of thymic involution occurs much earlier than most acknowledged features of aging and interestingly, microarray analysis of the aged thymic revealed limited overlap with genes normally associated with aging (7). Thus, we propose that there are at least two phases in thymic involution: the first occurring in early life which would be referred to as "growth-dependent thymic involution," as it is associated with this period of physiological growth and development and another termed "age-dependent thymic involution" linked to the age-related changes that are occurring in various body systems (85).

CONCLUDING REMARKS

Age-associated thymic involution represents one of the most recognizable features of the aging immune system and is believed to contribute significantly toward immunosenescence. Although the molecular triggers that instigate involution remain to be fully elucidated, both intrinsic and extrinsic factors are thought to contribute toward this process. Moreover, TEC offers a potential target for rejuvenation and requires further exploration. Given the alterations in thymic development in the aged, the evidence suggests that the RTE from the aging thymus are intrinsically defective and could further exacerbate peripheral immunosenescence. Finally, additional factors that are known to modulate thymic function such as pregnancy, infection, inflammatory status, and early life events; i.e., life history is also likely to have an impact on the rate of thymic involution (9, 90).

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T cell aging: a review of the transcriptional changes determined from genome-wide analysis

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Age carries a detrimental impact on T cell function. In the past decade, analyses of the genome-scale transcriptional changes of T cells during aging have yielded a large amount of data and provided a global view of gene expression changes in T cells from aged hosts as well as subsets of T cells accumulated with age. Here, we aim to review the changes of gene expression in thymocytes and peripheral mature T cells, as well as the subsets of T cells accumulated with age, and discuss the gene networks and signaling pathways that are altered with aging in T cells. We also discuss future direction for furthering the understanding of the molecular basis of gene expression alterations in aged T cells, which could potentially provide opportunities for gene-based clinical interventions.

Keywords: aging, thymocytes, T cells, naïve and memory T cells, CD28$^-$ T cells

INTRODUCTION

Age carries a detrimental impact on T cell function. Age-associated decline of T cell function is complex and occurs at multiple levels (Haynes and Swain, 2006). Alteration in transcription is arguably one of the most studied and identifiable age-associated change in T cells. As part of the adaptation to a changing microenvironment with age (Linton et al., 2005), T cells undergo substantial changes in gene expression, resulting in enhanced or reduced aspects of their functions (Pawelec et al., 2005; Weng, 2006). Reported age-associated alterations in T cell functions include: increased cytotoxicity, enhanced secretion of inflammatory cytokines, reduced antigen-induced proliferation, and a decrease in cell division. The precise details of age-associated changes in transcription are beginning to be revealed, but the functional significance of these transcriptional alterations and their contribution to the age-associated changes in T cell function have not been fully characterized. A better understanding of the transcriptional changes in T cells that are associated with aging is an essential first step toward further functional assessment and eventual clinical interventions for older adults.

The development of methods, such as microarrays, allow the genome-scale assessment of gene expression and provide a means to understand the scope and magnitude of gene expression changes during aging (Prolla, 2005). The current commercially available whole genome arrays (Agilent, Affymetrix, and Illumina) contain the information of over 30,000 transcripts with most of the annotated protein-coding genes of human and mouse, and readily assess RNAs isolated from a few thousand to millions of cells. With sufficient biological repeats and proper statistical tools (Hyatt et al., 2006), the global gene expression pattern and significantly enhanced or reduced expressed genes are promptly identified; this provides sufficient transcriptional details to match the complexity of the age-associated changes. However, connecting the individual altered expressed genes to the altered functional networks and signaling pathways with aging is a monumental task.

In the past a few years, studies of global gene expression in T cells with aging have evolved from analyzing whole organ/tissue/whole populations of T cells to comparing well-defined subsets of T cells from different lymphoid tissues or organs, and from identification of significantly altered genes to connecting the gene networks and signaling pathways that are associated with these altered genes. The cumulative evidence shows that age-related alterations of transcription in T cells can be found in thymocytes (Lustig et al., 2007, 2009; Griffith et al., 2012), in peripheral mature T cells (Remondini et al., 2010), and T cell subsets (Fann et al., 2005; Mirza et al., 2011). Although neither the causes nor the consequences of all the transcriptional changes in T cells with age are currently known, the identification and validation of these age-associated changes pave a new avenue for a better understanding of cellular and functional alterations associated with aging. This information will be essential for developing strategies to slow down or even reverse the course of T cell aging. The purpose of this review is to summarize the age-associated gene expression changes on a global level that have been observed in recent studies of T cells and their subsets. We also discuss a potential link between the transcriptional changes and functional changes in aged T cells.

TRANSCRIPTIONAL CHANGES IN AGED THYMUS AND PERIPHERAL BLOOD T CELLS

THYMOCYTES

Initial microarray experiments were focused on comparing gene expression of the total organ/tissue/T cells between young and old hosts (mouse and human). Global gene expression analysis using total thymus from mice revealed that the largest number of gene expression changes with age, within total thymus, occurred in

Table 1 | List of genes that are expressed either lower or higher in aged than in young T cells.

Down-regulated			Up-regulated		
Genes	**Subsets**	**Reference**	**Genes**	**Subsets**	**Reference**
T cell receptor signaling pathway			**Cytokine/chemokine network**		
AKT3	h CD8	Cao et al. (2010)	*ACVR2A, IL15, TNFSF14*	h CD8	Cao et al. (2010)
CD28	h CD4N, CD8CD28-	Weng et al. (2009), Czesnikiewicz-Guzik et al. (2008)	*Bmpr1a*	m CD4N, CD8N	Mirza et al. (2011)
CD3G	h CD8	Cao et al. (2010)	*Ccl1, Il5, Il9, Il3, Il21*	m s-CD4N & CD8N	Mirza et al. (2011)
DLG1	h CD8	Cao et al. (2010)	*Ccl17, Ccl20, Ccl22, Ccl24*	m s-CD4N	Mirza et al. (2011)
GRAP2	h CD8	Cao et al. (2010)	*Ccl21c, Ccl8, Csf2*	m CD8N	Mirza et al. (2011)
GRB2	h CD8CD28-	Lazuardi et al. (2009)	*CCL3/Ccl3*	h CD4, m CD4N	Mirza et al. (2011), Chen et al. (2003)
ITK	h CD8	Cao et al. (2010)	*CCL4*	h CD8CD28-	Fann et al. (2005)
LCK	h CD8	Cao et al. (2010)	*CCL5/Ccl5*	h CD8, m CD4N, CD8N	Cao et al. (2010), Mirza et al. (2011)
MAPK1, MAPK14	h CD8	Cao et al. (2010)	*CCR1*	h CD4	Mo et al. (2003), Yung and Mo (2003)
NCK1	h CD8	Cao et al. (2010)	*CCR2/Ccr2*	h CD4, m CD4N, CD8N	Mirza et al. (2011), Mo et al. (2003)
PDK1	h CD8CD28-	Lazuardi et al. (2009)	*CCR3*	h CD4	Yung and Mo (2003)
PIK3CD	h CD8CD28-	Lazuardi et al. (2009)	*CCR4/Ccr4*	h CD4, CD4N, m CD4N, s-CD8N	Czesnikiewicz-Guzik et al. (2008), Mirza et al. (2011), Mo et al. (2003), Yung and Mo (2003)
SOS2	h CD8	Cao et al. (2010)	*CCR5/Ccr5*	h CD4, CD4N, m CD4N/CD8N	Czesnikiewicz-Guzik et al. (2008), Mirza et al. (2011), Mo et al. (2003), Yung and Mo (2003)
			CCR6, CXCR2, CXCR5	h CD4	Mo et al. (2003)
Cytokine/chemokine network			*CCR8*	h CD4N	Czesnikiewicz-Guzik et al. (2008), Mo et al. (2003)
Ccl19	m s-CD4N	Mirza et al. (2011)	*CD70, IL17RB*	h CD4N	Czesnikiewicz-Guzik et al. (2008)
Ccl22	m CD8N	Mirza et al. (2011)	*CLCF1*	h CD8CD28-	Lazuardi et al. (2009)
Ccl24	m s-CD8N	Mirza et al. (2011)	*Csd2, Cxcl11*	m s-CD4N	Mirza et al. (2011)
Ccr9	m CD4N, CD8N	Mirza et al. (2011)	*Csf1, Cxcl10*	m CD4N	Mirza et al. (2011)
Cxcl1	m CD4N	Mirza et al. (2011)	*Csf2ra, Cxcl2*	m CD8N	Mirza et al. (2011)
Cxcl10, Cxcl12	m CD8N	Mirza et al. (2011)	*Csf3r*	m s-CD8N	Mirza et al. (2011)
CXCR7, CXCR9	h CD4, CD8	Mo et al. (2003)	*CX3CR1*	h CD8CD28-	Lazuardi et al. (2009), Fann et al. (2005)
Flt1	m s-CD8N	Mirza et al. (2011)	*CXCL12*	h CD4	Hernandez-Lopez et al. (2010), Cane et al. (2012)
Ifna1	m CD8N, s-CD4N	Mirza et al. (2011)	*Cxcl9*	m CD8N, s-CD4N	Mirza et al. (2011)
Ifna13	m CD8N	Mirza et al. (2011)	*CXCR3/Cxcr3*	h CD4, CD8, m CD4N, CD8N	Cao et al. (2010), Mirza et al. (2011), Mo et al. (2003)
Ifnab	m CD4N	Mirza et al. (2011)	*CXCR4*	h CD4	Mo et al. (2003), Hernandez-Lopez et al. (2010), Cane et al. (2012)
Il12b, Kit, Pdgfb	m s-CD4N	Mirza et al. (2011)	*FAS, IFNGR1*	h CD8CD28-	Lazuardi et al. (2009)

(Continued)

Table 1 | Continued

Down-regulated			Up-regulated		
Genes	**Subsets**	**Reference**	**Genes**	**Subsets**	**Reference**
Cytokine/chemokine network			**Cytokine/chemokine network**		
Il12rb2	m s-CD8N	Mirza et al. (2011)	*Ifng,* Il10, Il4	m CD4N, CD8N	Mirza et al. (2011)
Il17a, Il6	m s-CD8N	Mirza et al. (2011)	*Il12rb1, Il13, Il5ra*	m CD4N	Mirza et al. (2011)
Il6ra	m CD4N	Mirza et al. (2011)	*Il18r1, Il20ra, Il23r, Il7r*	m s-CD8N	Mirza et al. (2011)
Kit	m s-CD8N	Mirza et al. (2011)	*Il18rap, Il1b*	m CD8N	Mirza et al. (2011)
Ppbp	m CD4N	Mirza et al. (2011)	*Il19, Il24, Il2ra, Inhba*	m s-CD4N	Mirza et al. (2011)
Tnfrsf21	m s-CD8N	Mirza et al. (2011)	*Il1r2*	m CD4N, s-CD4N, CD8N	Mirza et al. (2011)
Tnfsf11a	m CD8N	Mirza et al. (2011)	*Il2, Il22, Il23a*	m s-CD8N	Mirza et al. (2011)
Tpo	m s-CD8N	Mirza et al. (2011)	*Ltbr*	m CD4N, CD8N	Mirza et al. (2011)
Xcr1	m CD4N, s-CD8N	Mirza et al. (2011)	*Tnfrsf13c*	m CD4N	Mirza et al. (2011)
Natural killer cell-mediated cytotoxicity			*Tnfsf13b, Tnfsf4, Tnfrsf17*	m CD4N, CD8N	Mirza et al. (2011)
Ifna1	m s-CD4N	Mirza et al. (2011)			
Klrd1, Klrk1	m s-CD4N	Mirza et al. (2011)	**Natural killer cell-mediated cytotoxicity**		
Nfatc4	m s-CD4N	Mirza et al. (2011)	*CD244*	h CD8	Cao et al. (2010)
			FAS	h CD8CD28-	Lazuardi et al. (2009)
Toll like receptor signaling pathway			*GZMB*	h CD8CD28-	Lazuardi et al. (2009)
Cd86	m CD8N	Mirza et al. (2011)	*IFNGR1*	h CD8CD28-	Lazuardi et al. (2009)
Cxcl10	m CD8N	Mirza et al. (2011)	*ITGB2*	h CD8	Cao et al. (2010)
Ifna1, Ifna13	m CD8N	Mirza et al. (2011)	*KIR2DL3, KIR2DL4*	h CD4N	Czesnikiewicz-Guzik et al. (2008)
Ifnab	m CD4N	Mirza et al. (2011)	*KIR2DL5A*	h CD8CD28-	Lazuardi et al. (2009)
Il6	m s-CD8N	Mirza et al. (2011)	*KIR2DS5*	h CD8CD28-	Lazuardi et al. (2009)
Irf7	m CD4N, s-CD8N	Mirza et al. (2011)	*KIR3DL1*	h CD8CD28-	Lazuardi et al. (2009)
Lbp	m CD4N	Mirza et al. (2011)	*KIR3DL2*	h CD4N, CD8CD28-	Czesnikiewicz-Guzik et al. (2008), Lazuardi et al. (2009)
Pik3cd	m CD4N	Mirza et al. (2011)	*KLRC3*	h CD8CD28-	Lazuardi et al. (2009)
Rela	m CD4N	Mirza et al. (2011)	*KLRD1*	h CD8	Cao et al. (2010)
Tlr5, Tlr7	m s-CD8N	Mirza et al. (2011)	*KLRG1*	h CD8	Henson and Akbar (2009)
			MAPK1	h CD8CD28-	Lazuardi et al. (2009)
Cell cycle regulator			*NKG2D*	h CD4	Alonso-Arias et al. (2011)
CCND2, CCNH	h CD8	Cao et al. (2010)	*PRF1*	h CD8CD28-	Lazuardi et al. (2009)
FZR1	h CD8	Cao et al. (2010)	*PTPN6*	h CD8	Cao et al. (2010)
MAD1L1	h CD8	Cao et al. (2010)	*VAV3*	h CD8CD28-	Lazuardi et al. (2009)
MYC	h CD8	Cao et al. (2010)			
RAD21	h CD8	Cao et al. (2010)	**MAPK signaling pathway**		
RB1, RBL2	h CD8	Cao et al. (2010)	*DUSP2, DUSP5,DUSP6, DUSP10*	h CD8	Cao et al. (2010)
SMAD2, SMAD4	h CD8	Cao et al. (2010)	*DUSP4*	h CD8CD28-	Lazuardi et al. (2009)
SMC3	h CD8	Cao et al. (2010)	*FAS*	h CD8CD28-	Lazuardi et al. (2009)
STAG1	h CD8	Cao et al. (2010)	*FGFR1*	h CD8	Cao et al. (2010)
TFDP2	h CD8	Cao et al. (2010)	*FLNA*	h CD8	Cao et al. (2010)
YWHAE, YWHAZ	h CD8	Cao et al. (2010)	*GNA12*	h CD8	Cao et al. (2010)
			MAP3K1	h CD8CD28-	Lazuardi et al. (2009)
Insulin signaling pathway			*MAP3K5*	h CD8	Cao et al. (2010)
AKT3	h CD8	Cao et al. (2010)	*MAP3K8*	h CD8	Cao et al. (2010)
BRAF	h CD8	Cao et al. (2010)	*MAP4K1*	h CD8	Cao et al. (2010)
FLOT2	h CD8	Cao et al. (2010)	*MAPK1*	h CD8CD28-	Lazuardi et al. (2009)
FOXO1	h CD8	Cao et al. (2010)	*NR4A1*	h CD8	Cao et al. (2010)
GRB2	h CD8CD28-	Lazuardi et al. (2009)	*RPS6KA5*	h CD8	Cao et al. (2010)

(Continued)

Table 1 | Continued

Down-regulated			Up-regulated		
Genes	**Subsets**	**Reference**	**Genes**	**Subsets**	**Reference**
Insulin signaling pathway			**MAPK signaling pathway**		
IRS1, IRS2	h CD8	Cao et al. (2010)	*RRAS2*	h CD8CD28-	Lazuardi et al. (2009)
MAPK1	h CD8	Cao et al. (2010)			
PDE3B	h CD8	Cao et al. (2010)	**Toll like receptor signaling pathway**		
PIK3CD	h CD8CD28-	Lazuardi et al. (2009)	*Ccl3*	m CD4N	Mirza et al. (2011)
PIK3R2	h CD8	Cao et al. (2010)	*Ccl5*	m CD4N	Mirza et al. (2011)
PRKACB, PRKAR1A	h CD8	Cao et al. (2010)	*Cd86*	m CD4N	Mirza et al. (2011)
RPS6KB1	h CD8	Cao et al. (2010)	*Cxcl10*	m CD4N	Mirza et al. (2011)
SOS2	h CD8	Cao et al. (2010)	*Tlr4*	m CD4N	Mirza et al. (2011)

h, Human; m, mouse; CD4N, CD4 naïve T cells; CD8N, CD8 naïve T cells; s-, TCR activated.

the categories of transcription regulators and enzymes, and were mostly involved with cell growth, proliferation, and death (Lustig et al., 2007). One of the more notable transcription regulators that decreased expression with age was the glucocorticoid receptor, which has been implicated in T cell development and survival within the thymus. Also detected was an increased expression of several heat shock proteins, which are necessary for cell survival.

A subsequent study focused specifically on thymocytes, demonstrated age-associated changes in the expression of genes involved in TCR signaling, antigen presentation, and lymphocyte development and function (Lustig et al., 2009). These age-related changes included increased expression of CXCR4 and CXCR6, as well as a decrease in CCL25. Another key change was an increase in Vav1, which is important for T cell development and activation. Genes involved with cell function included changes in various ribosomal proteins, which could indicate changes in translational activity with age, and a decrease in S100A, a calcium-binding protein involved in various cell functions. Interestingly, increased gene expression was consistent in genes associated with cancer.

An unexpected and significant difference was found in gene expression analyses of the immunoglobulin gene family from the thymus of old and young mice (Lustig et al., 2007). This observation has been noted in various gene expression studies (Schumacher et al., 2008; Swindell, 2009), even though some of these studies focused specifically on thymocytes or splenic T cells. Further analysis revealed the apparent presence of immunoglobulin on the cell surfaces of thymocytes as well as splenic T cells, but not intracellularly, indicating this might be an autoreactive phenomenon occurring with age (Lustig et al., 2009).

Not surprisingly, the age-associated change of the environment in which each T cell is located has an effect on the gene expression profile of thymocytes. Griffith et al. (2012) demonstrated that the most profound age-associated changes in gene expression (up to 30% of the genes) were found in the thymic stroma, and such changes affected gene expression changes in all subsets of thymocytes, which only had expression changes in about 3–4% of their total genes. However, the small proportion of changes within the developing thymocytes could be the most critical ones involved in T cell aging.

MATURE T CELLS IN THE PERIPHERY

Changes in gene expression with age, specifically within the murine spleen, mirrored the overall trends of other organs; these included an increase in immune response, stress, and apoptosis related genes, along with a decrease in genes involved in cell growth, energy utilization, and lipid and carbohydrate metabolism (Schumacher et al., 2008). An interesting observation was that many of these expression patterns were similar between mouse strains with shortened, normal, or longer life spans, but the changes occurred at a pace relative to the expected life span for each strain.

Sole focus on gene expression analyses of T cells purified from mouse spleens showed critical gene expression changes in the old mice that may be responsible for their alterations in T cell function (Han et al., 2006). The authors found that old T cells express higher *Socs3* and lower growth factor independent 1 (*Gfi-1*) compared to young T cells, which could contribute to the age-associated decline in proliferation. They also found the increase of apoptosis in old T cells could be explained by higher *Gadd45* and lower *Bcl2* in old T cells compared to young T cells. In addition, the authors observed an age-associated increase in many immunoglobulin-associated genes, as well as genes associated with innate immunity, such as lysozyme M and myeloperoxidase; moreover, this study showed a decrease in TCR-associated genes such as TCR-beta and various isoforms of *Cd3*, indicating that both the innate and adaptive arms of the immune system are involved in aging trends. Other categories of genes affected included structural proteins, calcium-binding proteins, and genes involved in electron transport.

Three basic patterns of changes in gene expression emerged from a study of T cells in peripheral blood of various aged adults: first, some genes increased in middle age then decreased; second, genes that decreased in middle age then increased, and lastly, some genes decreased with age (Remondini et al., 2010). Interestingly, they found no group of genes that only increased with age. Global clustering analysis revealed that the gene expression pattern in "successfully aged" adults (older than 90 years) more closely matched the patterns of the younger groups. Most of the genes involved in T cell signaling were in the group which increased in middle age, then decreased. Although it is difficult

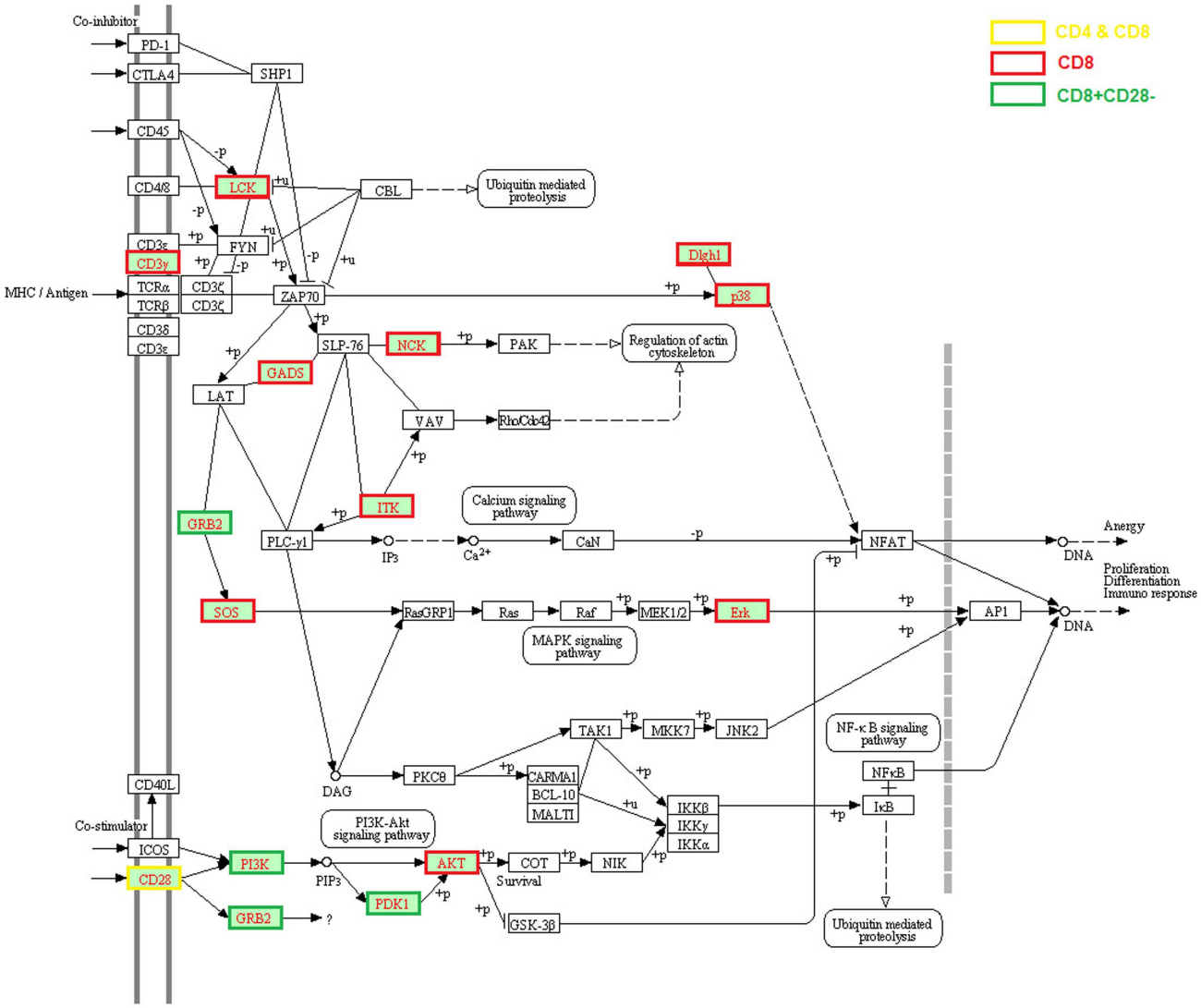

FIGURE 1 | Age-related alteration of gene expression in the TCR signaling pathway. The age-related genes were assembled from the literature based on the significant changes reported by the original articles which were applied for all figures (Chen et al., 2003; Mo et al., 2003; Yung and Mo, 2003; Fann et al., 2005; Czesnikiewicz-Guzik et al., 2008; Henson and Akbar, 2009; Lazuardi et al., 2009; Weng et al., 2009; Cao et al., 2010; Hernandez-Lopez et al., 2010; Alonso-Arias et al., 2011; Mirza et al., 2011; Cane et al., 2012). Three-hundred five up-regulated and 472 down-regulated genes in aged human T cells including CD4 and CD8 T cells were analyzed using KEGG analysis in the WEB-based GEne SeT AnaLysis Toolkit (WebGestalt). The entire TCR signaling pathway is included and the molecules with lower gene expression compared with young are highlighted in red text and different border color indicating specific T cell subsets. The altered expressed genes in the TCR signaling pathway include *CD3G, LCK, GRAP2* (encode GADS), *GRB2, ITK, NCK,* MAPK signaling pathway molecules *SOS2* and *MAPK1* (encode Erk), *CD28,* PI3K-AKT signaling pathway molecules *PI3K, PDK1,* and *AKT,* DLG1 (encode DLGH1) and *MAPK14* (encode p38).

to strictly compare the patterns between the human and mouse studies with age, some common genes are evident. Not surprisingly, these genes are associated with TCR signaling, cytokines and their receptors, and cancer-related genes. Other common genes between human and mouse that changed expression with age included those from more general signaling pathways, such as Jak/Stat, PPAR, and mTOR signaling. This agrees with the observation that changes in gene expression occurring within aging T cells mirror changes occurring in other cell types throughout the body.

Genome-wide analyses of transcription alterations identified the change associated with aging as a whole of either tissue or of total T cells. As lymphoid tissue consists of different types of cells (T and B cells, stromal cells, etc.), total T cells also consist of different subsets (naïve, effector, and memory), it is unknown whether the identified age-associated changes are a result of aging in all types of cells or in a specific type of cell or a subset of a cell type. Therefore, it is necessary to further analyze the gene expression changes with age in defined a defined cell type, such as T cells, and their subsets.

GENE EXPRESSION CHANGES OF T CELL SUBSETS IN AGED SUBJECTS

HUMAN CD4 T CELLS

Although a global comparison of gene expression in naïve CD4 T cells with age is not available, a study comparing global gene expression of memory CD4 T cells ($CD45RA^-$) between young (20–30 years old) and old (70–75 years old) human subjects show that the overall gene expression profiles of CD4 memory T cells are similar (Czesnikiewicz-Guzik et al., 2008). Based on the fold change (> or <1.25), 536 genes were identified as age-related, including up-regulated genes such as HLA region genes, *CCR4*, *CCR8*, *CD26*, *CD58*, *IL17Rb*, and *LAIT1*, and down-regulated genes such as *CD28*, *CCR6*, *KLRB1*, and *KLRC2*.

Another recent study compared gene expression of CD4 T cells between young and old donors after *in vitro* stimulation (Bektas et al., 2013). This study focused on examining changes in TCR-inducible gene expression via the NF-κB pathway, and found that most NF-κB target genes are not induced in a sustained manner in CD4 T cells from older compared to younger donors. Those genes that failed to exhibit sustained expression include *CXCL10*, *TNFAIP2*, *CCL2*, and *CXCL5*. On the other hand, a subset of NF-κB target genes such as *CXCL1*, *CXCL11*, *IL1a*, and *IL6*, continue to be up-regulated even in the absence of NF-κB induction. *IL1* and *IL6* are associated with a chronic pro-inflammatory state in the elderly and are dysregulated in CD4 T cells from old donors. In addition, the authors identified some immune function-related genes that were highly induced after 2 h of stimulation in old donors, including cell surface receptor and signaling molecules (*SAMD4A*, *CD83*, *KCTD12*, *SOCS1*, and *CTLA4*) and effector molecules (*GZMH*). Interestingly, some of the most noteworthy age-associated changes in murine models, such as up-regulation of the chemokines CCL3 and CCL4 and the cytokine IFN-γ, were not found in CD4 T cells of old donors in this study (Chen et al., 2003; Han et al., 2006; Mirza et al., 2011). Further studies are needed to determine the similarities and differences between mouse and human in age-associated changes in T cells.

The finding of low expression of CD28 in memory CD4 T cells from aged donors leads to further comparison of gene expression between $CD28^+$ and $CD28^-$ memory CD4 T cell subsets in selected old donors (65–75 years old) (Czesnikiewicz-Guzik et al., 2008). CD28 is a co-stimulatory receptor that offers a critical signal for complete T cell activation during T cell receptor initiation. Absence of the CD28 signal causes partial T cell activation or even anergy. Strikingly, it was found that the difference in gene expression between $CD28^+$ and $CD28^-$ memory CD4 T cells was greater than that of memory CD4 T cells between young and old subjects. $CD28^-$ memory CD4 T cells express high levels of several KIR genes and some surface receptors genes (*CD70*, *CD74*, and *CCR5*), but low levels of *TCRB*, *CD27*, *IL7R*, *IL9R*, and *ICAM2*. Although loss of CD28 protein expression with aging is mainly found in CD8 T cells, this study shows that reduced expression of CD28 mRNA is also found in CD4 T cells with aging.

HUMAN CD8 T CELLS

A global comparative analysis of gene expression in human CD8 T cells isolated from young (23–27 year old) and old (65–81 year old) adults reported the identification of a total of 754 genes (505 down-regulated and 258 up-regulated) using an expression fold change more than 1.5 (Cao et al., 2010). The functions of these genes are involved in the regulation of transcription, protein phosphorylation, ubiquitination, intracellular transport, immune response, and apoptosis. Intriguingly, the down-regulated genes in CD8 T cells from old donors affect multiple stages of gene transcription: chromatin structure, transcription initiation, elongation, RNA stabilization, and protein translation and translocation; this suggests the impaired regulation of these fundamental molecular events might be responsible for the declined immune function in the elderly. In addition, genes related to the signaling pathways are also found down-regulated in CD8 T cells from old donors, such as T cell receptor signaling, IL-2 signaling, IGF-1 signaling, insulin receptor signaling, JAK/Stat signaling, MAPK/JNK signaling, PI3K/AKT signaling, Wnt/β-catenin signaling, and ERK/MAPK signaling. On the other hand, oxidative phosphorylation and apoptosis signaling are up-regulated in CD8 T cells from old donors. These alterations of gene expression provide a mechanism for the functional decline of the immune response in the elderly.

$CD28^-$ CD8 T CELLS

Accumulation of $CD28^-$ CD8 T cells is one of the hallmarks of human aging. The $CD28^-$ population is considered as aged CD8 T cells in humans (Weng et al., 2009). The study of $CD28^+$ and $CD28^-$ memory CD8 T cells ($CD45RA^-$) from young and middle aged donors (25–45 years old) identified <100 altered genes/transcripts based on the criteria of the false discovery rate (FDR) <0.05 and fold change >2 (Fann et al., 2005). Among these 45 genes,$CD28^-$ CD8 T cells highly expressed natural killer cell receptors, *GZMB*, *PRF1*, *FASLG*, *IL12A*, *IL12*, *CCL4*, *CX3CR1*, and *CMKLR1*. In contrast, they had low expression of *IL3*, *IL23A*, *IL7R*, and *IL12RB2*.

A subsequent study comparing $CD28^+$ and $CD28^-$ CD8 T cells from young and old donors found that gene expression patterns of $CD28^-$ CD8 T cells from young and old subjects were similar (Lazuardi et al., 2009). In contrast, there were noticeable differences in gene expression of $CD28^+$ CD8 T cells between young and old subjects. From the gene expression similarities, the levels from $CD28^+$ CD8 T cells in old donors were located between young $CD28^+$ and $CD28^-$ (young and old) CD8 T cells. The highly expressed genes identified by Fann et al. (2005) in $CD28^-$ CD8 T cells were also found in this study. In addition, high expression of *PIK3CD*, *MAL*, *IL6R*, *CD62L*, and *CCR7* were found in $CD28^+$ CD8 T cells of young subjects whereas high expression of *GATA3*, *BIRC3*, *FAS*, *RGS1*, and *MAP3K1* were found in $CD28^+$ CD8 T cells of old subjects. This study reveals that $CD28^-$ CD8 T cells have a common pattern of gene expression regardless of whether they are isolated from young or old subjects. However, whether the difference in gene expression in $CD28^+$ CD8 T cells between young and old subjects is reflecting the impact of aging or the heterogeneous nature (different percentages of naïve and memory T cells) of $CD28^+$ CD8 T cells in young and old subjects remains to be determined.

Interestingly, similarly reduced expression of some co-stimulatory molecules including CD28, NK cell receptor, and chemokine receptors were found in both CD4 and CD8 $CD28^-$

T cells with some differences in the expression of *CD40L*, *KLRD1*, *KLRG1*, and *KLRK1* (Czesnikiewicz-Guzik et al., 2008). However, loss of CD28 surface expression was only prominent in CD8 T cells with aging. Further studies will be needed to compare the functional alteration of CD4 and CD8 subsets with aging.

MOUSE CD4 AND CD8 T CELLS

Studies in mouse T cells suggest that aging has a more profound detrimental impact on naïve compared to memory T cells (Ponnappan and Ponnappan, 2011). Gene expression comparison of naïve CD4 and CD8 T cells between young (3–4 months) and old (20 months) mice identified over 2000 age-associated genes in CD4 and CD8 T cells using a twofold change as the cutoff (Mirza et al., 2011). The functions of these genes are broad and involved in multiple cellular functions such as cell growth, cell cycle, cell death, inflammatory response, and cell trafficking. Some of those identified genes exhibited similar changes in both CD4 and CD8 T cells from old mice, such as *Anxa1*, *Ccl1*, *Ccl5*, *Ccr2*, *Il4*, *Havcr2*, and *Ltb4r1*. The enhanced expression of chemokines and chemokine receptors in aged mouse T cells was also observed in an earlier study (Mo et al., 2003). Some gene alterations are specific to either CD4 or CD8 T cells, such as *Jak3*, *Socs1*, and *Pi3kcd* in CD4, and *Penk*, *Nfc2*, and *Irak3* in CD8 T cells of old mice.

Gene expression analysis of *in vitro* stimulated naïve T cells between young and old mice further revealed some defects in T cell signaling, cytokine production, and differentiation into Th2 cells. Gata3 and c-Maf were found up-regulated post-activation in naïve aged CD4 T cells, which may be responsible for the imbalanced Th2 immune response in the elderly. *Ccl5* and *Tlt4* are up-regulated in aged CD8 T cells from pre- to post-activation, while *Ccl1*, *Ccl9*, *Il7r*, and other genes are only up-regulated post-activation. Genes such as *Tnfsf14* and *S100a9* are up-regulated in aged CD8 T cells only in pre- and 12 h post-activation. In CD4 aged T cells, *Ccl5* and *Tlr4* are up-regulated at all timepoints. *Rorc* is not differentially expressed before activation, however its expression was decreased in aged CD4 T cells compared with young ones after TCR activation. In the pattern of lower expression in the elderly at both pre- and post-activation, many genes associated with microtubules, cell cycle replication, migration, and other functions were found in both CD4 and CD8 naïve T cells. Taken together, the highly expressed and up-regulated specific cytokines, chemokines, and their receptors in aged naïve T cells indicate that naïve CD4 and CD8 T cells in old mice have a pro-inflammatory status.

GENE NETWORKS AND PATHWAYS ALTERED IN AGED T CELLS

A global view of gene expression profiles provides a means for examining the gene networks and signaling pathways of a defined biological process and function. Among the genes that were transcriptionally altered in aged T cells, substantial numbers of them are associated with basic cellular and molecular biological processes such as cell growth and proliferation, cell death and apoptosis, energy utilization and metabolism, and transcription regulation, which were also reported in other types of cells with aging (Kuilman et al., 2010). In **Table 1**, we combine the altered expressed genes from the literature based on their original selection and listed seven functional categories. We will focus on the molecular basis of three immune function-related alterations in T cells with aging in this review.

REDUCED EXPRESSION OF TCR AND CO-STIMULATION SIGNALING ASSOCIATED GENES

Impaired TCR signal transduction was observed in aged mice and humans two decades ago (Utsuyama et al., 1993; Whisler et al., 1996). Genes associated with several TCR signaling pathways were found to be expressed lower in aged hosts on both genome-scale (**Table 1**; **Figure 1**) and individual studies. First, *CD3G* expression was lower in human CD8 T cells (Cao et al., 2010), and phosphorylated Cd3z was decreased in aged mouse CD4 T cells, and almost absent in 18-month old mice (Garcia and Miller, 1997). Second, significantly reduced expression of CD28, particularly in CD8 T cells, was widely reported in humans with aging (Weng et al., 2009). Lower expression of CD28 was also found in human CD4 memory cells but to a much lower degree compared to CD8 T cells (Czesnikiewicz-Guzik et al., 2008). Intriguingly, several age-associated defects in TCR signaling occur at the post-translational level without obvious changes in the level of gene expression, such as: reduced phosphorylation of phospholipase Cγ1 (PLCγ1), JNK, second messengers such as inositol trisphosphate (IP3) and diacylglycerol (DAG), reduced influx of $Ca2^{+}$ (Utsuyama et al., 1997; Kirk et al., 1999) in T cells of aged mice, decreased LCK activity (Fulop et al., 1999), and ERK phosphorylation in T cells of aged humans (Li et al., 2012). The consequence of these age-associated changes in TCR signaling is observed at the transcriptional levels of their downstream targets, such as the decreased expression of *SOS*, *GADS*, *NKC*, *ITK*, *PI3K*, *PDK2*, *AKT*, *Erk*, *Dlgh1*, and *p38* (**Table 1**) (Utsuyama et al., 1993; Whisler et al., 1996). Certainly, more studies are needed to dissect the primary causes of the impaired expression of these age-related genes and their functional consequences.

ALTERATION OF CYTOKINES/CHEMOKINES NETWORK

Alteration of expression of various cytokines, chemokines, and their receptors in CD4 or CD8 T cells from old humans and mice are well documented (**Table 1**; **Figure 2**). Unlike the age-associated TCR signaling pathway, enhanced expression of various cytokines of the TNF and TGF-β families, chemokines (*CCL3*, *CCL4*, *CCL5*, *CXCL12*), and chemokine receptors (*CCR1*, *CCR2*, *CCR3*, *CCR4*, *CCR5*, *CCR6*, *CCR8*, *CXCR2*, *CXCR3*, *CXC4*, *CXCR5*) were reported in CD4 or CD8 T cells from old humans and mice (Mo et al., 2003; Yung and Mo, 2003; Fann et al., 2005; Lazuardi et al., 2009; Cane et al., 2012).

Age-related enhanced expression of cytokines and their receptors include *IL15*, *IL17RB*, TNF family members (*TNFSF7* and *TNFSF14*), and receptors (*FAS*, *TNFRSF18*, and *TNFSF1B*), IFN family receptor *IFNGR1*, and TGF-β family receptors *ACVR2A*. Most of these genes are involved in the inflammatory response, which could be the result of an increased inflammatory state during some chronic infections. Chemokine *CXCL12* and its receptor *CXCR4* are involved in regulating thymocyte development and differentiation from DN ($CD44^{-}CD25^{+}$, $CD44^{-}CD25^{-}$) to DP stages (Ara et al., 2003), but their roles in aged T

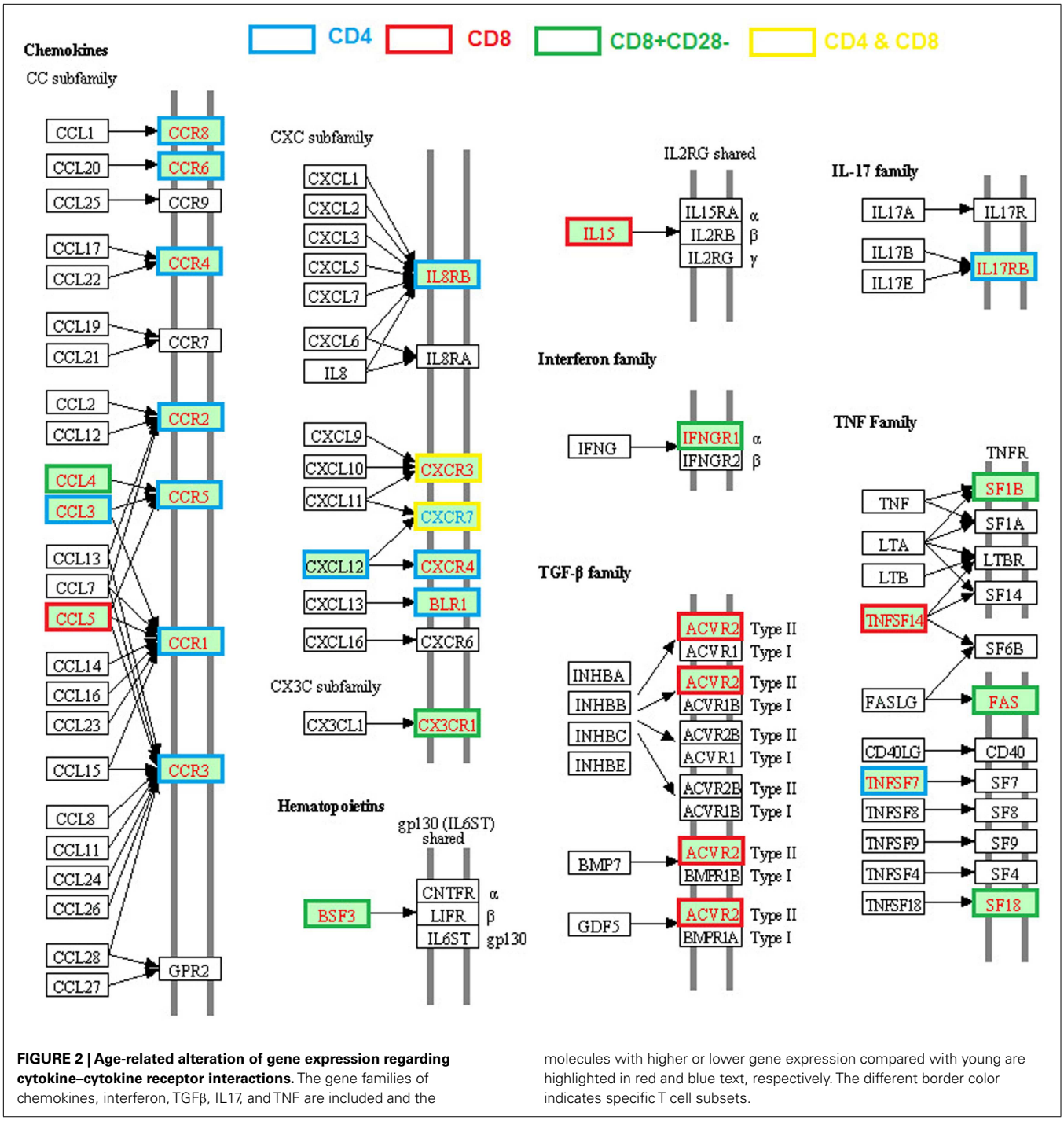

FIGURE 2 | Age-related alteration of gene expression regarding cytokine–cytokine receptor interactions. The gene families of chemokines, interferon, TGFβ, IL17, and TNF are included and the molecules with higher or lower gene expression compared with young are highlighted in red and blue text, respectively. The different border color indicates specific T cell subsets.

cells have not been determined. In general, the high levels of expression of chemokines/chemokine receptors lead to an enhanced T cell chemotactic response and different patterns of tissue migration/residency of T cells in the old subjects (Cane et al., 2012). It is unclear whether such changes are a result of an increased inflammatory state and/or some chronic infection. Regardless, it is apparent this age-related change in T cell chemokine expression has an important functional consequence.

ENHANCED GENE EXPRESSION RELATED TO NK CELLS FUNCTION

Enhanced expression of a cluster of genes associated with NK cell function is one of the most profound age-related changes in T cells, especially in human CD28$^-$ CD8 T cells (**Table 1**; **Figure 3**); it includes killer cell lectin-like receptors (*KLR*, *KLRC3*, *KLRD1*, and *KLRG1*), killer cell immunoglobulin-like receptors (*KIR2DL3*, *KIR2DL4*, *KIRDL5A*, *KIR2DS5*, *KIR3DL1*, and *KIR3DL2*), and *CD244*. Although these genes were primarily expressed in NK cells, aged memory, or CD28$^-$ T cells appear to increase their

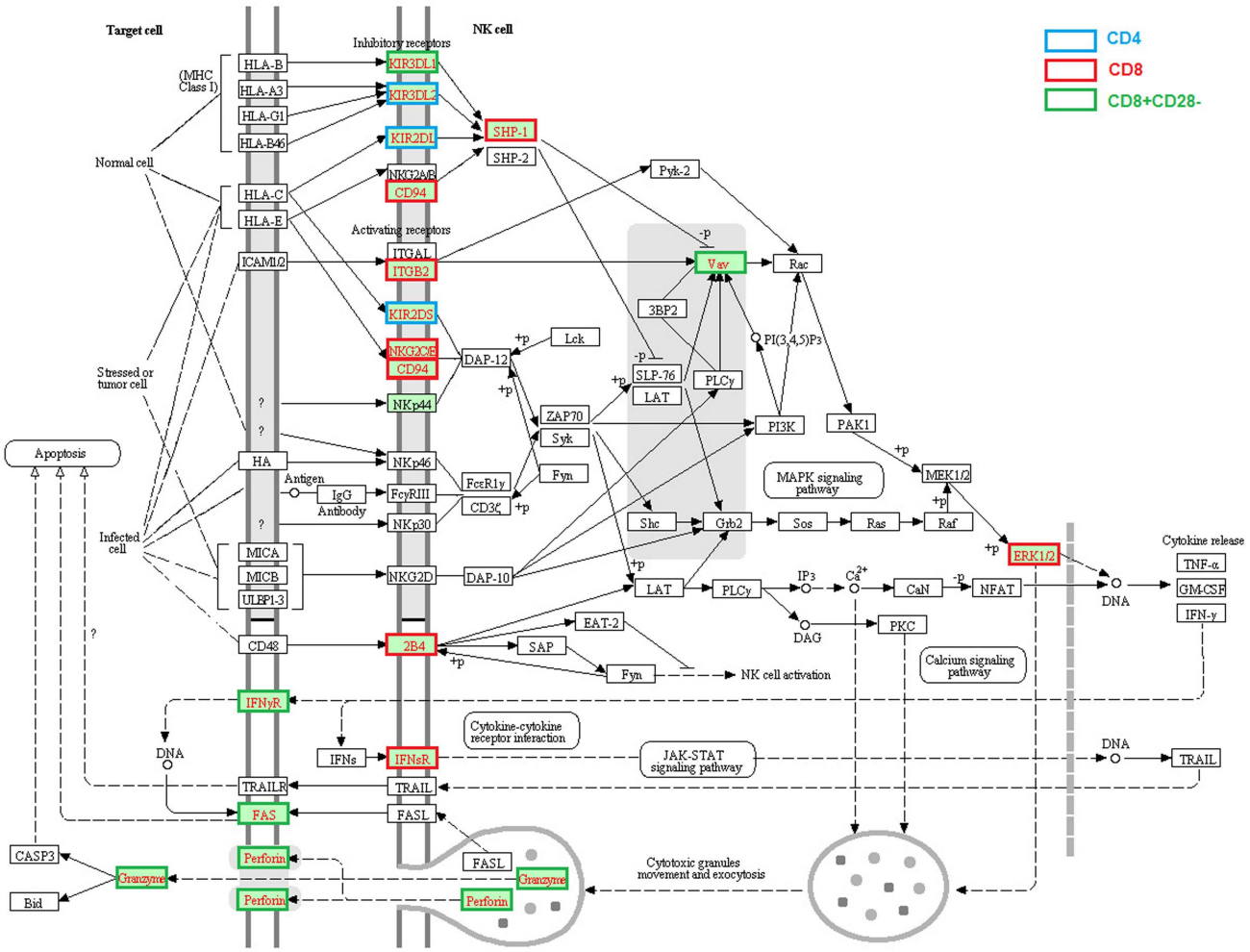

FIGURE 3 | Age-related alteration of gene expression regarding NK cell-mediated cytotoxicity. NK cell-mediated cytotoxicity was present and the molecules with higher gene expression compared with young were highlighted in red text. The different border color indicates specific T cell subsets. The altered expressed genes associated with NK mediated cytotoxicity in aged T cells include receptors *KIR3DL1*, *KIR3DL2*, *KIR2DL*, *KIR2DS*, *KLRD1* (encode CD94), *ITGB2*, *NKG2C/E*, *CD244* (encode 2B4), *IFNGR*, *IFNSR*, and *FAS*, signaling molecules *PTPN6* (*SHP-1*), *VAV* and *MARK1* (ERK1), and effector molecules *GZMB* and *PRF1* (encode Perforin).

expression. Since CD28$^-$ cells are a unique subset of T cells in older humans, the acquired expression of NK cell receptors and some cytotoxic molecules (*GZMB* and *PRF1*) might reflect chronic infection and an increased inflammatory state (Fann et al., 2005). Another possibility is that gaining expression of NK receptors, especially KIRs, might be a compensation for a shrinking TCR repertoire in aged hosts (Abedin et al., 2005; Vallejo, 2006) since KIR recognizes MHC-I/Ag complex with lower affinity compared with that of TCR and was found only in the later stage of proliferative lifespan of oligoclonal T cells (Snyder et al., 2004). If this is true, aged T cells with a high level of NK cell receptors can function in both an MHC-restricted and non-restricted manner.

CONCLUSION

In the past decade, we have learned enormously from the global gene expression analysis of aged T cells. Alteration of several gene networks and pathways that are associated with aged T cells have now been identified in humans and mice, including T cell receptor and activation-related molecules, alteration of chemokine/chemokine receptor expression, gain of NK cell receptors and functions, and etc. Whether these identified alterations of gene expressions occur in all cells or in subsets of defined T cell populations remains to be determined. It is sufficient to say these alterations contribute to the overall decline of T cell function.

It is worth mentioning that not all microarray data, especially for some of the early studies, have the highest quality. The shortcomings include: incomplete gene list of the array used, insufficient number of biological repeats, and lack of standardized selection criteria of significantly altered genes. It is necessary in future studies to use the whole genome microarray chips with better designed experiments, such as using defined cell populations, standardized stimulation conditions and time, sufficient numbers of the biological repeats, and proper statistical tools for selection of significantly altered genes. In addition, future studies will need to dissect which

specific stage of T cell development and differentiation these changes occurred. The precise relationship of gene expression change and function is essential for a better understanding these age-associated alterations in T cell functions.

The current commercial whole genome microarray chips offered by Agilent, Affymetrix, and Illumina have greatly improved from a few years ago, and become a standard method for global gene expression analysis. However, some shortcomings are also emerging with recent developments. Most of the microarray chips for gene expression analysis do not include miRNA and lncRNA, both of which serve critical biological functions and in which some alterations with aging and cell differentiation were recently reviewed (Gorospe and Abdelmohsen, 2011; Guttman et al., 2011). These microarray chips also do not provide information on different splicing isoforms of transcribed genes; thus, global transcriptional assessment by the traditional microarray method does not offer complete transcriptional information. RNA-Seq, a relatively new method based on next-generation sequencing, can readily address these shortcomings and provide more complete transcriptional analysis.

Identification of the alteration of several gene networks and pathways that are associated with aged T cells paves a way for further functional assessment and potential clinical interventions. Although the general approach such as supplementation of Vitamin E in mice showed some promising results, such as increased expression of genes involved in cell cycle regulation (*Ccnb2*, *Cdc2*, and *Cdc6*) and higher up-regulation of expression in old T cells following stimulation, a more focused approach targeting specific genes and gene networks that are altered in aged T cells may offer a more direct and specific treatment. With the advancement of the tools that can be used in manipulating gene expression in cells, experimental determination and verification of these specific genes/gene networks based therapeutic approach is feasible and will lead to the development of novel drugs that could postpone or even reverse the changes associated with aging in T cells.

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When aging reaches CD4+ T-cells: phenotypic and functional changes

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Beyond midlife, the immune system shows aging features and its defensive capability becomes impaired, by a process known as immunosenescence that involves many changes in the innate and adaptive responses. Innate immunity seems to be better preserved globally, while the adaptive immune response exhibits profound age-dependent modifications. Elderly people display a decline in numbers of naïve T-cells in peripheral blood and lymphoid tissues, while, in contrast, their proportion of highly differentiated effector and memory T-cells, such as the CD28^{null} T-cells, increases markedly. Naïve and memory CD4+ T-cells constitute a highly dynamic system with constant homeostatic and antigen-driven proliferation, influx, and loss of T-cells. Thymic activity dwindles with age and essentially ceases in the later decades of life, severely constraining the generation of new T-cells. Homeostatic control mechanisms are very effective at maintaining a large and diverse subset of naïve CD4+ T-cells throughout life, but although later than in CD8 + T-cell compartment, these mechanisms ultimately fail with age.

Keywords: immunosenescence, T-cells, IL-15, inflammation, CMV, NKRs

INTRODUCTION

Throughout life the aging of the immune system causes impairment of its defense capability, in a process known as immunosenescence. The aging process seems to alter the two branches of the immune system, the innate and the adaptive, in different ways. While the adaptive immune response undergoes profound age-dependent modifications (Haynes and Maue, 2009), innate immunity seems to be better preserved globally (Dace and Apte, 2008; Le Garff-Tavernier et al., 2010). The thymus, the development site of T-cells, atrophies with age (Dorshkind et al., 2009), with a direct impact on the proportions of naïve and memory T-cells. In aged animals and humans, the frequency of naïve CD4+ T-cells decreases, whereas the frequency of memory CD4+ T-cells increases (Nikolich-Zugich, 2005). Naïve and memory CD4+ T-cells are clearly distinct populations with unique cellular characteristics. Thus, any age-associated changes in CD4+ T-cell function including proliferation and cytokine production could be secondary to the alteration in the frequency of naïve and memory T-cells.

Despite CD4+ T-cells are more resistant to age-related phenotypic and functional changes than CD8+ T-cells (Weinberger et al., 2007), a progressive increase in the percentage of CD4+ T-cells that lack CD28 expression is common with increasing age in healthy individuals (Goronzy et al., 2007; Czesnikiewicz-Guzik et al., 2008) and in patients with chronic infections and autoimmune diseases (Fletcher et al., 2005; Thewissen et al., 2007). The accumulation of CD4 + CD28^{null} T-cells is partially explained by their reduced susceptibility to apoptosis and their oligoclonal expansions against *Cytomegalovirus* (CMV) and other chronic antigens (Almanzar et al., 2005; Pawelec and Derhovanessian, 2010). Loss of CD28 expression is a hallmark of the age-associated decline of CD4+ T-cell function. CD28 plays pivotal roles during T-cell activation, such as inducing cytokine production (IL-2) and promoting cell proliferation, so the lack of this costimulatory signal during activation results in a partial activation or even an anergic state of T-cells (Godlove et al., 2007). In this way, the accumulation of CD28^{null} T-cells is associated with a reduced overall immune response to pathogens and vaccines in the elderly (Saurwein-Teissl et al., 2002). In this way, CD4 + CD28^{null} T-cells can comprise up to 50% of the total CD4+ T-cell compartment in some individuals older than 65 years (Vallejo et al., 2000). CD4 + CD28^{null} T-cells acquire expression of several receptors commonly associated with natural killer (NK) cells, secrete large amounts of IFN-γ, and express perforin and granzyme B, which confer a cytotoxic capability on the cells (Appay et al., 2002b; van Leeuwen et al., 2004).

CD4+ T-CELL DIFFERENTIATION

Naïve CD4+ T-cells are activated after interaction with the antigen–major histocompatibility complex (MHC) complex and differentiate into specific subtypes depending mainly on the cytokine milieu of the microenvironment. The CD4+ T-cells carry out multiple functions, including activation of the cells of the innate immune system, B-lymphocytes, cytotoxic T-cells, as well as non-immune cells, and also play a critical role in suppressing the immune reaction. With the advent of multiparameter flow cytometry, it has become clear that individual cells can produce effector cytokines in different combinations (Seder et al., 2008), raising the question of whether there is heterogeneity within a lineage or whether each distinct cytokine combination represents a separate

lineage. Continuing studies have identified new subsets of CD4+ T-cells besides the classical T-helper 1 (Th1) and T-helper 2 (Th2) cells. These include T-helper 17 (Th17), T-helper type 22 (Th22), follicular helper T-cell (Tfh), induced T-regulatory cells (iTreg), and the regulatory type 1 cells (Tr1) as well as the potentially distinct T-helper 9 (Th9). The differentiation of the various lineages depends on the complex network of specific cytokine signaling and transcription factors followed by epigenetic modifications.

The differentiation of naïve CD4+ T-cells into effector and memory subsets is one of the most fundamental facets of T-cell-mediated immunity. CD4+ T-cells can be separated into functionally distinct populations using combinations of cell surface markers, such as the tyrosine phosphatase isoform CD45RA+ and the chemokine receptor CCR7 (**Figure 1**). With these markers, we subdivided the T-cells into naïve (NAÏVE; CD45RA + CCR7+), central memory (CM; CD45RA − CCR7+), effector memory (EM; CD45RA − CCR7-), and effector memory RA+ (EMRA; CD45RA + CCR7-) cells (Sallusto et al., 1999). EM is a heterogeneous population, and the staining of two additional markers, CD27 and CD28, has proved useful for identifying the less differentiated EM1 (CD28+ and CD27+) and EM4 (CD28+ and $CD27^{null}$) subsets, and the more differentiated EM3 cells ($CD27^{null}CD28^{null}$) (**Figure 2**). The EMRA subset can be further subdivided into very poorly differentiated pE1 (CD27 + CD28 +) and the most highly differentiated T-cell subset, E ($CD27^{null}CD28^{null}$) (Koch et al., 2008) (**Figure 2**). Differentiating CD4+ T-cells lose expression of CD27 first, then of CD28 in a later phase (Amyes et al., 2003; van Leeuwen et al., 2004). In contrast, CD8+ T-cells lose expression of CD28 first and then of CD27 (Gamadia et al., 2003).

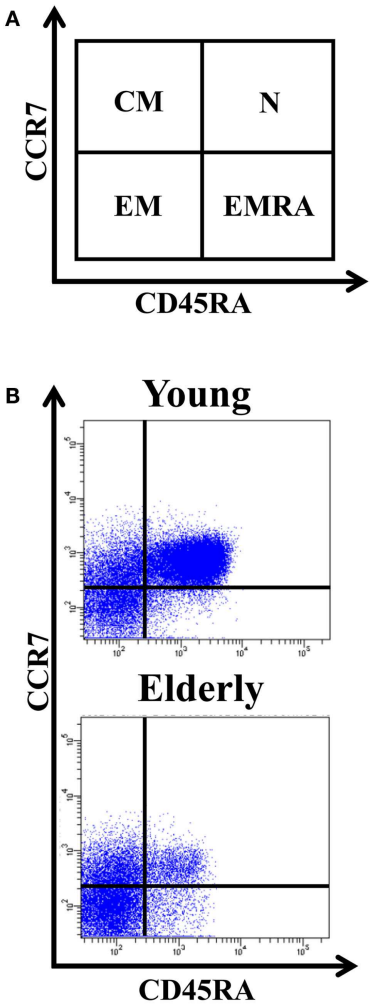

FIGURE 1 | Distribution of CD4+ T-cells into naïve, central memory, effector memory (EM), and effector memory RA (EMRA). **(A)** Schematic model of the T-cells differentiation subsets accordingly to CD4 + 5RA and CCR7 expression. **(B)** Dot-plots representatives of the naïve, CM, EM, and EMRA subsets in young people and elderly subjects into the CD4+ T-cells. Whole blood was stained with anti-CD45RA-FICT, anti-CD8-PE, anti-CD4-PerCP, and anti-CCR7-APC, and 10^5 cells were acquired in each experiment.

NAÏVE CD4+ T-CELLS

Naïve T-cells are characterized by the expression of surface markers CD45RA, CD27, CD28, CD62L, CCR7, and the IL-7 receptor (De Rosa et al., 2001; Swainson et al., 2006). Naïve T-cells exit the thymus following maturation and are enriched for T-cell receptor excision circles (TREC) and express the surface marker CD31 (Kimmig et al., 2002). Naïve T-cells circulate between the blood and the lymphoid tissue driven by cell surface markers CD62L and CCR7 (Sallusto et al., 1999). The number of naïve T-cells in the blood remains relatively constant throughout adult life despite continuous stimulation with foreign antigens and a dramatic reduction in thymic output with age. Although *thymic involution* is a well-known phenomenon, no satisfactory explanation for its existence has been offered (Lynch et al., 2009). Several hypotheses have argued that this age-related change is adaptive rather than detrimental (Aronson, 1991; O'Leary and Hallgren, 1991; Dowling and Hodgkin, 2009). Accordingly, thymic involution may represent a mechanism for how the body is able to achieve the remarkable balancing act of avoiding autoimmunity and maintaining a sufficiently diverse repertoire to combat a large number of potential pathogens. Some possible causes of thymic involution may be the blocking of the rearrangement of T-cell receptor (TCR) genes (Aspinall, 1997), self-peptide MHC-decreased molecules (Lacorazza et al., 1999), and loss of T-cell progenitors (Zoller et al., 2007). The importance of the thymus for developing adequate cellular immunity can be studied in the context of several disease states (associated with thymic ablation or hypoplasia). Young people who were thymectomized within 2 weeks of birth display several immunological alterations, including lower CD4+ or CD8 + T-cell counts, reduced proportions of recent thymic emigrants and naïve cells, accumulation of oligoclonal memory T-cell populations, and increased levels of inflammation markers (Sauce et al., 2009; Zlamy and Prelog, 2009).

Exposure to the cytokine IL-7 and contact with MHC molecules presenting self-peptides through the TCR within secondary lymphoid tissue are both essential for naïve T-cell homeostasis (Brocker, 1997; Tan et al., 2001; den Braber et al., 2012). When these naïve T-cells do encounter antigens on activated dendritic cells (DCs) in central lymphoid organs, they proliferate

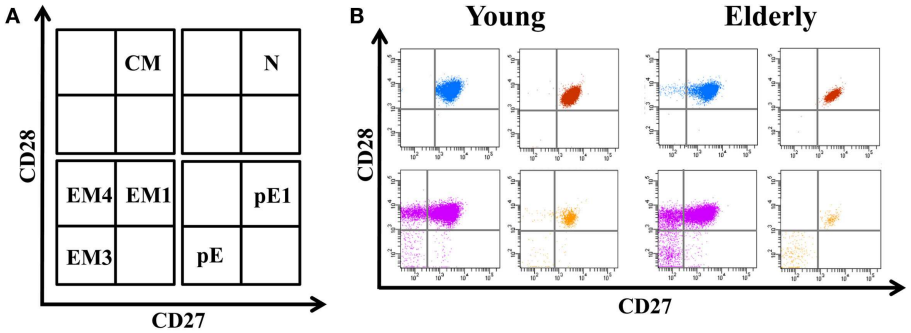

FIGURE 2 | Distribution of EM and EMRA in CD4+ T-cells into subsets defined by CD28 and CD27 expression. **(A)** Schematic model of the EM and EMRA CD4+ T-cells differentiation subsets accordingly to CD27 and CD28 expression. EM T-cells can be divided into EM1 (CD27 + CD28 +), EM2 (CD27 + $CD28^{null}$, only in CD8 + T-cells), EM3 ($CD27^{null}CD28^{null}$), and EM4 ($CD27^{null}$CD28 +). Similarly, EMRA can be divided into pE1 (CD27 + CD28+) and pE2 (CD27 + $CD28^{null}$, only in CD8 T-cells) and E ($CD27^{null}CD28^{null}$). **(B)** Dot-plots representatives of the EM and EMRA subsets in young people and elderly subjects into the CD4+ T-cells.

and differentiate into effector T-cells. When the antigen has been cleared, a contraction phase follows, during which time the number of effector cells declines through apoptosis, leaving behind some survivors that go on to differentiate into memory T-cells.

CENTRAL MEMORY

Human CM are CD45R0+ memory cells that constitutively express CCR7 and CD62L, two receptors that are also characteristic of naïve T-cells, and which are required for cell extravasation through high endothelial venules (HEV) and migration to T-cell areas of secondary lymphoid organs (Campbell et al., 1998; Forster et al., 1999). Homeostatic proliferation ensures the longevity of CM T-cells by inducing cell proliferation in the absence of cellular differentiation or activation. This process is governed mainly by IL-7. Nonetheless, CM T-cells can also be stimulated via engagement of the TCR, leading to proliferation but also activation and differentiation (Bosque et al., 2011). Compared with naïve T-cells, CM have higher sensitivity to antigenic stimulation, are less dependent on costimulation, and upregulate CD40L to a greater extent, thus providing more effective stimulatory feedback to DCs and B cells. Following TCR triggering, CM produce mainly IL-2, but after proliferation they efficiently differentiate into effector cells and produce large amounts of IFN-γ or IL-4.

EFFECTOR MEMORY

Human EM are memory cells that have lost the constitutive expression of CCR7, are heterogeneous for CD62L expression, and display characteristic sets of chemokine receptors and adhesion molecules that are required for homing to inflamed tissues. Compared with CM, EM cells are characterized by a rapid effector function. They produce IFN-γ, IL-4, and IL-5 within hours of antigenic stimulation. The relative proportions of CM and EM in blood vary in the CD4+ and CD8+ T-cells. CM is predominant in CD4+ and EM in CD8+ (Taylor and Jenkins, 2011). Within the tissues, CM cells are enriched in lymph nodes and tonsils, whereas lung, liver, and gut contain greater proportions of EM (Campbell et al., 2001). Increasing evidence indicates the existence of highly heterogeneous functional EM subpopulations: EM1, which is very similar to EM4, and EM3. EM1 and EM4 are memory-like, and EM3 is effector-like. Taken together, these data are consistent with the model according to which there is a differentiation pathway with progressive loss of CCR7, CD27, and CD28 cell surface expression concomitant with upregulation of cytolytic capacity (Appay et al., 2002a).

EFFECTOR MEMORY RA

Persistent viral infections and inflammatory syndromes induce the accumulation of T-cells with characteristics of terminal differentiation or senescence. However, the mechanism that regulates the end-stage differentiation of these cells is unclear. EMRA T-cells have features of telomere-independent senescence that are regulated by active cell signaling pathways that are reversible. These EM T-cells that re-express CD45RA (CCR7-CD45RA+; EMRA) have many characteristics of end-stage differentiation. The EMRA subset can be further subdivided into very poorly differentiated pE1 (CD27 + CD28+) and the most differentiated T-cell subset, E ($CD27^{null}CD28^{null}$). However, the exact nature of these T-cells is not clear.

PHENOTYPIC AND FUNCTIONAL CHANGES ASSOCIATED WITH AGING

As we age, the CD4+ T-cells are repetitively stimulated by a large number of different antigens and as a consequence, CD4+ T-cells become refractory to telomerase induction, suffer telomere erosion, and enter replicative senescence. Replicative senescence is characterized by the accumulation of highly differentiated T-cells with newly acquired functional capabilities, which can be caused by aberrant expression of genes normally suppressed by epigenetic mechanisms in CD4+ T-cells. Age-dependent demethylation and overexpression of genes normally suppressed by DNA methylation have been demonstrated in senescent subsets of T-lymphocytes (Lu et al., 2003; Liu et al., 2009). There are some major features of CD4+ T-cell that are acquired as they age: loss of proliferative capacity and telomerase activity, TCR restriction, low production of and response to IL-2, high response to IL-15, loss of expression of CD28 molecule, expression of NK cell-related receptors

Table 1 | Functional differences between naïve and late-memory CD4+ T-cells.

Naïve	CD4+	Late-memory
↑↑↑	Proliferative capacity	↓↓
↑↑↑	Telomerase activity	↓↓
–	TCR restriction	↑↑
↑↑↑	IL-2 production and response	↓↓
–	Response to IL-15	↑↑
↑↑↑	CD28 expression	↓↓
–	NKRs	↑↑
↑↑	IFN-γ production	↑↑↑
–	Cellular cytotoxicity (perforin and granzyme)	↑↑

(NKRs), production of molecules involved in cellular cytotoxicity (perforin and granzyme), and a substantial increase in the production of IFN-γ (Appay et al., 2008) (**Table 1**).

THE COSTIMULATORY MOLECULE CD28 IN AGED CD4+ T-CELL

Several studies have demonstrated that expression of TCR in CD4+ T-cells are not altered in the elderly (Bazdar et al., 2009), however, costimulatory molecules required for lymphocyte activation appear to be altered. One of the major costimulatory molecules present in T-lymphocytes is the CD28 molecule, and its loss as individuals age is well-documented in CD4+ T-cells (Weyand et al., 1998). Loss of CD28 has been associated with a loss of immune system responsiveness in the elderly. These cells are less able to proliferate than are CD4 + CD28+ T-cells, have a diminished antigenic recognition repertoire, and gain a very powerful cytotoxic capacity (Bryl and Witkowski, 2004). CD28 downregulation occurs with T-cell activation, involving transcriptional repression and increased protein turnover. This is thought to be a negative feedback mechanism (Swigut et al., 2001). When CD4+ T-cells recognize an antigen, CD28 expression decreases rapidly, but immediately returns to normal levels. However, with sustained stimulation over time, the expression of CD28 decreases and may be lost. CD28 can be initially reinduced by IL-12 (Warrington et al., 2003) or with treatment with anti-TNF agents (Rizzello et al., 2006), but once firmly established, its loss is irreversible in the majority of $CD28^{null}$ T-cells, suggesting active transcriptional silencing.

ACQUISITION OF NEW AGING MARKERS

Although CD28 is a major costimulatory molecule, these $CD28^{null}$ T-cells remain functionally active; other molecules must be able to maintain responsiveness and survival in these cells. Therefore, alternative receptors must exist to prevent these cells entering into a state of anergy. CD4 + $CD28^{null}$ T-cells are resistant to apoptosis (Vallejo et al., 2000), which is one possible cause of its accumulation throughout life (Posnett et al., 1994). An explanation of why these cells are able to be activated, is the *de novo* expression of several NKRs (Abedin et al., 2005). Among the best studied are the receptors CD16, CD56, CD94, KLRG1, several members of the NK receptor G2 (NKG2), and the killer cell immunoglobulin (Ig)-like receptor (KIR) families. CD94, KLRG1, and the NKG2s are lectin-like receptors, and CD16 and CD56 are receptors belonging to the superfamily of immunoglobulins, and are the prototypic NKRs that are normally used to identify NK cells (**Figure 3**). The functional roles of CD16, CD56, and CD94 on senescent T-cells are still unknown. The KLRG1 receptor seems to influence the state of CD4+ T-cell senescence due to their ability to inhibit proliferation via TCR (Hayhoe et al., 2010; Di Mitri et al., 2011). KLRG1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain and has been shown to be a receptor for some members of the cadherin family of proteins (Grundemann et al., 2006). It is an inhibitory receptor and its presence in CD4+ T-cells blocks the costimulatory activities mediated by Akt, such as proliferation (Henson et al., 2009). Among NKG2s receptors, only NKG2D is expressed in CD4 + $CD28^{null}$ aged T-cells, its expression becoming present for the first time in CD4 + $CD28^{null}$ T-lymphocytes as people age. This novel age-marker was recently described by our laboratory (Alonso-Arias et al., 2011b). It has been implicated in NK-mediated anti-viral immunity and in TCR-independent cytotoxic activity in CD4+ and CD8+ T-cells. The regulation of KIRs seems to differ in NK cells and T-lymphocytes (Xu et al., 2005). The KIR repertoire in T-cells is very restricted (Abedin et al., 2005), being limited to memory T-cells, mainly $CD28^{null}$ T-lymphocytes. In addition, the same population of T-lymphocytes with the same TCR specificity may have different combinations of KIRs on their surface (Vely et al., 2001; van Bergen et al., 2009). It seems quite clear that the expression of NKRs differs in oligoclonal and senescent T-cells. The expression of these molecules appears to represent a different way of diversifying the immune repertoire, i.e., an oligoclonal population of T-lymphocytes for a particular TCR can express a wide diversity of receptor NKRs codominantly (Tarazona et al., 2000) (**Figure 3**). In the case of arterial disease and CMV infection, the expression of KIR receptors in CD4 + $CD28^{null}$ T-cells is broadly accepted as being responsible for their functionality (Zal et al., 2008; van Bergen et al., 2009). The appearance of these "aberrant" molecules in senescent T-cells could help maintain the adequate homeostasis of T-cells and would be a way to stay functionally active, independent of TCR activation.

FUNCTIONAL PROPERTIES ACQUIRED BY AGING CD4+ T-CELLS

A defining feature of the eukaryotic genome is the presence of linear chromosomes. This arrangement, however, poses several challenges with regard to chromosomal replication and maintenance. Telomeric DNA is lost due to the incomplete terminal synthesis of the lagging DNA strand during cell division. Immune cells must be able to grow exponentially and die when no longer needed. They support an extremely high replicative rate, so their telomeres suffer great stress. The lymphocytes are capable of upregulating telomerase, an enzyme that elongates telomeres and can therefore prolong the life of the cell (Klapper et al., 2003; Andrews et al., 2010). Signaling via the TCR and other costimulatory molecules, such as CD28, are necessary for inducing telomerase activity with a peak of activation at 4–5 days after stimulation and a decrease in activity at 10 days (Macallan et al., 2004; Fritsch et al., 2005). In the absence of mechanisms that compensate for telomere shortening, growth arrest of the cells occurs when progressive telomere erosion reaches a critical point known as replicative senescence (Hodes et al., 2002). The overall finding from several different studies is

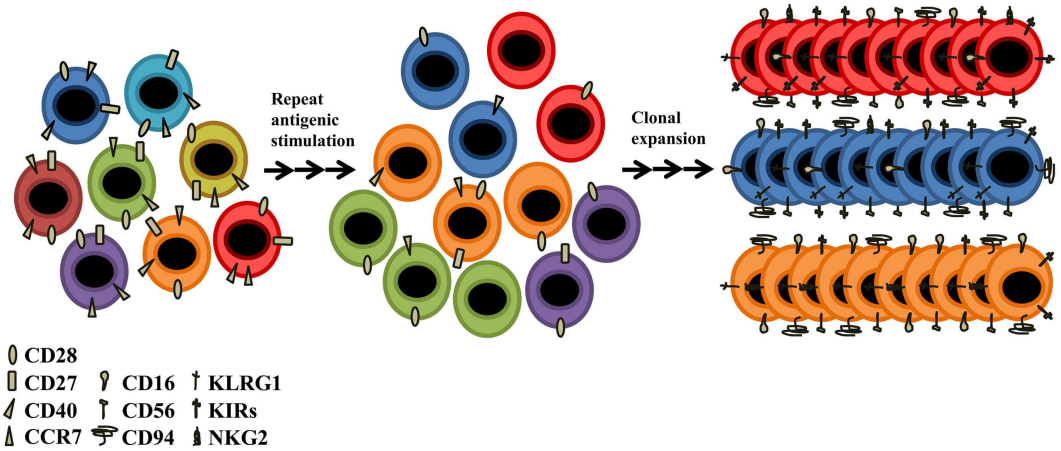

FIGURE 3 | Older people present a different receptor repertoire from that of young individuals. Despite the decline of thymic function and the low TCR diversity, the elderly CD4+ T-cells present novel functions attributed to their acquisition of NK-related receptors (NKRs) such as KIR, CD94, CD16, CD56, NKG2, and KLRG1. CD4+ T-cells undergo senescence due to lifetime exposure to persistent pathogens and to homeostatic proliferation. With advancing age, the T-cell repertoire becomes populated with highly oligoclonal, long-lived T-cells, most of which have lost the ability to express CD28. Such $CD28^{null}$ T-cells have limited proliferative capacity, but are functionally active. They are generally long-lived and functionally active.

that human T-cells can undergo a limited number of divisions, after which they cease dividing (Perillo et al., 1989). Importantly, the arrival of T-cells at a stage of replicative senescence does not imply the loss of cell viability. In fact, under appropriate conditions senescent cells remain alive and metabolically active for a long period (Wang et al., 1994). In cultures where CD4+ and CD8+ T-cells of the same subject are stimulated identically, CD8+ T-cells were unable to upregulate telomerase after the fourth encounter with the antigen. In contrast, the CD4+ T-cells from the same donor had a high level of telomerase activity induced by antigen (Valenzuela and Effros, 2002). Several studies have shown that telomerase activity is preserved and replicative senescence is delayed if telomere length is stabilized (Dagarag et al., 2004; Choi et al., 2008). The inhibition of cytokines involved in shortening telomeres, such as TNF-α, could delay telomeric loss. One of the main causes of cell division is the interaction of TCR and CD28 receptors that leads to the production of cytokines. One of the best studied is IL-2, which is produced in an autocrine form and that causes upregulation of its own receptor (IL-2R), composed of three subunits (α, β, and γ) (Almeida et al., 2002). The γ-chain is common to other cytokine receptors such as IL-7, IL-15, and IL-21, and the differences in the responses elicited by these cytokines must lie in the other two chains that form the receptor. The main molecules involved in signaling via IL-2 are Janus kinases (Jaks) and signal transducer and activator of transcription (STATs) (Johnston et al., 1996). One of the first indications that the immune system of the elderly has impaired functionality was the reduction in the production of IL-2 (Caruso et al., 1996). Several studies have demonstrated that levels of TCR in T-cells are not altered in the elderly, for which reason it is thought that the problem may be to do with intracellular signaling (Bazdar et al., 2009). Alterations in intracellular signaling may partly explain the lack of production of IL-2 in the elderly.

CD4+ T-cells have not been classically considered as cytotoxic cells, although intracytoplasmic stores of granzyme B and perforin have been previously described in CD4 + $CD28^{null}$ T-cells (Appay et al., 2002b). Granzyme B and perforin expression in CD4+ T-cells are closely associated with the loss of CD28 from the cell surface. These CD4 + $CD28^{null}$ T-cells resemble cytotoxic CD8+ T-cells, because their cytotoxic capacity is mediated by TCR stimulation. In addition, they lack costimulatory molecule requirements (Appay et al., 2002a). The expression of NK molecules described above is associated with increased cytotoxic capacity with high levels of expression of intracytoplasmic perforin and granzyme (Brown et al., 2012). The expression of these NK receptors in CD4+ T-cells probably serves to regulate their cytotoxicity, and even cytokines involved in NK cell activation, such as IL-15, can enhance their cytotoxic ability. The expansion of these cells not only occurs in the elderly, but also under other clinical conditions involving chronic activation of the immune system, such as viral infections, autoimmune and rheumatic diseases, certain tumors, and coronary artery disease (Thewissen et al., 2007; Alonso-Arias et al., 2009). CD4 + $CD28^{null}$ T-cells also secrete large amounts of IFN-γ. CD4 + $CD28^{null}$ T-lymphocytes have been described as being antigen-specific cells against chronic viral antigens, mainly in some autoimmune diseases (Thewissen et al., 2007). IFN-γ expression is present at all stages of CD4+ T-cell differentiation, but is mostly improved in late-differentiated cells that lack IL-2-production (Yue et al., 2004; Harari et al., 2005). The dominant IFN-γ CD4+ T-cell response is associated with models of antigen persistence and high antigen levels.

It has been hypothesized that CD4 + $CD28^{null}$ T-cells might play a role in containing viral infections tropic for HLA class II cells, such as EBV in B cells, HIV-1 in activated CD4+ T-cells, monocytes and DCs, and CMV in endothelial cells. However, the presentation mechanism of this antigen is not currently known.

In the case of CMV infection, endothelial cells are poor antigen-presenting cells under normal conditions in a classical immune response because they lack costimulatory molecules. Nevertheless, since the $CD28^{null}$ T-cells do not require costimulation and have a low activation threshold, antigen presentation could be rendered effective by non-professional cells such as endothelial cells. This hypothesis is supported by the fact that the class II pathway may be preferentially targeted, since both EBV and CMV prevent normal MHC class I expression as part of their strategies of immune evasion (Alcami and Koszinowski, 2000).

IMMUNE FUNCTIONAL (DIS)ABILITY

The human immune system progressively deteriorates with age, leading to a greater incidence or the reactivation of infectious diseases, as well as to the development of autoimmune disorders and cancer (DelaRosa et al., 2006; Prelog, 2006). These defective immune responses are also manifested in a reduced capacity to induce immunological memory to vaccines and infections. In fact, the incidence of acute transplantation rejections is significantly lower in elderly transplant patients (Bradley, 2002; Deng et al., 2004; Trzonkowski et al., 2010). Immunological impairment may be partially due to the restriction of antigen recognition (**Figure 3**). Protection from pathogens and tumor development depends on the generation and maintenance of a diverse TCR repertoire. CD4+ and CD8 T-cells undergo the same principal phenotypic shifts; however, the rate at which they occur or accumulate with age is vastly different. Diminution of naïve cells with age is drastic for CD8 T-cells, but relatively minor for CD4+ T-cells. Homeostatic control of the CD4+ compartment is much more robust than that of CD8 T-cells. In spite of the majority of naïve T-cells in the adult being generated by IL-7- and IL-15-induced division of pre-existing cells, the diversity of the naïve CD4+ T-cell repertoire is maintained up to the age of 65 years (Prlic and Jameson, 2002; Naylor et al., 2005). At older ages, TCR diversity is remarkably reduced by accumulation of clonal cells in both naïve and memory compartments (Vallejo, 2007). Elderly donors display a marked increase in the proportion of highly differentiated effector and memory T-cells due to a lifetime of exposure to a variety of pathogens. Accumulation of these highly differentiated T-cells is partially explained by their reduced susceptibility to apoptosis and their oligoclonal expansions against CMV and other chronic antigens (Vescovini et al., 2004; Almanzar et al., 2005; Vasto et al., 2007; Derhovanessian et al., 2009). Persistent viral infections and/or the pro-inflammatory cytokines produced during some infectious processes may drive their differentiation. Another possible explanation is the corroborated fact that advanced age is accompanied by low-grade, chronic upregulation of inflammatory responses, evidence for which is provided by increased serum levels of pro-inflammatory cytokines (IL-6, IL-15, IL-8), coagulation factors, and reactive oxygen species (ROS) (Mari et al., 1995; Forsey et al., 2003; Zanni et al., 2003; Ferrucci et al., 2005; Wikby et al., 2006; Giunta et al., 2008). Since the number of circulating T-cells is maintained over the lifespan, a compensatory mechanism may give rise to an increase in highly differentiated memory cells in parallel with the reduction in naïve cell proliferation. Even the higher absolute counts of highly differentiated CD8+ T-cells could modulate the levels of CD4+ T-cells. Experienced T-lymphocytes, mainly CD8+, may fill the immunological space, and homeostatic mechanisms block the generation of new naïve cells to maintain the numbers of peripheral T-lymphocytes (Alonso-Arias et al., 2013). These mechanisms make it difficult to preserve the T-cell repertoire diversity that combats new pathogens and the host's ability to mount vigorous recall responses to recurrent infections (Nikolich-Zugich, 2008). Another of the most prominent changes during T-cell aging in humans is the change in the functional ability of the T-cells with a high degree of differentiation. CD28 is pivotal in T-cell activation, doing such things as inducing cytokine production (IL-2) and promoting cell proliferation, so the lack of this costimulatory signal during activation results in a partial activation or even an anergic state of T-cells (Godlove et al., 2007). In contrast, CD4 + $CD28^{null}$ T-cells have a low activation threshold, which could play a part in their predisposition to the breakage of self-tolerance (Yung et al., 1996). In this way, the accumulation of $CD28^{null}$ T-cells, particularly within the CD8 subset, is associated with a reduced overall immune response to pathogens and vaccines in the elderly (Saurwein-Teissl et al., 2002; Alonso-Arias et al., 2013).

EFFECT OF IL-15 HOMEOSTATIC CYTOKINE ON HIGHLY DIFFERENTIATED CD4+ T-CELLS

It is widely accepted that IL-7 signaling through the IL-7 receptor (IL-7R), is essential for prolonged survival and proliferation of naïve and memory T-cells. Naïve T-cells rely on survival signals through contact with self-peptide-loaded MHC molecules plus interleukin IL-7. On the other hand, antigen-experienced (memory) T-cells are typically MHC-independent and survive and undergo periodic homeostatic proliferation through contact with both IL-7 and IL-15 (Boyman et al., 2012) (**Figure 4**). Both cytokines seem equally essential to enable these cells to undergo basal homeostatic proliferation (Lenz et al., 2004; Purton et al., 2007), but IL-15 has a less prominent role for memory CD4+ cell homeostasis than for NK and memory CD8+ cells (Surh and Sprent, 2008). Memory CD4+ T-cells compete less effectively for IL-15 than the latter cells since they have much lower levels of expression of the IL-15 receptor (Lenz et al., 2004). Homeostatic proliferation of T-cells can be one cause of the age-associated loss of CD28 expression, since CD8+ memory T-cells in the presence of IL-15 alone, without TCR stimulation, lose CD28 expression and proliferate at a similar rate to CD8+CD28+ T-cells (Chiu et al., 2006). In contrast, IL-15 does not induce loss of CD28 expression in CD4+ T-cells, although recent studies have shown that IL-15 does in fact play an appreciable role in CD4+ memory T-cell proliferation under physiological conditions and after *in vitro* stimulation (Geginat et al., 2001; Lenz et al., 2004; Alonso-Arias et al., 2011b) (**Figure 4**). CD4+ memory T-cells rely on STAT5, the downstream signaling molecule used by IL-15, considerably more than do effector CD4+ T-cells (Purton et al., 2007; Tripathi et al., 2010). IL-15 increased the cytolytic properties of CD4 + $CD28^{null}$ T-cells and enhanced their antigen-specific responses (Alonso-Arias et al., 2011b). Although the role of CD4+ T-cells as cytotoxic effector cells is not well understood, the enhancing effector activity of IL-15 may have a substantial impact, since CD4 + $CD28^{null}$ T-cells are mainly specific against chronic contact antigens. Moreover, IL-15 plays a critical role in

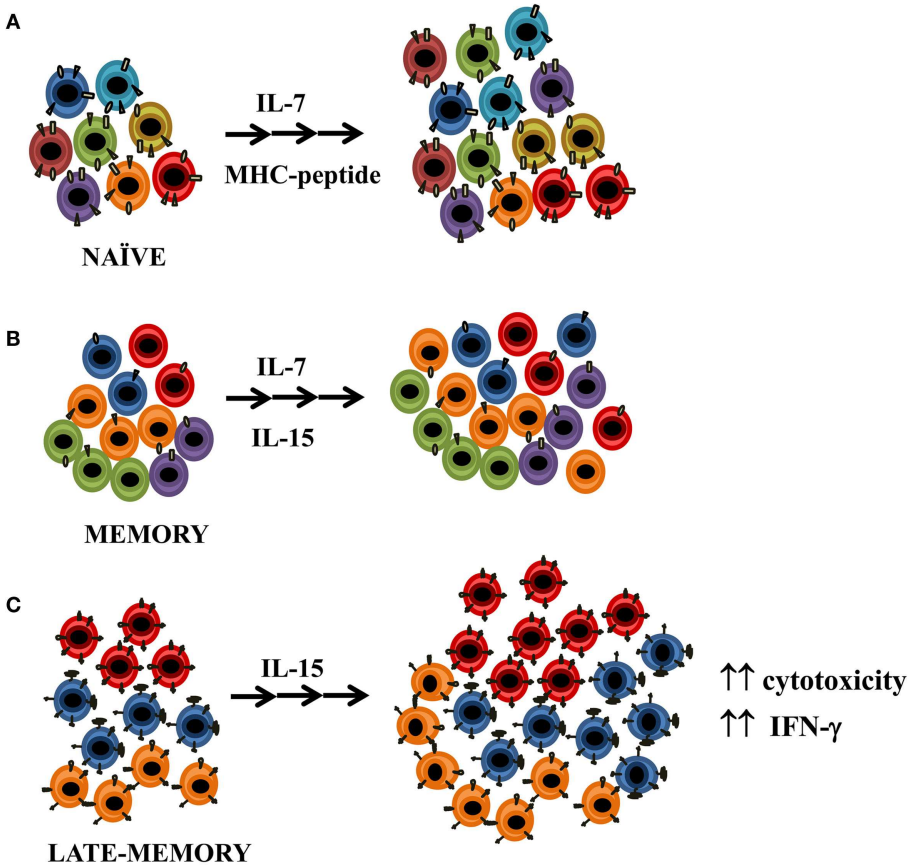

FIGURE 4 | Effect of IL-15 homeostatic cytokine on CD4+ T-cells. **(A)** It is widely accepted that IL-7 signaling through the IL-7 receptor is essential for prolonged survival of naïve and memory T-cells. Naïve T-cells rely on survival signals through contact with self-peptide-loaded major histocompatibility complex (MHC) molecules plus interleukin IL-7. **(B)** Antigen-experienced (memory) T-cells are typically MHC-independent. They survive and undergo periodic homeostatic proliferation through contact with both IL-7 and IL-15. **(C)** IL-15 promotes the proliferation of late-memory CD4+ T-cells and enhances the proliferative response of $CD28^{null}$ cells with respect to CD28+ CD4+ T-cells. IL-15 increases the cytolytic properties of CD4 + $CD28^{null}$ T-cells and enhances their antigen-specific responses.

the immune responses to early infection and chronic inflammation by amplifying the effects of pro-inflammatory cytokines on IFN-γ secretion and by enhancing the antigen-specific responses of CD4 + $CD28^{null}$ (Smeltz, 2007; Alonso-Arias et al., 2011b).

INFLAMMATION AND CMV AS INDUCTORS OF CD4+ T-CELL AGING

The degree of immunosenescence varies greatly, even among age-matched elderly individuals (Alonso-Arias et al., 2011a). This may mean that individual or environmental factors influence immunological status in different ways. In younger individuals, the inflammatory response is necessary to protect against infectious and damaging agents, but it can be detrimental in later life (Franceschi et al., 2007). As a result of continual antigenic stress throughout life, chronic low-grade inflammation develops, and this is considered to be a major contributor to age-associated frailty, morbidity, and mortality (Franceschi et al., 2000). Progressive T-cell differentiation and low-grade inflammation are two processes that occur simultaneously and/or enhance each other. Highly differentiated cells help increase the levels of pro-inflammatory cytokines, whereas inflammatory mediators are involved in the development of differentiated T-cell phenotypes. The ability to prevent or block this inflammatory status may be responsible for the differences seen between individuals. In centenarians, who are commonly considered a paradigm of "successful aging," the chronic pro-inflammatory state of aging is countered by increased expression of anti-inflammatory cytokines. In this way, the frequency of the IL-10 (−1082GG) genotype, associated with increased production of this anti-inflammatory cytokine, is higher in centenarians than in younger controls (Lio et al., 2004). In parallel, their immune system exhibits no signs of a T-cell Immune Risk Profile (IRP), comprising a group of immune parameters which has been defined as an inverted CD4/CD8 ratio, an accumulation of CD8 + $CD28^{null}$ T-cells, and CMV infection (Olsson et al., 2000). The inverted CD4/CD8 ratio was the sole marker significantly associated with the IRP. Subsequently, CMV infection has been shown to exert a major impact on the immunosenescence process (Hadrup et al., 2006). The Swedish OCTO and NONA immune longitudinal studies were able to identify and confirm the IRP predictive of an increased 2-year mortality in very old individuals,

86–94 years of age. Recently, a similar study conducted in subjects aged 66, indicates that the IRP could be also associated with increased mortality in hexagenerians. Therefore, it will be important to examine morbidity and mortality to assess whether the immune profile also is an IRP in the hexagenerians (Strindhall et al., 2012).

One of the main factors affecting longevity could be represented by a well-functioning immune system that prevents the main age-related chronic diseases such as atherosclerosis, type 2 diabetes, and Alzheimer's disease (Pradhan et al., 2001; Libby et al., 2002; Griffin, 2006). Even depression and frailty (the latter an emerging clinical entity occurring late in life), which are correlated with increased morbidity and mortality within a few years, have a major inflammatory component (De Martinis et al., 2006; Raison et al., 2006). These pathologies are all also characterized by important alterations in the CD4+ T-cell compartment, resulting in lower proportions of naïve cells and higher proportions of late-differentiated cells (Dumitriu et al., 2009; Giubilato et al., 2011; Pellicano et al., 2012). Recently, CMV has been linked to this range of chronic diseases with an inflammatory component (Harkins et al., 2002; Aiello et al., 2006; Simanek et al., 2009; Moro-Garcia et al., 2012). The specific mechanisms responsible for these associations are not fully determined but are likely to have an inflammatory and immune component. After infection, the virus establishes lifelong latency within the host and periodically reactivates. Reactivation from latency is a key step in the pathogenesis of the infection and can be detected in response to inflammation, infection, stress, or immunosuppression (Kutza et al., 1998; Prosch et al., 2000). Activation of protein kinase C and NF-κB by TNF-α and increasing concentrations of cyclic AMP by stress hormones and prostaglandins promotes viral reactivation and replication. Reactivation of CMV is more frequent in the elderly and the virus, in turn, may result in increased levels of pro-inflammatory molecules such as IL-6, TNF-α, and C-reactive protein (CRP) (Stowe et al., 2007), contributing to the increase in the inflammatory status. These more frequent and/or intense reactivations in the elderly may be a consequence rather than the cause of immunosenescence. Furthermore, reactivations imply repetitive reencounters between specific T-cells and CMV antigens, leading to their activation and proliferation and consequent aging.

Despite the evidence suggesting that CMV induces aging of T-lymphocytes, more frequent and/or intensive reactivations in the elderly may be a consequence rather than the cause of immunosenescence. CMV seropositivity and anti-CMV antibody titers are related to the degree of differentiation of CD4+ T-cells and to the other IRP parameters of elderly people (Olsson et al., 2000; Alonso-Arias et al., 2013). Differences between elderly and young individuals in highly differentiated and naïve CD4+ T-cells become more marked, depending on their anti-CMV antibody titers.

Strategies directed at counteracting the inflammatory status in the elderly have been evaluated. Cross-sectional studies reveal an association between physical inactivity and low-grade systemic inflammation in elderly people (Wannamethee et al., 2002; King et al., 2003). Sedentary elderly individuals have a greater risk of mortality than those doing intermediate or high levels of physical activity, who have a reduced risk of coronary heart disease, neurodegeneration, cancer incidence, and disability (functional impairment) (Hambrecht et al., 2000; Melzer et al., 2004; Lautenschlager et al., 2011; Speelman et al., 2011). Elderly individuals with functional disability, which implies mobility impairment, even to the point of not being able to perform all daily activities adequately, also have smaller naïve CD4+ T-cell subpopulations and higher percentages of effector cells, together with reduced anti-CD3 responses. However, their responses to CMV gradually increase. The underlying mechanisms conferring protection are not known but it is thought that the anti-inflammatory role of moderate physical activity may be an influence (Pedersen and Saltin, 2006; Walsh et al., 2011; Warren et al., 2011). This anti-inflammatory effect of exercise may be responsible for the beneficial effects of exercise on health, and may play important roles in the protection against aging of the immune response and diseases associated with low-grade inflammation.

CONCLUDING REMARKS

Changes similar to those observed in CD8+ T-cells during aging appear, albeit belatedly, in the compartment of CD4+ T-lymphocytes. These aged CD4+ T-cells can be found in the elderly and in individuals under inflammatory and/or antigenic stress due to autoimmune or chronic infectious processes. All these events in CD4+ T-cells appear at late stages in life, correlating with the impaired health status in elderly people. This impairment may be the result of their restricted immune response, as reflected by their reduced ability to fight against pathogens and poorer response to vaccination. A possible field of action to prevent the deterioration of the adaptive immune response would be the "rejuvenation" of the CD4+ T-cell population. Preclinical and clinical studies on the T-cell reconstitution effects of sex steroid ablation, keratinocyte growth factor, the growth hormone pathway, and the cytokines IL-7, IL-12, and IL-15 indicate that these strategies may be used to alleviate the effects of T-cell deficiencies in the aging immune system.

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The impact of aging on regulatory T-cells

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Age-related deviations of the immune system contribute to a higher likelihood of infections, cancer, and autoimmunity in the elderly. Senescence of T-lymphocytes is characterized by phenotypical and functional changes including the loss of characteristic T-cell surface markers, while an increase of stimulatory receptors, cytotoxicity as well as resistance against apoptosis is observed. One of the key mediators of immune regulation are naturally occurring regulatory T-cells (T_{regs}). T_{regs} express high levels of CD25 and the intracellular protein forkhead box P3; they exert their suppressive functions in contact-dependent as well as contact-independent manners. Quantitative and qualitative defects of T_{regs} were observed in patients with autoimmune diseases. Increased T_{reg} activity was shown to suppress anti-tumor and anti-infection immunity. The effect of aging on T_{regs}, and the possible contribution of age-related changes of the T_{reg} pool to the pathophysiology of diseases in the elderly are still poorly understood. T_{reg} homeostasis depends on an intact thymic function and current data suggest that conversion of non-regulatory T-cells into T_{regs} as well as peripheral expansion of existing T_{regs} compensates for thymic involution after puberty to maintain constant T_{reg} numbers. In the conventional T-cell subset, peripheral proliferation of T-cells is associated with replicative senescence leading to phenotypical and functional changes. For T_{regs}, different developmental stages were also described; however, replicative senescence of T_{regs} has not been observed yet.

Keywords: FOXP3, regulatory T-lymphocyte, aging, cellular senescence, thymus, suppressor cells

INTRODUCTION

The immune system combats against infectious agents and depletes damaged or transformed cells, whereas intact self-components are usually ignored. Nevertheless, clinical manifestations of autoimmunity occur in at least 5% of the general population. The exact causes of autoimmune diseases are elusive; however, genetic and environmental risk factors as well as an insufficient elimination of cells bearing autoreactive T-cell receptors (TCRs) in the thymus contribute to the evolvement of disease (1, 2). To prevent autoimmunity, tolerance mechanisms including clonal deletion, induction of apoptosis, or anergy of self-reactive T-cells are essential. In addition, regulatory T-cells (T_{regs}) were identified as sentinels of the immune response keeping aberrant/exaggerated immune reactions in balance. Several distinct T-cell subsets with regulatory function have been identified so far including natural T_{regs}, adaptive or induced T_{regs} (iT_{reg}), type 1 regulatory T-cells (Tr1), T helper 3 cells (Th3), double-negative (dn) T-cells, γδ T-cells, and iNKT cells. In a number of autoimmune diseases a diminished prevalence and/or impaired function of T_{regs} were observed (3). As several autoimmune disorders (such as rheumatoid arthritis or vasculitis) occur more frequently in the elderly, the question arises whether aging is linked to quantitative and/or qualitative defects of the T_{reg} pool (4–6).

In this review we summarize current data about the effects of aging on T_{regs} and highlight the possible mechanisms leading to senescence of T_{regs}.

CHARACTERIZATION OF T_{REGS}

DEFINITION AND PHENOTYPE

Natural T_{regs} develop in the thymus through recognition of self-antigen presented by thymic epithelial or dendritic cells. For this process CD28 co-stimulation is required, whereas IL-2 and TGF-β are less important as indicated by knock-out mice models (7).

Today, there is still no consensus on the reliable identification of T_{regs} by flow cytometry. A variety of cell surface molecules have been proposed as specific T_{regs} markers such as glucocorticoid-induced tumor necrosis factor receptor (GITR), cytotoxic T-lymphocyte associated antigen-4 (CTLA-4), the co-receptors Neuropilin-1 and PD-1, the adhesion molecule CD62L, major histocompatibility complex (MHC) class II DR, or CD45 isoforms. The type I cytokine receptor CD127 is a negative marker of T_{regs} and the absence of this molecule is frequently used for T_{reg} identification (8).

The forkhead transcription factor FoxP3 was proposed as the most specific marker of T_{regs} as FoxP3 expression is essential for T_{reg} development and function (9): T_{regs} were unable to develop in a mouse receiving FoxP3-deficient progenitor cells from another animal (10) and retroviral expression of FoxP3 in human and murine T-cells enabled the conversion of non-regulatory naïve T-cells into a T_{reg}-like phenotype with suppressive activity and surface expression of CD25 (9). A mutation of the *FoxP3* gene in humans results in the fatal autoimmune syndrome IPEX (immune dysregulation, polyendocrinopathy, X-linked) (11). For experimental studies, however, FoxP3 appears not to be an optimal T_{reg}

marker because first, permeabilization of T-cells is necessary to stain FoxP3 and cells are thus not viable anymore and second, newer data indicate that human FoxP3 is up-regulated in activated T-cells without suppressive function as well (12).

The Ikaros family transcription factor Helios was proposed as an alternative indicator of human T_{regs} with a higher specificity compared to FoxP3. Recent data, however indicate that Helios is also up-regulated in activated non-regulatory T-cells (13). In summary, there is currently no specific marker of human T_{regs} available limiting the validity of studies investigating qualitative and/or quantitative changes of the T_{reg} pool.

MECHANISM OF SUPPRESSION

The mechanisms of T_{reg} mediated immunosuppression are still unclear. Most likely, T_{regs} have multiple functions with direct and indirect inhibitory effects on antigen-presenting cells (APCs) and T-cells such as the following (14, 15): (a) expression of the surface molecule CTLA-4 directly suppressing the activity of T-cells, (b) indirect inhibition of effector cells by the induction of anti-inflammatory biochemical pathways in APC, (c) direct or indirect killing of effector cells and APCs, and/or (d) production of immunoregulatory cytokines such as TGF-β and IL-10 (16).

Interestingly, a recent study reported that human T_{regs} are able to induce senescence of naïve and memory responder T-cells *in vitro* and *in vivo*. The resulting senescent T-cell subset had an altered phenotype and revealed potent suppressive functions. The mechanisms leading to senescence of non-regulatory T-cells were not completely understood; however, the phosphorylation of p38 and ERK1/2 signaling pathways inhibiting naïve T-cell growth and cell-cycle regulation appeared to play a role (17).

THE EFFECT OF AGING ON T_{REG} PREVALENCES AND FUNCTION

A prevalence of approximately 0.6–15% out of the $CD4^+$ T-cell pool has been reported for T_{regs} in healthy adults and mice (4, 18). The influence of aging on T_{reg} prevalence in humans has been rarely studied so far and available reports suggest only minor changes of the circulating T_{reg} pool through age (19). Higher proportions of T_{regs} were only found in cord blood samples suggesting a pivotal role of T_{regs} during homeostatic proliferation of naïve T-cells in the fetal life (20, 21). During the first 36 months of life T_{reg} levels decline rapidly (22) and remain relatively stable thereafter.

Mouse studies showed increased T_{reg} prevalences in lymphoid organs of aged compared to young animals, whereas frequencies in circulating blood and thymus were unchanged (23, 24). This finding led to the hypothesis that during aging T_{regs} accumulate in lymphoid tissues; hypothetically explaining the increased susceptibility to infections and reduced vaccine response in elderly animals. The accumulation of T_{regs} has further been observed in the skin of aged persons possibly resulting in a higher risk of skin cancer as T_{regs} reduce local anti-tumor immune responses (25–27).

In animals, T_{reg} function seems to decrease with advancing age. The transfer of $CD25^+$ T_{regs} from aged mice into young animals for example resulted in a lower suppression of delayed type hypersensitivity responses compared to the infusion of young T_{reg} cells (23). Another study found that $CD4^+CD25^{high}$ T_{regs} from aged animals less efficiently inhibited the proinflammatory activity of $IL\text{-}17^+$ T-cells compared to T_{regs} from young mice (28). In human studies it was observed that T_{regs} from young and elderly individuals similarly inhibited the proliferation of responder cells whereas the production of the anti-inflammatory cytokine IL-10 was reduced in cells from the older group. The phenotype of T_{regs} including expression of CD25, FoxP3, IL-7Rα, or chemokine receptor expression, however, was unchanged (29). In conclusion T_{regs} from aged individuals are less efficient in preventing the occurrence of autoimmunity, while their number remains unaltered.

On the other hand, cancer and infections occur more commonly in the elderly suggesting increased T_{reg} responses (see also above) (29–31). One mouse study found an increase of T_{reg} prevalences in aged animals correlating with a defective tumor clearance. CD25-depletion restored the anti-cancer immune response (32). Similarly, CD25-depletion in aged mice reduced the lesion size in a *Leishmania major* infection model (24). Others reported that the depletion of T_{regs} with denileukin diftitox improved tumor-specific immunity only in young mice whereas tumor growth was unaffected in aged mice. This was explained by increased numbers of myeloid-derived suppressor cell (MDSC) in aged animals, and upon depletion of these cells tumor-specific immunity was restored (33).

In summary, current data on age-related changes of T_{reg} prevalences and function are conflicting and do not completely explain the simultaneously increased risk of autoimmunity (suggesting lower T_{reg} function), cancer, and infections (indicating increased T_{reg} responses) in the elderly. Apart from the difficulty of a reliable identification of T_{regs} the possible accumulation of T_{regs} in lymphoid organs and/or tissues during aging might lead to an underestimation of the total T_{reg} pool in current human studies. Future studies investigating tissue samples from immune-organs of elderly individuals would be desirable to better understand the role of T_{regs} in the pathogenesis of age-related diseases.

T_{REG} DEVELOPMENT AND HOMEOSTASIS

Development of natural T_{regs} in the thymus depends on a positive selection process including high affinity interactions of the TCR to cortically expressed host antigens. Thymic stromal lymphopoietin activated CD11c-positive dendritic cells (34), co-stimulatory molecules including CD28, PD-1, CD40L (35) as well as the cytokine IL-2 were all shown to be crucial for thymic T_{reg} generation (36–38). Besides, the Nr4a nuclear receptors (involved in apoptosis, proliferation, DNA repair, inflammation, and others) were recently reported to contribute to T_{reg} development. Mice lacking these receptors in T-cells were unable to produce T_{regs} and died early from systemic autoimmunity (39).

During aging a progressive degeneration of the thymus occurs leading to a substantial loss of its capacity to generate and export new T-cells (40, 41). Throughout middle age thymic epithelial space and the functional unit of thymopoiesis (and thus the production of T-cells) decline by approximately 3% per year until the age of 45 when only an irrelevant level of functional thymic tissue remains. The total number of T-cells in the periphery nevertheless is unchanged and peripheral mechanisms of T-cell renewal have to compensate for progressive thymic failure (42–44).

Parallel to the overall reduction of thymic T-cell output the production of thymically derived T_{regs} decreases with age (45). Alternative mechanisms such as increased surveillance of T_{regs} in the elderly (46) as well as peripheral T_{reg} generation may compensate for the loss of thymic function to maintain a sufficient T_{reg} pool (see **Figure 1**). Indeed, numerous studies indicate a possible conversion of non-regulatory $CD4^+CD25^-$ T-cells into T_{regs} *in vitro* and *in vivo* (47, 48). Moreover, mouse studies showed that peripheral self-antigen-driven proliferation of T_{regs} is a thymus-independent mechanism to maintain T_{regs} (49–51). The proportion of conventional T-cells differentiating into T_{regs} as well as the relative contribution of homeostatic T_{reg} proliferation to the overall T_{reg} pool in elderly individuals are unknown.

Peripheral mechanisms of T-cell renewal (particularly homeostatic expansion of existing T_{regs}) are probably not infinite. Normally, T-cells proliferate beyond the seventh decade of life. Thereafter, telomere lengths are usually contracted to levels known as the "Hayflick limit". At this stage, non-regulatory T-cells do not proliferate anymore and undergo phenotypical and functional changes such as down-regulation of CD28 and acquisition of cytotoxic potential (4, 52, 53). Due to the fact that T_{regs} display even shorter telomeres than non-regulatory T-cells, it is conceivable that peripherally proliferating T_{regs} reach the "Hayflick limit" even earlier (54). Impaired T_{reg} homeostasis may then result in immune dysfunction with increased risk of immune-mediated disorders.

In addition to the shortened telomere length, TCR diversity is also contracted to at least 100-fold in elderly individuals (55). This has been explained by the observation that homeostatic proliferation of T-cells is antigen dependent. Thus, T-cells with a high affinity TCR to self-antigens or antigens deriving from chronic virus infections have a survival advantage over other T-cells (42, 56). Given that similar mechanisms drive peripheral proliferation of non-regulatory T-cells and T_{regs}, a reduction of T_{reg} TCR diversity (with a skew to certain antigens) can be expected in the elderly. Consequently, T_{regs} could mediate increased immunosuppression in response to specific self- (even if transformed) or viral antigens with increased incidence of malignancies and infections in the elderly. At the same time the reduced diversity of T_{regs} could result in decreased protection from autoimmunity (3).

DEVELOPMENT AND CELLULAR SENESCENCE OF T_{REGS}

FROM NAÏVE TO MEMORY CELL STATUS

Similar to the developmental stages known for non-regulatory T-cells (development form $CD45RA^+$ naïve to $CD45RO^+$ memory and finally to $CD28^-$ memory effector T-cells), different cellular subsets of T_{regs} were also observed. In humans, $CD4^+foxP3^+$ T_{regs} may have either a "naïve-like" phenotype characterized by the expression of $CD25^+CD45RA^+$ or a $CD25^{hi}CD45RO^+$ "memory-like" phenotype (54). In mice, naïve-like T_{regs} were characterized by the expression of CD25, CD62L, and CCR7 and by preferential homing to antigen-draining lymph nodes, where they

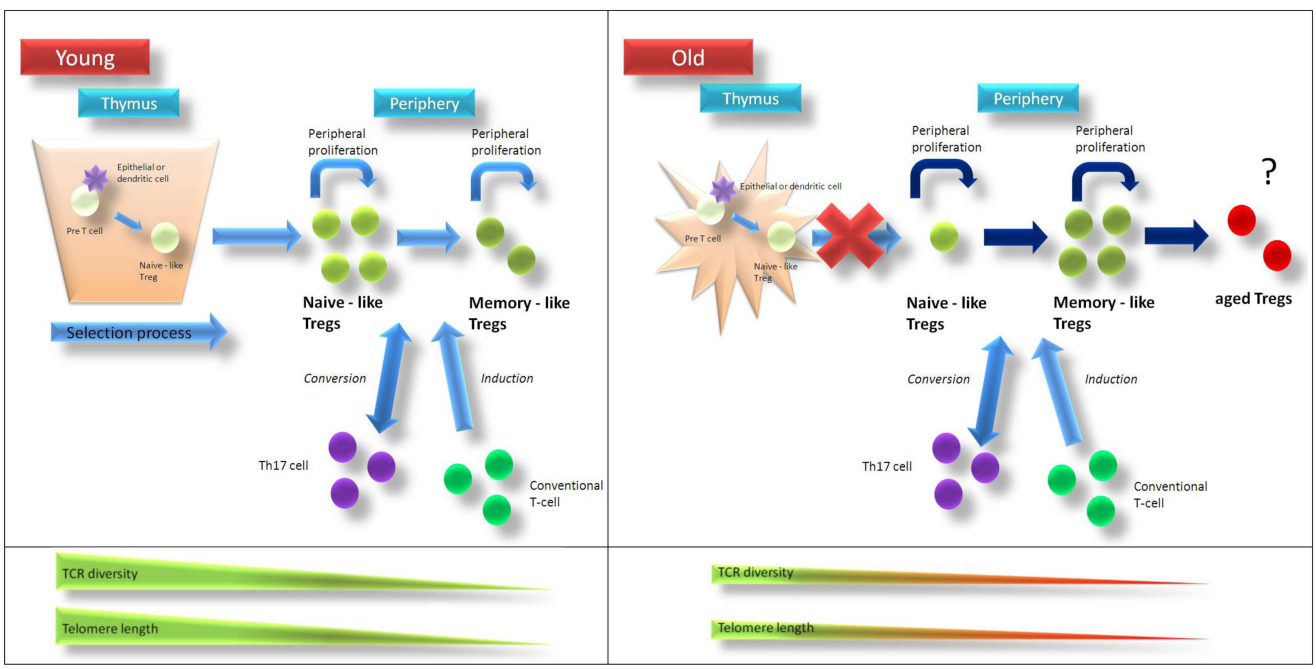

FIGURE 1 | Age-related changes of T_{reg} homeostasis. In young individuals T_{regs} are generated in the thymus and are released as "naïve-like" T_{regs} into circulation. After antigen-contact, T_{regs} develop into a "memory-like" phenotype. T_{reg} homeostasis is supported by homeostatic proliferation of "naïve-like" and "memory-like" T_{regs} as well as conversion of non-regulatory T-cells into T_{regs}. Telomere length and T-cell receptor diversity is higher in naïve-like compared to memory-like T_{regs}. After puberty thymic function is progressively lost and in aged individuals homeostatic proliferation of existing T_{regs} as well as conversion of non-regulatory T-cells into T_{regs} compensate for thymic failure to maintain T_{reg} pool. Due to ongoing homeostatic replication telomere length and T-cell receptor diversity of T_{regs} from elderly people are contracted compared to those from young individuals. Recurrent stimulation of T_{regs} might then lead to a status of "terminal-differentiation" with altered phenotype and function. T_{reg} *regulatory T-cell,* TCR . . . *T-cell receptor.*

were able to inhibit the induction of inflammation (10, 57, 58). Memory/effector-like T_{regs} (characterized by expression of CD29, CD44, ICOS, and LFA-1) migrated into non-lymphogenic tissues and sites of inflammation; a local down-regulation of immune reactions was shown (57, 58).

In humans, the highest prevalence of naïve-like T_{regs} were found in cord blood and it was assumed that these naïve-like T_{regs} are produced in the thymus (20, 59). The prevalence of memory-like T_{regs} increases rapidly during childhood and it was demonstrated that these memory-like T_{regs} have shorter telomeres and a lower content of TCR excision circles (Trecs) compared to naïve-like T_{regs} reflecting a longer replicative history (54). The mechanisms mediating the transition of a naïve-like T_{reg} into a memory-like phenotype still have to be explored; however, it is believed that antigen experienced dendritic cells migrating to secondary lymphoid tissues are involved. T_{regs} proliferate upon stimulation with autologous immature and mature dendritic cells (54, 60). A low surface expression of CD45RB on memory-like T_{regs} further supports the hypothesis of an antigen-driven development of naïve-like T_{regs}. CD45RB is normally down-regulated after repeated antigen-contact (61).

Human adult peripheral blood usually contains both, naïve-like and memory-like T_{regs}. Parallel to the reduction of total naïve T-cells, the quantity of naïve-like T_{regs} declines with age whereas the prevalence of memory-like T_{regs} increases (29, 62). The total pool of circulating T_{regs}; however, remains unchanged as mentioned above (19). As naïve-like T_{regs} exhibit a higher proliferative potential *in vitro* compared to memory-like T_{regs} it can be expected that the capacity of the immune system to downregulate abnormal immune responses declines with age (54).

END-DIFFERENTIATED T_{REGS} AND ASPECTS OF T_{REG} SENESCENCE

Replicative senescence of T-cells is a prominent feature of aging resulting from homeostatic proliferation and repetitive antigen exposure (63). The most important phenotypic feature of senescent T-cells is the loss of the type I transmembrane protein CD28, a major co-stimulatory molecule (64). From the functional perspective, non-regulatory $CD28^-$ T-cells produce large amounts of interferon γ, perforin, and granzyme B, providing them with the ability to lyse target cells (65). Another feature of $CD28^-$ T-cells is their longevity and persistence that can be explained by defects in the apoptotic pathway with upregulation of bcl-2 and Fas-associated death domain like IL-2-converting enzyme-like inhibitory protein (FLIP) (66, 67). Terminally differentiated T-cells also acquire new stimulatory receptors including killer cell immunoglobulin-like receptors (KIRs) and Toll-like receptors (TLRs) (68, 69). Thus, activation of $CD4^+CD28^-$ T-cells no longer depends on professional antigen-presenting cells, rather it is promoted by stress molecules as well as bacterial and/or viral products (65). The frequency of terminally differentiated $CD4^+CD28^-$ T-cells is increased in old individuals as well as in younger patients with autoimmune diseases such as rheumatoid arthritis or spondyloarthritis (70). Given that T_{regs} proliferate in the periphery to maintain the total T_{reg} pool after thymic failure it is plausible to hypothesize that T_{regs} may undergo terminal-differentiation as well.

Interestingly, a proportion of T_{regs} from aged mice showed decreased expression of CD25 (46, 71). These $CD25^{low}$ T_{regs} occurred predominantly in the spleen (24) but had comparable functional properties to $CD25^+$ T_{regs}. A similar $CD4^+CD25^-foxP3^+$ T_{reg} population has been observed in SLE patients. SLE patients are known to have a prematurely aged immune system (72) with accumulation of $CD28^-$ T-cells. A detailed characterization of $CD4^+CD25^-FoxP3^+$ T_{regs} regarding the expression of naïve/memory T-cell markers or determination of telomere lengths was unfortunately not performed. Further evidence for the occurrence of T_{reg} senescence was found in a study on healthy aged individuals reporting the occurrence of a $CD8^+CD25^+$ T_{reg} population lacking CD28 expression. These regulatory cells shared phenotypic and functional features with $CD4^+$ T_{regs} from the same population (73). The occurrence and possible characteristics of terminally differentiated $CD4^+$ T_{regs} is an interesting issue that has to be investigated by future studies.

CONCLUSION

Accumulating evidence suggests age-associated changes of T_{reg} prevalence and/or T_{reg} function. Due to involution of thymus after puberty peripheral mechanisms including homeostatic proliferation of T_{regs} or conversion of non-regulatory T-cells into T_{regs} compensate for the decreasing generation of new T_{reg} cells. However, these peripheral mechanisms are limited; this leads to altered composition of the T_{reg} pool. Age-related changes of T_{regs} are suspected to increase the risk of autoimmunity, cancer, and infections in the elderly; however, the exact mechanisms are still poorly understood. Current studies are limited by the difficult identification of human T_{regs} and the uncertainty whether circulating T_{regs} reflect the total T_{reg} pool or a cellular subset only. Future studies are required to investigate cellular senescence of T_{regs} and possible therapeutic approaches targeting T_{regs} in aged individuals.

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IL-15 fosters age-driven regulatory T cell accrual in the face of declining IL-2 levels

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We and others have shown that regulatory T cells (T_{reg}) accumulate dramatically with age in both humans and mice. Such T_{reg} accrual contributes to age-related immunosenescence as they reduce the response to tumors and parasite infection. While we reported earlier that aged T_{reg} have decreased expression of the pro-apoptotic molecule Bim and germline deletion of Bim promoted earlier accumulation of T_{reg}, it remains unclear whether the effects of Bim are: (i) T_{reg} intrinsic and (ii) dominant to other BH3-only pro-apoptotic molecules. Further, the mechanism(s) controlling Bim expression in aged Treg remain unclear. Here we show that T_{reg}-specific loss of Bim is sufficient to drive T_{reg} accrual with age and that additional loss of the downstream apoptotic effectors Bax and Bak did not exacerbate T_{reg} accumulation. Further, our results demonstrate that a subpopulation of T_{reg} expands with age and is characterized by lower expression of CD25 (IL-2Rα) and Bim. Mechanistically, we found that IL-2 levels decline with age and likely explain the emergence of $CD25^{lo}Bim^{lo}$ T_{reg} because T_{reg} in $IL\text{-}2^{-/-}$ mice are almost entirely comprised of $CD25^{lo}Bim^{lo}$ cells, and IL-2 neutralization increases $CD25^{lo}Bim^{lo}$ T_{reg} in both young and middle-aged mice. Interestingly, the T_{reg} population in aged mice had increased expression of CD122 (IL-2/IL-15Rβ) and neutralization or genetic loss of IL-15 led to less T_{reg} accrual with age. Further, the decreased T_{reg} accrual in middle-aged $IL\text{-}15^{-/-}$ mice was restored by the additional loss of Bim ($IL\text{-}15^{-/-}Bim^{-/-}$). Together, our data show that aging favors the accrual of $CD25^{lo}$ T_{reg} whose homeostasis is supported by IL-15 as IL-2 levels become limiting. These data have implications for manipulating T_{reg} to improve immune responses in the elderly.

Keywords: CD25, Bim, IL-2, IL-15, aging, T_{reg}

INTRODUCTION

Aging is associated with declining immune function, which contributes to increased infectious diseases, increased cancer, and decreased vaccine efficacy in the elderly. The aging immune system is characterized by a progressive deterioration in the adaptive immune system, particularly affecting T cells (Linton and Dorshkind, 2004; Maue et al., 2009). Factors contributing to T cell dysfunction with age include: (i) thymic involution and decreased naïve T cell production (Sempowski et al., 2002; Hale et al., 2006); (ii) impaired TCR signaling and immune synapse formation (Miller et al., 1997; Miller, 2000; Tamir et al., 2000; Garcia and Miller, 2002); (iii) impaired T cell proliferation (Murasko et al., 1987; Haynes et al., 2005); (iv) $CD8^+$ T cell clonal expansion driven by chronic infections (Khan et al., 2002; Ouyang et al., 2003; Clambey et al., 2005). While many of these effects are T cell intrinsic, recent work has found that regulatory T cell (T_{reg}) frequencies increase dramatically with age in both mice and humans and may contribute substantially to impaired T cell responses in aged hosts (Valmori et al., 2005; Nishioka et al., 2006; Sharma et al., 2006; Lages et al., 2008; Agius et al., 2009).

Regulatory T cells, a specialized subset of $CD4^+FoxP3^+$ T cells, are known to control the intensity of immune responses through modulating the activation and function of both effector T cells and antigen-presenting cells (Miyara and Sakaguchi, 2007; Onishi et al., 2008). The functionality of aged T_{reg} have not been well studied, however we have shown that aged $FoxP3^+$ T_{reg} have an equal or increased *in vitro* suppressive capacity compared to young T_{reg} (Lages et al., 2008). *In vivo*, depletion of $CD25^+$ T_{reg} allowed for a more robust $CD4^+$ T cell response against *Leishmania major* in aged mice, suggesting increased T_{reg} in the aged can dampen effector T cell activation (Lages et al., 2008). Additionally, T_{reg} accrual with age has been shown to inhibit anti-tumor responses (Sharma et al., 2006). Thus, aged T_{reg} appear to be functional *in vivo* and T_{reg} accrual may contribute significantly to immunosenescence in aging.

Many studies have looked at the factors involved in T_{reg} homeostasis in young mice, particularly the γc cytokines IL-2, IL-7, and IL-15. The receptor for IL-2 is comprised of CD25 (IL-2Rα), CD122 (IL-2/15Rβ), and CD132 (IL-2/15/7Rγ). IL-2 shares the IL-2/15Rβ receptor with IL-15, and the γc receptor (CD132) with

both IL-15 and IL-7. IL-2 is the dominant cytokine required for T_{reg} survival and homeostasis, as the loss of IL-15 or IL-7 signaling does not substantially affect the frequency of $CD4^+$ cells that are T_{reg} when IL-2 is present (Burchill et al., 2007; Bayer et al., 2008; Vang et al., 2008). However, CD132 and to a lesser extent CD122-deficient mice have a more profound loss of T_{reg} compared to IL-2 or CD25 deficient mice, suggesting that IL-15 and/or other γc cytokines also contribute to T_{reg} homeostasis (Fontenot et al., 2005a). All of these studies examining the requirements for cytokine signaling in T_{reg} development and survival have been done in young mice, and the role for the γc cytokines in aged T_{reg} homeostasis is unclear.

Our previous study showed that Bim plays a major role in T_{reg} homeostasis and that Bim levels decline significantly in aged T_{reg} (Chougnet et al., 2011). Further germline deletion of Bim led to significantly faster accrual of T_{reg} (Chougnet et al., 2011). Here, we found that T_{reg}-specific loss of Bim was sufficient to drive T_{reg} accrual and that Bim was the dominant pro-apoptotic molecule driving T_{reg} accrual. Further, decreased Bim levels in aged T_{reg} is reflected by decreased Bim mRNA and increased Bim turnover. Additionally, declining IL-2 levels with age resulted in reduced levels of CD25 and increased levels of CD122 which foster T_{reg} dependence upon IL-15, which, in turn, functions to restrain the remaining Bim in aged T_{reg}.

MATERIALS AND METHODS

MICE

C57BL/6 mice were purchased from either Taconic Farms (Germantown, NY, USA) or the National Institutes of Aging colony located at Charles River Laboratories (Wilmington, MA, USA). B6.129P2-*Il2*^{tm1Hor}/J (IL-$2^{-/-}$) mice and their C57BL/6 controls were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ mice on the C57BL/6 background were purchased from The Jackson Laboratory and aged in house. $Bim^{-/-}$ mice have been backcrossed to C57BL/6 mice for at least 20 generations. $Bim^{f/f}$ mice were generated at the Walter and Eliza Hall Institute as part of a collaborative effort with Dr. P. Bouillet. Briefly, a targeting vector was created by flanking coding exons 2, 3, and 4 of *Bim* with loxP sites. The vector was electroporated into C57BL/6 embryonic stem (ES) cells and homologously recombined ES cells were selected with hygromycin. The hygromycin cassette was removed by crossing the $Bim^{f/f}$ mice with B6.Cg-Tg(ACTFLPe)9205Dym/J (Jackson Labs) to generate a Bim floxed allele that could be crossed to tissue – specific cre transgenic mice to achieve tissue-specific deletion of Bim. Offspring from this cross were screened for removal of the Hygromycin cassette and maintenance of the conditional Bim allele. Mice were then bred to Cre-expressing mice and offspring screened for lack of the ACTFLPe allele. Lck-Cre $Bax^{f/f}Bak^{-/-}$ mice were a gift from Dr. S. Korsmeyer and were previously described (Takeuchi et al., 2005). IL-15-deficient mice on the C57BL/6 background were purchased from Taconic Farms, mated with the $Bim^{-/-}$ mice to generate IL-$15^{-/-}$ $Bim^{-/-}$ mice, and aged in house. FoxP3-IRES-DTR-GFP knock-in C57BL/6 mice (Kim et al., 2007) and FoxP3-Cre mice (Rubtsov et al., 2008) were a gift from Dr. A. Rudensky. FoxP3-IRES-DTR-GFP mice were aged in house. FoxP3-Cre mice were mated with $Bim^{f/f}$ mice and aged in house. Mice were housed under specific pathogen-free conditions. All animal protocols were reviewed and approved by our Institutional Animal Care and Use Committee.

FLOW CYTOMETRY

Spleens, lymph nodes (inguinal, axillary, and brachial), and thymi were harvested and crushed through 100 μm filters (BD Falcon) to generate single-cell suspensions. About 1×10^6 cells were surface stained with Abs against CD4 (BD Biosciences, San Diego, CA, USA), CD25 (eBioscience, San Diego, CA, USA), CD44 (eBioscience), CD122 (Biolegend, San Diego, CA, USA), CD45.2 (eBioscience) and intracellularly for FoxP3 (eBioscience), Bim (Cell Signaling Technology, Beverly, MA, USA), and Ki67 (eBioscience). All intracellular stains were performed using the eBioscience FoxP3 staining protocol. For detection of Bim, secondary anti-rabbit IgG Abs were used (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Invitrogen, Carlsbad, CA, USA; or Cell Signaling Technology). Data were acquired on an LSRII flow cytometer (BD Biosciences), analyzed using FACSDiva software (BD Biosciences), and histogram overlays were prepared using FlowJo software (Tree Star).

ADOPTIVE TRANSFERS

Spleen cells from young (3–4 months) and old (19–23 months) FoxP3-IRES-DTR-GFP mice were enriched for $CD4^+$ cells using the negative selection MACS $CD4^+$ T cell Isolation Kit II (Miltenyi Biotec, Auburn, CA, USA) and stained with CD4 antibody. $CD4^+FoxP3^{GFP+}$ cells were then sorted by FACSAria (BD Biosciences), and >85% purity was obtained. About 5×10^5 cells were injected i.v. into young (2 months) or aged (15 months) C57BL/6 congenic CD45.1 recipient mice. The recipient mice were sacrificed either 1.5 or 10 days post-transfer, spleens were harvested, and single-cell suspensions were stained for CD4, CD45.2, CD25, and intracellularly for FoxP3. Cells were fixed with 4% methanol-free formaldehyde (Polysciences, Warrington, PA, USA), instead of the eBioscience FoxP3 fix-perm buffer, to better retain GFP within the cells.

IN VIVO CYTOKINE CAPTURE ASSAY

IL-2 *in vivo* cytokine capture assay (IVCCA) was performed as described (Finkelman and Morris, 1999; Finkelman et al., 2003). Briefly, young (2–5 months), middle-aged (11–12 months), and old (>15 months) C57BL/6 mice were injected i.v. with 10 μg of biotinylated anti-IL-2 capture antibody (JES6-5H4-BD Biosciences), or with PBS as a control, and mice were bled 24 h later and serum was collected. Ninety-six-well Costar plates were coated overnight with anti-IL-2 JES61A12 (eBioscience) and then a luminescent ELISA was performed. For the cytokine:anti-cytokine mAb standard, 100 ng of recombinant mouse IL-2 (R&D) was incubated with 10 μg of the anti-IL-2 capture antibody (JES6-5H4) for 10 min, and 100 ng/ml aliquots were stored in −80° C freezer. The IL-2 concentration obtained from our PBS control was subtracted from our young and old samples to remove the ELISA background.

IN VIVO CYTOKINE NEUTRALIZATION

Anti-IL-2 Ab (clones S4B6 and Jes61A12) and rat IgG2A isotype control (2A3) were purchased from BioXcell (West Lebanon, NH,

USA). Anti-IL-15 (M96) was a kind gift from Amgen (Seattle, WA, USA). For IL-2 neutralization: 3- and 12-month-old mice were injected i.p. with 170 μg of S4B6 and Jes61A12, or with isotype control (2A3), on days 0, 1, 2, 4, 6, and sacrificed on day 7 and spleens were harvested. For IL-15 neutralization: 3- and 12-month-old mice were injected i.p. with 25 μg of M96 on days 0, 2, 4, 6, and sacrificed on day 7. IL-15 neutralization was confirmed by assessing natural killer cells, which showed >60% deletion in the spleen (data not shown).

CYCLOHEXIMIDE ASSAY

Splenocytes from either young (3 months) or old (22 months) C57BL/6 mice were cultured with or without cycloheximide (20 μM; Sigma-Aldrich, St. Louis, MO, USA) for 8 h at 37° C. Cells were stained for CD4, FoxP3, and Bim and analyzed by flow cytometry. Percent decrease in Bim expression was determined by comparing Bim expression between cells cultured with our without cycloheximide.

QUANTITATIVE REAL-TIME PCR

Spleen cells from young (3 months) and old (22 months) FoxP3-IRES-DTR-GFP mice were enriched for $CD4^+$ cells using the negative selection MACS $CD4^+$ T cell Isolation Kit II (Miltenyi Biotec) and stained with CD4 antibody. $CD4^+FoxP3^{GFP+}$ and $CD4^+FoxP3^{GFP-}$ cells were then sorted by FACSAria (BD Biosciences) and >90% purity was obtained for both populations. RNA was isolated from cells using the RNeasy Mini Kit (Qiagen) and converted into cDNA using Superscript II Reverse Transcriptase (Invitrogen, CA, USA). The primers used for Bim were: 5′-ACAAACCCCAAGTCCTCCTT-3′ and 5′-GTTTCGTTGAACTCGTCTCC-3′; and for the internal control L19: 5′-CCTGAAGGTCAAAGGGAATGTG-3′ and 5′-GCTTTCGTGCTTCCTTGGTCT-3′. Quantitative real-time PCR was performed with Roche LightCycler 480 SYBRGreen 1 Master Mix using the Roche LightCycler 480 II instrument (Roche Diagnostics).

RESULTS

BIM PROGRESSIVELY DECREASES IN T_{reg} WITH AGE

We have previously shown that T_{reg} in aged mice have decreased expression of Bim, and $Bim^{-/-}$ mice accrued T_{reg} more rapidly, together suggesting loss of Bim expression drives T_{reg} accrual with age (Chougnet et al., 2011). Here we investigated this in more detail and found that the decrease in Bim expression with age is a progressive process (**Figure 1A**). The major loss in Bim expression is observed by 12 months (threefold; fourfold by 21 months), and most significantly within the $CD4^+FoxP3^+$ population, not in the $CD4^+FoxP3-$ population (**Figure 1A**) (Chougnet et al., 2011).

As Bim is subject to transcriptional, translational, and post-translational control, we next determined the relative contribution of these mechanisms. First, $CD4^+FoxP3^{GFP+}$ T_{reg} and $CD4^+FoxP3^{GFP-}$ non-T_{reg} were sorted from young (3 months) and old (22 months) $FoxP3^{GFP}$ reporter mice (Kim et al., 2007). T_{reg} cells from old mice showed significantly decreased Bim mRNA (~2-fold), which was observed to a lesser extent in non-T_{reg} (**Figure 1B**). Next, to examine Bim protein turnover, splenocytes from young and old mice were cultured with cycloheximide, an antibiotic that inhibits translation. Interestingly, old $FoxP3^+$ T_{reg} cells had significantly greater Bim turnover compared to young T_{reg} (30 vs. 18%, respectively; **Figure 1C**). These data indicate that both decreased Bim transcription and increased Bim turnover likely contribute to the decreased Bim protein levels observed with age.

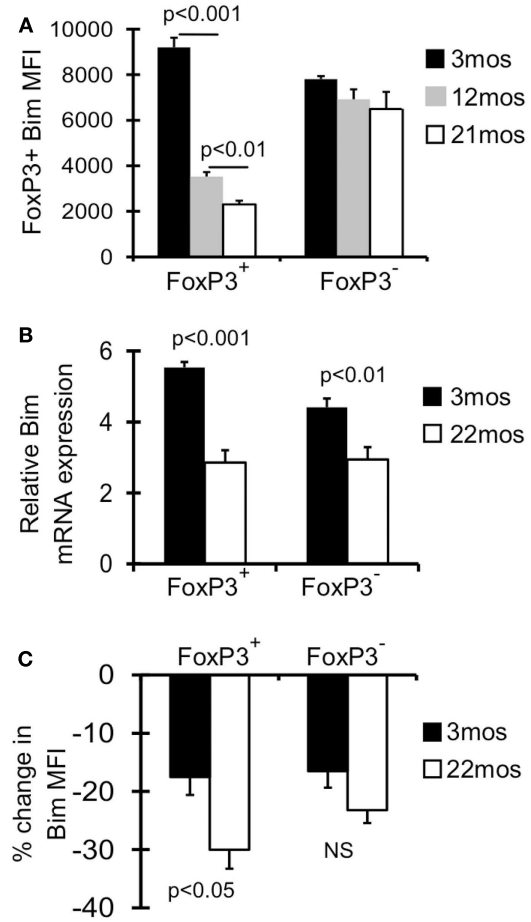

FIGURE 1 | Bim levels in T_{reg} are progressively decreased with age. (A) Splenocytes from 3 month ($n=4$), 12 month ($n=4$), and 21-month-old ($n=5$) mice were stained with Abs against CD4, FoxP3, and Bim and analyzed by flow cytometry. Results show the average mean fluorescent intensity (MFI) of Bim in $CD4^+FoxP3^+$ T cells (±SE). The p values represent the difference between 3 and 12 months; 12 and 21 months (Student's t test). **(B)** $FoxP3^{GFP}$ reporter mice were used to sort $CD4^+FoxP3^{GFP+}$ T_{reg}, $CD4^+FoxP3^{GFP-}$ non-T_{reg} from young (3 months; $n=4$) and old (22 months; $n=4$) mice. Results show the relative expression of Bim mRNA in 3 and 22 months mice (±SE). The p values represent the difference between 3 and 22 months (Student's t test). **(C)** Splenocytes from 3 months ($n=4$) and 22 months ($n=3$) mice were cultured with or without cycloheximide for 8 h at 37° C, and then stained for CD4, FoxP3, and Bim and analyzed by flow cytometry. Results show the percent decrease in Bim expression within $CD4^+FoxP3^+$ and $CD4^+FoxP3-$ cells cultured with cycloheximide (±SE). Results are representative of at least two independent experiments.

T_{reg} INTRINSIC LOSS OF BIM EXPRESSION PROMOTES ACCRUAL WITH AGE

It remains unclear whether the effects of Bim on T_{reg} accrual are T_{reg} intrinsic. To address this question, we crossed FoxP3-Cre mice

(Rubtsov et al., 2008) with mice that have loxP flanked Bim on both alleles ($Bim^{f/f}$) (**Figure 2A**), and aged these FoxP3-Cre $Bim^{f/f}$ mice to 6 months. Bim staining in FoxP3-$Cre^{+}Bim^{f/f}$ mice was comparable to $Bim^{-/-}$ mice, confirming efficient deletion of Bim (**Figure 2B**). There was a small fraction of $CD4^{+}FoxP3^{-}$ cells with somewhat reduced expression of Bim, suggesting that some cells may have deleted one or both alleles of Bim (**Figure 2B**). However, we did not observe loss of Bim in non-T cell populations (i.e., $CD11c^{+}$ or $B220^{+}$ cells, data not shown). The frequency of T_{reg} in FoxP3-$Cre^{+}Bim^{f/f}$ mice was increased at 2 months of age, compared to either FoxP3-Cre^{-} $Bim^{f/f}$ or $Bim^{-/-}$ mice (**Figure 2C**). This increase in T_{reg} at 2 months of age may be due to increased thymic output in FoxP3-$Cre^{+}Bim^{f/f}$ mice, as we see increased frequencies and numbers of $FoxP3^{+}$ cells in the thymi of FoxP3-$Cre^{+}Bim^{f/f}$ mice compared to FoxP3-Cre^{-} $Bim^{f/f}$ (data not shown). Importantly, by 6 months of age the frequency of T_{reg} in FoxP3-$Cre^{+}Bim^{f/f}$ mice increased even further, reaching levels observed in $Bim^{-/-}$ mice (**Figure 2C**). These data show that T_{reg} intrinsic loss of Bim is sufficient to promote T_{reg} accumulation with age.

BIM IS THE MAJOR PRO-APOPTOTIC PROTEIN RESPONSIBLE FOR T_{reg} HOMEOSTASIS

Although our data incriminate Bim as the major regulator of T_{reg} accumulation with age, we addressed the contribution of other pro-apoptotic proteins in T_{reg} survival, by using mice with T cell specific deletion of Bax and Bak, Lck-$Cre^{+}Bax^{f/f}Bak^{-/-}$ mice. Bax and Bak are the downstream mediators of pro-apoptotic proteins, and the deletion of both genes eliminates apoptosis through the intrinsic pathway (Youle and Strasser, 2008). If other pro-apoptotic proteins were critical in limiting T_{reg} survival, then T_{reg} accrual in Lck-Cre^{+} mice would be significantly greater than that observed in $Bim^{-/-}$ mice. One caveat of comparing T_{reg} accrual between Lck-$Cre^{+}Bax^{f/f}Bak^{-/-}$ mice and $Bim^{-/-}$ mice is that non-T_{reg} loss of Bim could affect T_{reg} homeostasis; however, our data in FoxP3-$Cre^{+}Bim^{f/f}$ show that T_{reg}-specific loss of Bim is sufficient to drive accrual to levels similar to those observed in $Bim^{-/-}$ mice. T cell loss of Bax and Bak resulted in increased T_{reg} accumulation at 15 months of age compared to Lck-Cre^{-} mice (20% increase compared to 10% increase, respectively; **Figure 2D**). Importantly, the frequency of T_{reg} was similar in 15-month-old Lck-$Cre^{+}Bax^{f/f}Bak^{-/-}$ mice and age-matched $Bim^{-/-}$ mice, demonstrating that Bim is the major pro-apoptotic protein controlling T_{reg} survival.

BOTH SURVIVAL-DEPENDENT AND – INDEPENDENT MECHANISM(S) CONTRIBUTE TO THE EMERGENCE OF BIM^{LO} T_{reg}

Old Bim^{lo} T_{reg} have increased survival *ex vivo* (Chougnet et al., 2011); however, it remains unclear if T_{reg} with lower Bim expression emerge *in vivo* because they are afforded a selective survival advantage. To test this, we examined Bim levels in Lck-$Cre^{+}Bax^{f/f}Bak^{-/-}$ T_{reg} as their levels of Bim will be largely irrelevant to their survival. Notably, the levels of Bim were significantly increased in Lck-Cre^{+} mice compared to Lck-Cre^{-} mice, indicating T_{reg} are able to tolerate higher levels of Bim in the absence of Bax and Bak (**Figure 3A**). As expected, Bim levels in T_{reg} from

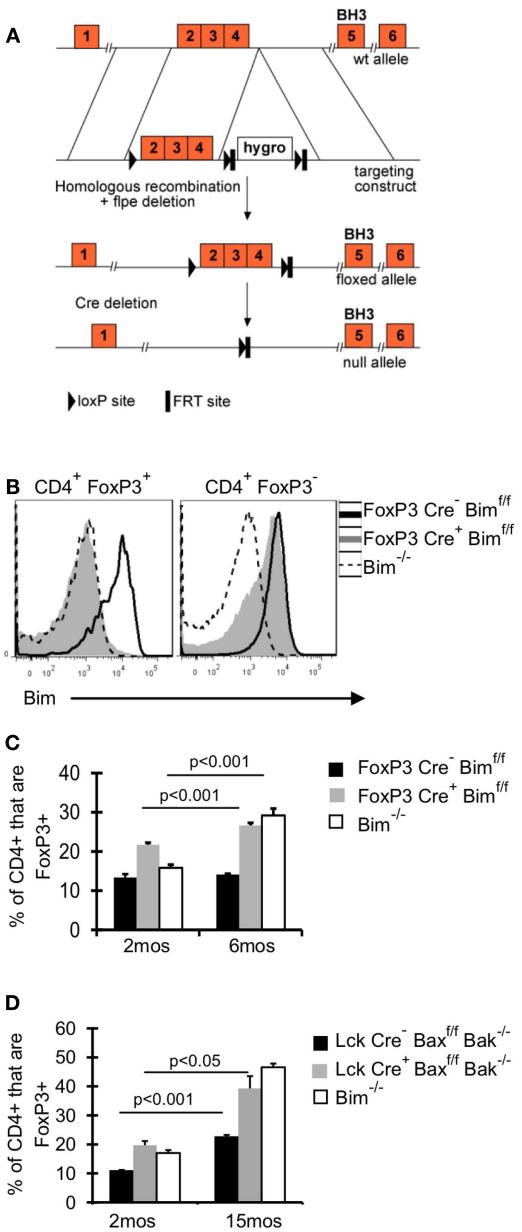

FIGURE 2 | Bim is the dominant BH3-only molecule in T_{reg} and its effects on T_{reg} homeostasis are T_{reg} intrinsic. (A) A conditional *Bim* allele was generated by targeting loxP sites upstream of exon 2 and downstream of exon 4. **(B,C)** Cells from the lymph nodes of 2- and 6-month-old FoxP3-Cre^{-} $Bim^{f/f}$, FoxP3-$Cre^{+}Bim^{f/f}$, and $Bim^{-/-}$ mice were stained for CD4, FoxP3, and Bim and analyzed by flow cytometry. **(B)** Histograms show the expression of Bim in $CD4^{+}FoxP3^{+}$ and $CD4^{+}FoxP3^{-}$ T cells from 2-month-old mice. **(C)** Results show the frequency of $CD4^{+}$ cells that are $FoxP3^{+}$ in 2 month ($n = 5$/group) and 6-month-old mice ($n = 3$–4/group; ±SE). The *p* values represent the difference between 2 and 6 months (Student's *t* test). Results are representative of two independent experiments. **(D)** Splenocytes from Lck-$Cre^{-}Bax^{f/f}Bak^{-/-}$ ($n = 3$–5/group), Lck-$Cre^{+}Bax^{f/f}Bak^{-/-}$ ($n = 3$–5/group), and $Bim^{-/-}$ ($n = 3$–4/group), mice were stained for CD4, FoxP3, and Bim and analyzed by flow. The $Bim^{-/-}$ mice were analyzed in an independent experiment. Results show the frequency of $CD4^{+}$ cells that are $FoxP3^{+}$ in 2- and 15-month-old mice (±SE). The *p* values represent the difference between 2 and 15 months (Student's *t* test).

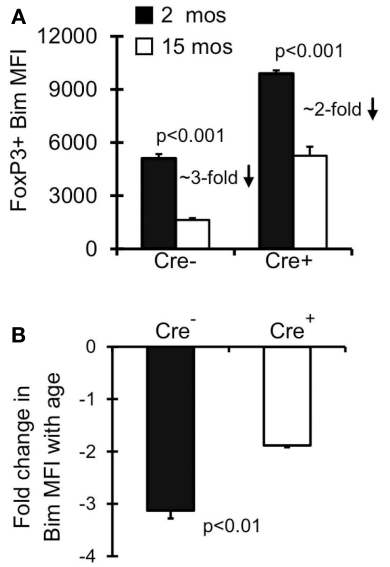

FIGURE 3 | Both environment and survival contribute to the emergence of Bim^{lo} T_{reg}. Splenocytes from Lck-Cre^{-}Bax$^{f/f}$Bak$^{-/-}$ ($n = 3$–5/group) and Lck-Cre^{+}Bax$^{f/f}$Bak$^{-/-}$ ($n = 3$–5/group) mice were stained for CD4, FoxP3, and Bim and analyzed by flow. **(A)** Results show the Bim MFI in CD4^{+}FoxP3^{+} cells (±SE). The *p* values represent the difference between 2 month-old and 15 month-old mice (Student's *t* test). **(B)** Results show the fold change in CD4^{+}FoxP3^{+} Bim expression in 15 month Lck-Cre^{-}Bax$^{f/f}$Bak$^{-/-}$ and Lck-Cre^{+}Baxf^{f}Bak$^{-/-}$ mice compared to their 2 month counterparts. The *p* values represent the difference between Lck-Cre^{-} and Lck-Cre^{+} (Student's *t* test).

15-month-old Lck-Cre^{-} mice was significantly decreased compared to their 2-month-old counterparts (**Figures 3A,B**). Bim was also decreased in 15-month-old Lck-Cre^{+} mice (**Figures 3A,B**), but the fold-decrease in Bim expression in Lck-Cre^{-} mice was greater than in Lck-Cre^{+} mice (threefold vs. twofold, respectively; **Figure 3B**). Together, these data suggest that Bim levels are controlled in T_{reg} by both survival-dependent and independent mechanisms.

AN AGED ENVIRONMENT PROMOTES THE EMERGENCE OF CD25^{LO} T_{reg}

T_{reg} are described in the literature as CD4^{+}FoxP3^{+} CD25^{hi}, however there is a substantial population of CD4^{+}FoxP3^{+} CD25^{lo} cells in young mice (∼10–20% of FoxP3^{+} cells; **Figures 4A,B**). Interestingly, this population of CD25^{lo} T_{reg} expands with age, comprising 50% of the T_{reg} population by middle-aged (**Figures 4A,B**) (Nishioka et al., 2006; Lages et al., 2008). Importantly, while both CD25^{lo} and CD25^{hi} T_{reg} expand with age, the relative increase in CD25^{lo} T_{reg} is much greater (**Figure 4C**). To assess whether the aged environment drives CD25^{lo} T_{reg} accumulation, we adoptively transferred young (3–4 months) and old (19–23 months) T_{reg} into young (2 months) and aged (15 months) recipient mice (**Figure 4D**). At 1.5 and 10 days later, recipient mice were sacrificed and the frequency of CD25^{lo} T_{reg} from donors was assessed (**Figure 4E**). Interestingly, when young cells were placed into aged recipients, we observed an emergence of CD25^{lo} T_{reg}. Similarly, CD25^{lo} T_{reg} were enriched slightly if old T_{reg} were transferred into aged mice. Increases in CD25^{lo} T_{reg} are likely not due to increased proliferation as there was an equivalent percentage of CD25^{lo} and CD25^{hi} T_{reg} that were Ki67^{+} after transfer into aged mice (**Figure A1A** in Appendix). Further, the total numbers of CD25^{lo} T_{reg} were affected as the ratio of CD25^{hi} to CD25^{lo} T_{reg} decreased by day 10 post-transfer (**Figure A1B** in Appendix), suggesting that CD25^{hi} T_{reg} may lose expression of CD25. On the other hand, when young or old T_{reg} were placed into young donors, we did not see an enrichment of CD25^{lo} T_{reg}. Together, these data strongly suggest that environmental changes with age support the emergence of CD25^{lo} T_{reg}.

DECLINING IL-2 LEVELS WITH AGE FOSTERS THE EMERGENCE OF CD25^{LO}BIM^{LO} T_{reg}

IL-2 signaling is known to promote expression of its own receptor CD25 (IL-2Rα) (Liao et al., 2013), and could be critical for maintaining CD25^{hi} T_{reg}. As assessed in the serum, levels of IL-2 were significantly decreased in middle-aged and old mice compared to young mice (**Figure 5A**). Furthermore, T_{reg} in IL-2$^{-/-}$ mice were comprised almost entirely of CD25^{lo} cells (**Figure 5B**). Interestingly, CD25^{lo} T_{reg} were Bim^{lo} at any age (**Figure 5C**), and this lower expression of Bim may promote their increased accrual with age. To assess whether IL-2 also controls Bim expression we measured Bim levels in T_{reg} from IL-2$^{-/-}$ mice. Strikingly, Bim levels were significantly decreased in T_{reg} from IL-2$^{-/-}$ mice (**Figure 5D**). This loss of Bim expression occurred in the periphery, as Bim levels in thymic T_{reg} were similar between IL-2$^{-/-}$ and wild-type mice (data not shown). To independently test the role of IL-2 in maintaining CD25^{hi}Bim^{hi} T_{reg}, we neutralized IL-2 in young mice (3 months), which resulted in decreased expression of Bim and an enrichment of CD25^{lo} T_{reg} (**Figures 5E,F**). Also, the total number of T_{reg} were not decreased after IL-2 neutralization, suggesting that CD25^{hi} T_{reg} lose expression of CD25 (data not shown). Taken together, these data show that IL-2 is critical for maintaining T_{reg} with a CD25^{hi}Bim^{hi} phenotype, both in young and aged mice. These data also suggest that reduced IL-2 with age promotes the emergence of CD25^{lo}Bim^{lo} cells.

IL-15 CONTRIBUTES TO T_{reg} HOMEOSTASIS IN MIDDLE-AGED MICE

We next examined the expression of receptors for other common γ cytokines which can function redundantly with IL-2 to support T_{reg} homeostasis and survival in young mice (Fontenot et al., 2005a; Burchill et al., 2007; Bayer et al., 2008; Vang et al., 2008). Although the expression of IL-7Rα (CD127) increases with age, we previously showed that blocking CD127 signaling alone did not affect T_{reg} survival in 12-month-old mice (Chougnet et al., 2011). IL-2Rβ (CD122) is a part of both the IL-2 and IL-15 receptor, and its expression was significantly upregulated on T_{reg} by 12 months of age (**Figure 6A**). Additionally, CD122 expression was increased twofold on T_{reg} in IL-2$^{-/-}$ mice (AVG MFI: WT = 1265; IL-2$^{-/-}$ = 2378; $p < 0.001$) (**Figure 6B**). We therefore assessed the role of IL-15 in T_{reg} accumulation in middle-aged mice, as increased T_{reg} CD122 expression and increased CD25^{lo} T_{reg} by 12 months of age may reflect a change in T_{reg} cytokine dependency. While there was no difference in T_{reg} frequency in young IL-15$^{-/-}$ and wild-type mice, the frequency of T_{reg} was significantly reduced in IL-15$^{-/-}$ mice by 12 months of age (**Figure 6C**).

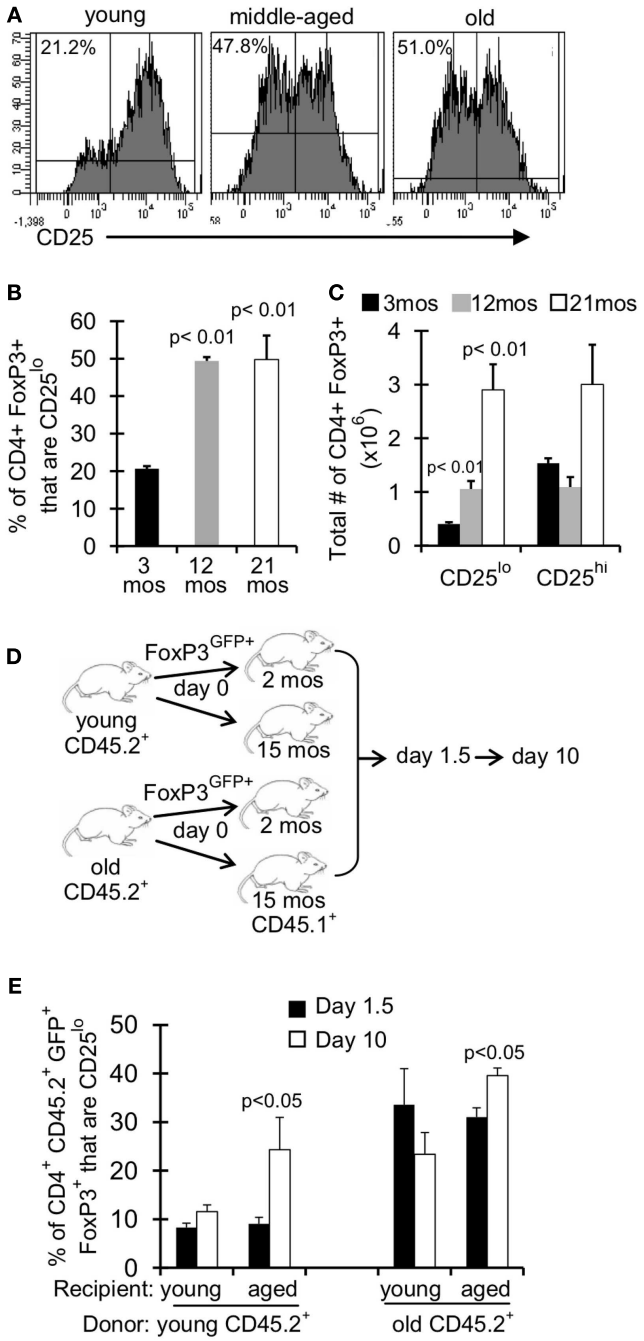

FIGURE 4 | An aged environment promotes emergence of CD25^{lo} T_{reg}. **(A–C)** Splenocytes from young (3 months; $n = 4$), middle-aged (12 months; $n = 4$), and old (21 months; $n = 5$) mice were stained for CD4, FoxP3, and CD25 and analyzed by flow. **(A)** Histograms show the expression of CD25 on CD4$^+$FoxP3$^+$ cells. Percentages are the frequencies of CD4$^+$FoxP3$^+$ that are CD25^{lo}. **(B)** Results show the frequency of CD4$^+$FoxP3$^+$ cells that are CD25^{lo} (±SE). The p values represent the difference compared to 3-month-old mice (Student's t test). **(C)** Results show the total number of CD4$^+$FoxP3$^+$ cells that are CD25^{lo} or CD25^{hi}. The p values represent the difference compared to 3-month-old mice (Student's t test). Results are representative of at least three independent experiments. **(D,E)** CD4$^+$FoxP3^{GFP+} T cells were sorted from splenocytes of young (3–4 months) *(Continued)*

FIGURE 4 | Continued
and old (>18 months) CD45.2 FoxP3^{GFP} reporter mice. About 5×10^5 CD4$^+$FoxP3^{GFP+} cells were injected i.v. into young (2 months) and aged (15 months) congenic CD45.1 mice. Mice were sacrificed at day 1.5 ($n = 3$–5/group) and day 10 ($n = 3$–5/group) post-injection. Cells from the spleen were stained for CD4, FoxP3, CD25, and CD45.2 and analyzed by flow. **(E)** Results show the frequency of CD4$^+$CD45.2$^+$FoxP3$^+$GFP$^+$ cells that are CD25lo (±SE). The p values represent the difference between day 1.5 and 10 (Student's t test).

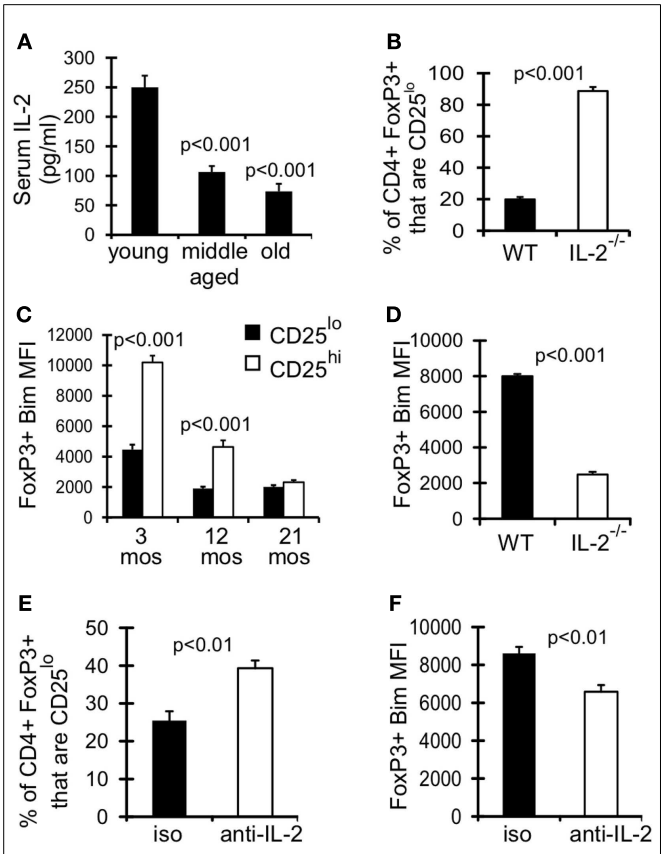

FIGURE 5 | Declining IL-2 levels drives emergence of CD25^{lo}Bim^{lo} T_{reg}. **(A)** Serum IL-2 was determined by IVCCA. Results show the average serum IL-2 levels from young (3–4 months; $n = 10$), middle-aged (11–12 months; $n = 11$), and old (>15 months; $n = 9$) mice (±SE). Results are representative of 3 independent experiments. The p values represent the difference compared to young (Student's t test). **(B,D)** Splenocytes from 5-week-old C57BL/6 wild-type (WT; $n = 4$) and IL-2$^{-/-}$ ($n = 3$) mice were stained for CD4, FoxP3, CD25, and Bim and analyzed by flow. **(B)** Results show the frequency of CD4$^+$FoxP3$^+$ cells that are CD25lo (±SE) and are representative of two independent experiments. **(C)** Splenocytes from 3 month ($n = 4$), 12 month ($n = 4$), and 21-month-old ($n = 5$) mice were stained for CD4, FoxP3, CD25, and Bim and analyzed by flow. Results show the Bim MFI of CD4$^+$FoxP3$^+$CD25^{lo} cells and CD4$^+$FoxP3$^+$CD25^{hi} cells (±SE). The p values represent the difference between CD25^{lo} and CD25^{hi} T_{reg} (Student's t test). **(D)** Results show the Bim MFI of CD4$^+$FoxP3$^+$ T_{reg} from WT and IL-2$^{-/-}$ mice (±SE) and are representative of two independent experiments. **(E,F)** Three-month-old mice were injected with IL-2 neutralizing antibodies or IgG isotype control ($n = 4$/group). Spleens were harvested at day 7 and stained for CD4, FoxP3, CD25, and Bim. **(E)** Results show the frequency of CD4$^+$FoxP3$^+$ cells that are CD25^{lo} (±SE). **(F)** Results show the Bim MFI of CD4$^+$FoxP3$^+$ T_{reg} (±SE).

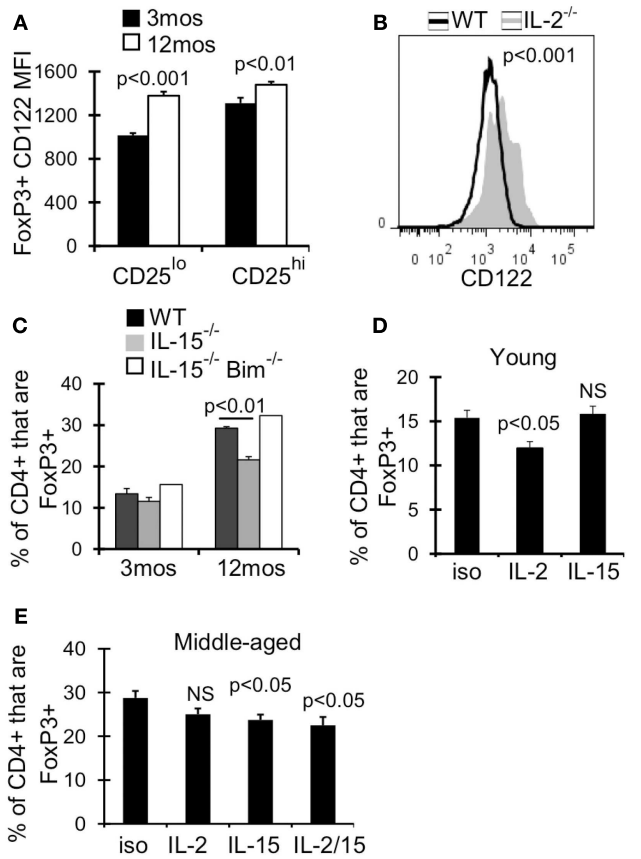

FIGURE 6 | IL-15 antagonizes Bim to promote T_{reg} homeostasis in middle-aged mice. Splenocytes from 3 month ($n = 4$) and 12-month-old ($n = 4$) mice **(A)** or 5-week-old WT ($n = 4$) and IL-$2^{-/-}$ ($n = 3$) mice **(B)** were stained for CD4, FoxP3, and CD122, and analyzed by flow. **(A)** Results show the MFI of CD122 on $CD4^+FoxP3^+$ cells (±SE). **(B)** Histograms show CD122 expression on $CD4^+FoxP3^+$ cells. The *p* value represents the difference between the average CD122 MFI from WT and IL-$2^{-/-}$ mice (Student's *t* test). **(C)** Splenocytes from WT ($n = 3$–4/group), IL-$15^{-/-}$ ($n = 3$/group), and IL-$15^{-/-}$ Bim$^{-/-}$ ($n = 2$/group) were stained for CD4 and FoxP3 and analyzed by flow. Results show the frequency of $CD4^+$ cells that are $FoxP3^+$ (±SE) and are representative of two independent experiments. Young (3 months; $n = 4$/group) **(D)** and middle-aged (12 months; $n = 5$/group) **(E)** mice were injected with IL-2 and/or IL-15 neutralizing antibody or with IgG isotype control. Spleens were harvested at day 7 and stained for CD4, FoxP3, CD25, and Bim. **(D,E)** Results show the frequency of $CD4^+$ cells that are $FoxP3^+$ (±SE). The *p* values represent the difference between antibody treated and isotype control (Student's *t* test).

Importantly, the additional loss of Bim (IL-$15^{-/-}$Bim$^{-/-}$), rescued the loss of T_{reg} in middle-aged IL-$15^{-/-}$ mice (**Figure 6C**), suggesting that IL-15 supports T_{reg} homeostasis in aged mice by combating Bim-mediated cell death.

To test whether IL-15 promotes T_{reg} survival when IL-2 levels decline, we neutralized IL-2 and/or IL-15 in young and middle-aged mice. IL-2 neutralization in young mice (3 months) but not middle-aged mice (12 months) resulted in a significant loss of T_{reg} (**Figures 6D,E**), supporting that IL-2 is less critical for T_{reg} homeostasis in an aged environment. Conversely, IL-15 neutralization in middle-aged mice resulted in a significant loss of T_{reg}, which was not observed in young mice (**Figures 6D,E**). Importantly, neutralization of IL-2 and IL-15 did not result in further loss of T_{reg} compared to IL-15 neutralization alone (**Figure 6E**). Together, our data suggests that in an aging environment, IL-15 supports T_{reg} survival in the face of declining IL-2.

DISCUSSION

Age-related immunosuppression is multi-factorial, encompassing cell-intrinsic defects within T and B cells, as well as population based defects such as a loss of naïve T cells and increased levels of regulatory T cells (Valmori et al., 2005; Nishioka et al., 2006; Sharma et al., 2006; Lages et al., 2008; Agius et al., 2009). Increased levels of T_{reg} are observed in both mice and humans and functionally suppress T cell responses to both parasites (*L. major*) and tumors (Sharma et al., 2006; Lages et al., 2008). We also recently showed that Bim is a key controller of T_{reg} homeostasis as T_{reg} accrual is accelerated in Bim-deficient (Bim$^{-/-}$) mice (Chougnet et al., 2011); although it was unclear if this was due to T cell specific effects of Bim in peripheral T_{reg}. Here, we focused on further understanding the mechanisms controlling T_{reg} accrual in aging.

First, the germline deletion of Bim results in loss of Bim from all tissues and it was possible that non-T cell expression of Bim controls T_{reg} homeostasis. In particular, the increased lifespan of Bim-deficient dendritic cells (DC) (Chen et al., 2007) could contribute to T_{reg} accrual as DC can promote T_{reg} homeostasis and inducible T_{reg} development (Yamazaki et al., 2003; Belkaid and Oldenhove, 2008). Second, Bim$^{-/-}$ thymocytes are resistant to negative selection (Bouillet et al., 2002) and such altered thymic development in Bim$^{-/-}$ mice could skew the levels of T_{reg} in Bim$^{-/-}$ mice. By using FoxP3-Cre, which would likely not turn on until after cells have committed to the T_{reg} lineage and largely at a point beyond negative selection (Fontenot et al., 2005b), we avoided the effects of Bim in other tissues and the effects of Bim on thymic development. Thus, our data clearly show that T_{reg}-specific loss of Bim is sufficient to drive T_{reg} accrual with age and strongly suggest that the normal decline of Bim expression seen in wild-type mice promotes T_{reg} accrual in aging mice.

While our data support Bim as a major controller of T_{reg} homeostasis, we could not exclude potentially redundant roles for other pro-apoptotic Bcl-2 family members. For instance, the additional loss of Puma in Bim-deficient mice enhances T cell survival, suggesting a redundant role for Puma with Bim in certain T cell contexts (Erlacher et al., 2006; Gray et al., 2012). Moreover, we have found that, like Bim, Puma levels are normally decreased as mice age (data not shown). However, mice singly deficient in either Bmf, Puma, Noxa, or Bad have no difference in T_{reg} frequencies in young mice (Tischner et al., 2012), while young Bim-deficient mice have subtle, but significant increases in T_{reg} (Zhan et al., 2011; Tischner et al., 2012). Further, our finding that T_{reg} accrual was similar in aged Bim$^{-/-}$ mice and Lck-$Cre^+Bax^{f/f}Bak^{-/-}$ mice strongly suggest that Bim is the main negative regulator of T_{reg} homeostasis with age.

IL-2, on the other hand, is a major positive regulator of T_{reg} homeostasis as IL-2-deficient mice have significantly reduced T_{reg} (Fontenot et al., 2005a; Burchill et al., 2007; Barron et al., 2010). Although it has been accepted for many years that IL-2 levels decrease with age, this assumption was largely based upon a

reduced production of IL-2 after *in vitro* stimulation of aged T cells (Thoman and Weigle, 1981). To the best of our knowledge, our study is the first demonstration that *in vivo* IL-2 levels are decreased in aged mice. We acknowledge that serum IL-2 levels may not fully reflect the *in vivo* bioavailability of IL-2, as IL-2 can bind to heparin sulfate on extracellular matrices (Wrenshall and Platt, 1999), but we did not observe substantially increased staining for IL-2 on spleen tissue from old mice (data not shown). We previously proposed that continued IL-2 signaling drives the expansion of Bim^{lo} T_{reg}, as exogenous IL-2 preferentially induced proliferation and expansion of Bim^{lo} T_{reg} (Chougnet et al., 2011). We note that these experiments were performed in young mice that were given supraphysiologic levels of IL-2. Given that we now know that IL-2 levels decline by middle-age, this model seems less likely. Further, our new data clearly show that neutralization of IL-2 and/or loss of IL-2 both lead to the accumulation of $CD25^{lo}$ T_{reg} and decreased the levels of Bim within these cells. It does seem paradoxical that reduced levels of IL-2 in aged mice would be associated with T_{reg} accrual. However, our and others data suggest that IL-2 and Bim antagonize one another to regulate T_{reg} homeostasis. For example, the absence of Bim can restore T_{reg} in IL-2-deficient mice (Barron et al., 2010) and we found that limiting or restricting IL-2 results in the accrual of Bim^{lo} T_{reg}. Thus, while it has been shown in cell lines that acute withdrawal of IL-2 leads to increased Bim expression (Stahl et al., 2002), chronic limiting IL-2 may select for Bim^{lo} T_{reg}. Further, we show that the lack of IL-15 reduces the age-related accumulation of T_{reg} and that the additional loss of Bim restores T_{reg} accrual. We did not observe an effect of IL-15 on Bim expression directly, but we cannot exclude that IL-15 acts to restrain the remaining levels of Bim through another mechanism (i.e., Bim phosphorylation and turnover, induction of another anti-apoptotic molecule). As IL-2 and IL-15 both signal through CD122 (IL-2/15Rβ) and CD132 (common γ chain), it is possible that the decline of IL-2 favors the emergence of $CD25^{lo}Bim^{lo}$ T_{reg} because these cells are selected to survive and are maintained by IL-2-independent mechanisms, such as IL-15 (Fontenot et al., 2005a; Burchill et al., 2007).

While IL-2 and IL-15 signal through the same receptors, they can have divergent effects on T cells. For example, both IL-2 and IL-15 can activate the STAT5 and PI-3K/AKT pathways (Waldmann, 2006). In $CD8^+$ T cells, the magnitude of STAT5 activation by IL-2 and IL-15 is similar, although IL-2 drives more prolonged STAT5 activation (Castro et al., 2011). Further, while both IL-2 and IL-15 can activate PI-3K/AKT/mTOR, IL-2 drives both an increased magnitude and persistence of mTOR activation compared to IL-15 (Cornish et al., 2006; Castro et al., 2011). Recent work has shown that unrestrained activation of mTORC1 in T cells lead to significantly increased Bim expression (Yang et al., 2011). Thus, it is possible that the differential activation of mTORC1 by IL-2 and IL-15 may underlie Bim regulation in T_{reg}. However, it remains to be determined whether or not the degree to which the mechanisms are operative in T_{reg} cells.

Bim is also regulated at the transcriptional level by the FOXO family of transcription factors. FOXO transcription factors promote Bim expression in T cells in response to cytokine withdrawal (Stahl et al., 2002; Salih and Brunet, 2008). FOXO(s) are inhibited by PI-3K/AKT activation, which leads to FOXO phosphorylation and sequestration from the nucleus (Salih and Brunet, 2008). Consistent with this, we found that *in vivo* IL-2 administration to young mice resulted in accrual of Bim^{lo} T_{reg} (Chougnet et al., 2011). However, in aged mice we found decreased *in vivo* IL-2 levels and *decreased* Bim expression, which are inconsistent with a role for FOXO transcription factor control of Bim. Interestingly, we also find that FOXO1 and FOXO3a levels themselves are decreased in aged T_{reg} (data not shown). As mentioned earlier, levels of Puma, another FOXO target are also decreased in aged T_{reg}, raising the possibility that decreased levels of FOXO molecules may contribute to decreased expression of Bim.

Certainly, a survival advantage contributes to the accrual of T_{reg} with low Bim expression as T_{reg} with higher levels of Bim accumulate in mice whose T_{reg} cannot undergo apoptosis (i.e., T cell-specific Bax/Bak-deficient mice). One factor that may promote selection of Bim^{lo} T_{reg} is the decreased expression of Bcl-2 and Mcl-1 observed in aged T_{reg} (Chougnet et al., 2011). Indeed, Bcl-2 is the major anti-apoptotic reported to combat Bim-mediated death in T cells (Wojciechowski et al., 2007), and Bim and Bcl-2 have been shown to affect the expression of each other (Jorgensen et al., 2007). Further, we recently showed in $CD8^+$ T cells that inhibition or loss of Bcl-2 selected for effector $CD8^+$ T cells expressing low levels of Bim (Kurtulus et al., 2011). Thus, decreased expression of Bcl-2 with age may prompt the selection of Bim^{lo} T_{reg}. However, it is also clear that, relative to their young counterparts, Bim levels decline even in T cell-specific Bax/Bak-deficient mice, suggesting that levels of Bim may be controlled by non-survival related mechanisms as well. An alternative, and not mutually exclusive, explanation is that decreased Bim mRNA levels in aged T_{reg} may be due to epigenetic modification of the Bim promoter. The Bim promoter is CpG rich, and there is evidence that Bim expression can be repressed through hypermethylation (Paschos et al., 2009; San Jose-Eneriz et al., 2009; De Bruyne et al., 2010; Richter-Larrea et al., 2010). Furthermore, increased CpG methylation is a trait observed in aged cells (Golbus et al., 1990; Issa, 2003). Thus, as epigenetic modifications can be inherited, Bim promoter methylation may aid in the prolonged survival of aged T_{reg}. We are currently determining whether epigenetic and/or transcriptional repression affects Bim expression in aged T_{reg}.

While the exact mechanisms leading to decreased T_{reg} Bim expression with age are still unclear, progressive loss of Bim expression occurs in both $CD25^{lo}$ and $CD25^{hi}$ T_{reg} and this loss is likely sufficient to drive accrual of both populations with age. Furthermore, it is likely the lower expression of Bim in $CD25^{lo}$ T_{reg} that affords these cells a survival advantage over $CD25^{hi}$ T_{reg}, especially when IL-2 becomes limiting, and promotes increased $CD25^{lo}$ T_{reg} accrual. Indeed, not until IL-2 levels have declined do we begin to see the emergence of $CD25^{lo}$ T_{reg} in middle-aged mice. Because IL-2 promotes the expression of CD25 (Liao et al., 2013), there is likely still enough bioavailable IL-2 present in aged mice to maintain CD25 expression on the accumulated $CD25^{hi}$ T_{reg} population. Thus, it is likely that both reduced IL-2 signaling as well as other mechanism(s) (i.e., Bim promoter epigenetics) contribute to the control of Bim expression with age.

In this study, we show that T_{reg} loss of Bim drives accrual with age, while IL-15 is critical for aged T_{reg} survival in the face of declining IL-2. We have shown here that T_{reg} accrual is driven by several non-mutually exclusive mechanisms, including: (i) decreased Bim transcription, (ii) decreased Bim protein half-life, (iii) selection of Bim^{lo} T_{reg} due to a survival advantage, and (iv) a switch in cytokine dependency (from IL-2 to IL-15) with age. Future studies will focus on the mechanisms driving decreased Bim gene expression and protein half-life (i.e., promoter epigenetics, AKT/FOXO pathway), which may expose potential therapeutic targets for manipulating Bim expression and T_{reg} accrual. The ability to moderately manipulate T_{reg} homeostasis and accrual, without inducing inflammation and autoimmune responses, may provide a potential therapy for enhancing immune competence in the elderly.

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APPENDIX

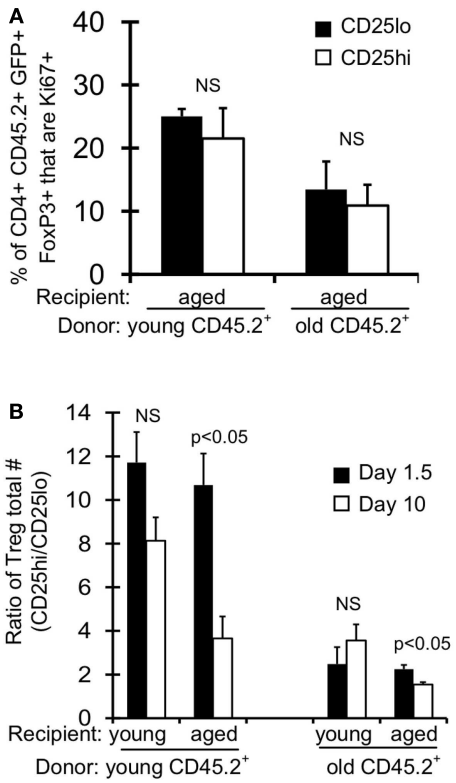

FIGURE A1 | Absolute numbers and proliferation of Treg post-adoptive transfer. CD^+FoxP3^{GFP+} T cells were sorted from splenocytes of young (3–4 months) and old (>18 months) CD45.2 $FoxP3^{GFP}$ reporter mice. About 5×10^5 $CD4^+FoxP3^{GFP+}$ cells were injected i.v. into young (2 months) and aged (15 months) congenic CD45.1 mice. Mice were sacrificed at day 1.5 ($n = 3$–5/group) and day 10 ($n = 3$–5/group) post-injection. Cells from the spleen were stained for CD4, CD25, CD45.2, and intracellularly for FoxP3 and Ki67 and analyzed by flow. **(A)** Results show the frequency of $CD4^+CD45.2^+FoxP3^+GFP^+CD25^{lo}$ and $CD25^{hi}$ cells that are $Ki67^+$ on day 10 post-transfer into aged recipient mice (±SE). **(B)** Results show the numbers ratio of $CD25^{hi}$ to $CD25^{lo}$ $CD4^+CD45.2^+FoxP3^+GFP^+$ cells (±SE).

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The Janus head of T cell aging – autoimmunity and immunodeficiency

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Immune aging is best known for its immune defects that increase susceptibility to infections and reduce adaptive immune responses to vaccination. In parallel, the aged immune system is prone to autoimmune responses and many autoimmune diseases increase in incidence with age or are even preferentially encountered in the elderly. Why an immune system that suboptimally responds to exogenous antigen fails to maintain tolerance to self-antigens appears to be perplexing. In this review, we will discuss age-associated deviations in the immune repertoire and the regulation of signaling pathways that may shed light on this conundrum.

Keywords: immunosenescence, autoimmunity, inflammation, pathogenesis, DNA damage response, T cell receptor signaling, rheumatoid arthritis, giant cell arteritis

INTRODUCTION

With increasing age, the ability of the immune system to protect against infection or to mount adaptive immune responses after vaccinations declines (Thompson et al., 2003; Jefferson et al., 2005; Nichol et al., 2007; Targonski et al., 2007). The mechanisms of this immune dysfunction are multidimensional. However, at the core is an inability of T cells to translate recognition of antigenic peptide in the context of the appropriate HLA molecule into productive T cell activation, clonal expansion, and differentiation into effector cells that provide help for B cells to differentiate or that exert effector function (Weng, 2006). We have recently shown that this dysfunctionality is, in part, conferred by the activation of negative feedback mechanisms in T cell signaling that raise the T cell receptor activation threshold or that negatively control sustained activation and lead to the early termination of differentiation pathways (Goronzy et al., 2012). While immune aging is perceived as a loss in effectiveness, it also brings along increased inflammation and a higher susceptibility to develop autoimmune diseases (**Figure 1**) (Goronzy and Weyand, 2012). Increased titers of autoantibodies, such as rheumatoid factor or antinuclear antibodies after the age of 60 years are well recognized and often are an indicator of increased autoreactivity without disease relevance (Moulias et al., 1984; Ruffatti et al., 1990a,b). More importantly, many autoimmune diseases show bimodal age of onset distributions with the first peak in young adulthood and the second peak in older age; or, alternatively, they occur in late adulthood and then increase in incidence with age (Cooper and Stroehla, 2003; Crowson et al., 2011). Typical examples are rheumatoid arthritis that occurs in females preferentially after menopause with steadily increasing incidence in the sixth and seventh decades of age (Doran et al., 2002). Even more dramatically, polymyalgia rheumatica and giant cell arteritis (GCA) are only found in individuals older than 50 years and the risk of developing GCA continues to increase into the 1980s (Weyand and Goronzy, 2003; Kermani et al., 2010; Mohan et al., 2011).

In the current paradigm, autoimmunity develops when self-reactive T and B cells recognize a self-antigen and differentiate into memory and effector cells. Recognition of self-antigens by self-reactive T cells is by virtue of the thymic selection process a low affinity interaction, which should be most affected by the negative regulatory signaling pathways, deviations of which we have discovered to occur in aging T cells (Goronzy et al., 2012). The co-occurrence of declining immunocompetence and increasing autoimmune susceptibility therefore appears to be contradictory. In this review, we will discuss possible models that overcome this apparent paradox and ultimately explore the hypothesis that the same defects that account for the decreased ability to generate protective immune responses also contribute to the increased risk of autoimmunity.

PERIPHERAL REPERTOIRE SELECTION WITH AGE AS A RISK FACTOR FOR AUTOIMMUNITY

The T cell receptor repertoire is essentially fully established in the first 20 years of life when new T cells are generated in the thymus. Thymic activity is the highest immediately after birth and then steadily declines. It is debatable whether in healthy individuals any thymic T cell generation occurs after the age of 20 years. Ongoing thymic activity is necessary to maintain a naïve T cell compartment in the mouse where the decline in thymic output is responsible for the eventual loss in naïve T cells (den Braber et al., 2012). In contrast, a fundamentally different rule appears to apply to T cell homeostasis in men where virtually all naïve T cell generation in

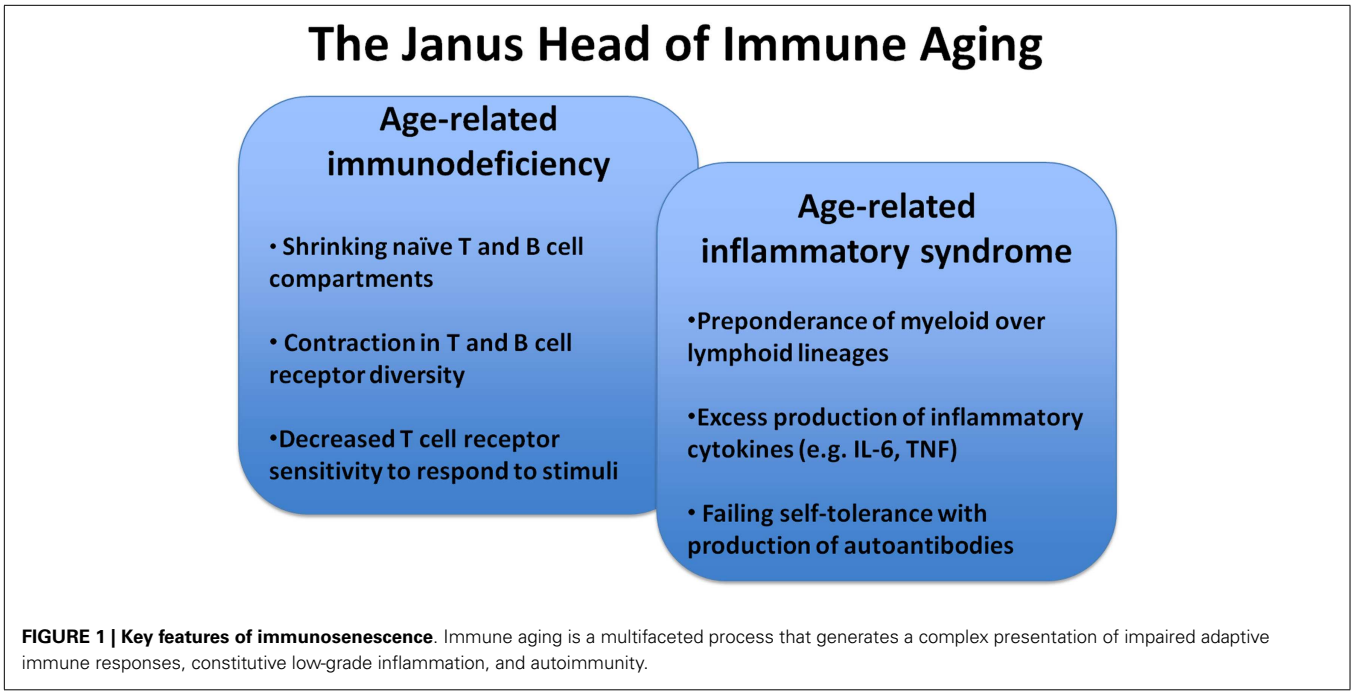

FIGURE 1 | Key features of immunosenescence. Immune aging is a multifaceted process that generates a complex presentation of impaired adaptive immune responses, constitutive low-grade inflammation, and autoimmunity.

humans after the age of 20 arises from self-renewal of the existing T cell pool (den Braber et al., 2012). This model is consistent with our recent *in silico* simulation of T cell homeostasis with progressive age (Johnson et al., 2012). The simulation was most consistent with a model in which thymic output was neither necessary nor beneficial for maintaining a diverse naïve T cell repertoire with age. In fact, virtual rejuvenation of thymic activity was not able to prevent or restore a contraction in diversity. Most factual observations on human T cell homeostasis are consistent with or explained by these virtual data (Goronzy and Weyand, 2005). The size of the naïve CD4 T cell compartment in humans only moderately shrinks with aging and the T cell receptor diversity is very well maintained up to the eighth decade of life when it abruptly collapses (Czesnikiewicz-Guzik et al., 2008). In our model, this abrupt contraction is best reproduced by cumulative changes in growth behavior and peripheral selection. In bone marrow transplant studies, reactivation of the thymus and repopulation of the peripheral T cell compartment was no longer achievable in the majority of individuals older than 40–50 years (Hakim et al., 2005). Finally, there is no evidence for compensatory increase in peripheral T cell turnover between the age of 20 and 70 years, consistent with thymic production already being irrelevant in the 20s (Naylor et al., 2005). Similar to men, increased turnover rates in non-human primates have only been noted in relatively old animals, probably in response to critical lymphopenic thresholds (Cicin-Sain et al., 2007).

Homeostatic proliferation is therefore the major driving force of T cell generation in humans and generally sufficient to maintain sizable numbers of naïve T cells, in particular of CD4 T cells. It is, however, non-random and is subject to peripheral selection pressures that impose cumulative effects with progressive age (**Table 1**). Peripheral recognition of self MHC/peptide complexes provides necessary signals for naïve T cell survival. In murine lymphopenia models, accelerated homeostatic proliferation is associated

Table 1 | Reshaping of the peripheral T cell repertoire in the aging host – a risk factor for autoimmunity.

Homeostatic proliferation with selection for self recognition
Imbalance of pro- and anti-apoptotic molecules prolonging T cell survival
Cytokine-driven T cell expansion (IL-7, IL-15, IL-21) with selection for cytokine responsiveness
Lymphopenia

with the selection of T cells recognizing self with higher affinity (Kassiotis et al., 2003; Kieper et al., 2004). Nikolich-Zugich and colleagues have studied the repertoire of CD8 T cells specific for foreign antigen in unprimed mice at different ages and have found a repertoire contraction with selection for antigen-specific T cells that presumably have been selected on self because they respond to lymphopenia with a higher proliferative rate than random T cells (Rudd et al., 2011). Evidence for repertoire skewing due to homeostatic maintenance has also been provided for the human system. CD4 T cells expressing the CD31 marker more frequently carry T cell receptor excision circle episomes than CD31-negative naïve CD4 T cells (Kimmig et al., 2002), suggesting a lesser replicative history. By comparing the repertoire of CD31-positive and CD31-negative naïve CD4 T cells, Thiel and colleagues clearly demonstrated a skewing of the repertoire in the CD31-negative population (Kohler et al., 2005). One possible outcome of cumulative homeostatic proliferation is the selection of a T cell receptor repertoire that is prone to autoreactivity (Goronzy and Weyand, 2001). In support of this finding, the naïve CD4 TCR repertoire of RA patients has been reported to differ in the frequencies of TCR Vβ–Jβ combinations compared to HLA-DRB1 matched healthy individuals (Walser-Kuntz et al., 1995). The age-associated repertoire skewing in these RA patients may be accelerated by

increased T cell loss due to defective DNA repair mechanisms and compensatory increased peripheral replication leading to telomere shortening and TCR repertoire contraction (Goronzy et al., 2006).

REDUCED T CELL RECEPTOR SIGNALING STRENGTH – A RISK FACTOR FOR AUTOIMMUNITY?

Intuitively, one would predict that hyperactivity of the TCR signaling pathway confers autoimmunity. Stimulation of the TCR initiates a cascade of tyrosine phosphorylation events that is regulated by an intricate network of tyrosine kinases and tyrosine phosphatases. Although both can have activating as well as inhibiting function depending on the phosphotyrosine targeted, quiescence is mainly regulated by phosphatase activity. Indeed, mutation or deletion of several phosphatases has been shown to cause autoimmunity (Zikherman and Weiss, 2009). A classic example is the moth-eaten mouse in which SHP-1 (PTPN6) is mutated (Shultz et al., 1997). Also, deletion of PTPN2 in T cells leads to spontaneous autoimmunity in mice (Wiede et al., 2011). Moreover, a PTPN2 variant is associated with type I diabetes mellitus, RA, and celiac disease in men (Consortium, 2007; Parkes et al., 2007; Franke et al., 2008). Similarly, a variant PTPN22 that undergoes faster degradation is associated with multiple human autoimmune diseases (Stanford et al., 2010; Zhang et al., 2011). Paradoxically, however, in several model systems autoimmunity is a result of decreased TCR signaling (**Figure 2**). The classical example is the SKG mouse in which a mutation of the ZAP70 gene dampens the activation-induced TCR signaling cascade; however, that mouse develops a Th17-mediated autoimmune disease that resembles features of RA (Hirota et al., 2007).

The pathogenesis of autoimmune disease in the SKG mouse remains unexplained. Positive, as well as negative, selection in the thymus appears to be influenced by the mutation (Sakaguchi et al., 2003; Hsu et al., 2009). The mutation impairs the association of ZAP70 with the T cell receptor zeta-chain leading to impaired TCR signaling induction in peripheral T cells and reduced proliferative responses after TCR stimulation. However, peripheral tolerance appears to be unstable. Germ-free SKG homozygous mice do not develop disease, but stimulation of pattern-recognition receptors induces onset of disease which is T cell-dependent (Yoshitomi et al., 2005). In contrast, SKG heterozygous mice develop spontaneous autoimmune disease emphasizing the impact of graded TCR stimulation (Tanaka et al., 2010). Autoimmune manifestations are also seen in ZAP70 hypomorphic mutants that allow the study of the impact of graded T cell receptor signaling strengths (Siggs et al., 2007). Mice with a partial, but not mice with a severe, defect in ZAP70 signaling developed increased Th2 polarization with the production of antinuclear antibodies. Similarly, an LAT mutation that inhibits PLCγ activation but leaves ERK phosphorylation intact, results in a multi-organ inflammatory disease with the production of antinuclear antibodies (Sommers et al., 2005; Genton et al., 2006). Overall, autoimmune disease mouse models with hyperactive TCR activation pathways appear to be the exception rather than the norm while several examples exist where selected changes of the TCR signaling complex that cause a reduced quantitative response or a shift in kinetics increases the risk for autoimmunity. While effects on thymic selection cannot be excluded as the driving pathomechanisms, there clearly needs to be a peripheral trigger. Peripheral tolerance mechanisms appear to be less stable in these mice. Presumably, the defect is associated

Comparison of Immune Aging with Murine Autoimmune Models and Human Autoimmune Disease

Mechanism	Aging host	Mouse model of autoimmunity	Human autoimmune disease
Lymphopenia-induced proliferation	Progressive loss of naïve T/B cells	Lymphopenia-induced autoimmunity in NOD and SKG mice	T cell loss due to defective DNA repair
T/B cell hyper-responsiveness	Not described	PTPN2 -/- mice PTPN6-mutant mice	PTPN2 and PTPN22 variants BRAF and KRAS over-expression
TCR hypo-responsiveness	DUSP6 upregulation	SKG mice ZAP70- or LAT-mutant mice	Defective TCR signaling in SLE

FIGURE 2 | Comparison of immune aging manifestations and autoimmune pathomechanisms. The figure highlights mechanistic parallels between immune aging, animal models of autoimmune diseases, and human autoimmune diseases. Few selected animal models and human diseases representative of many others were chosen to illustrate the mechanistic path ways involved.

with the establishment of a signaling equilibrium at rest that is less stable and more prone to non-linear transitions. Of note, signaling networks are in a constitutively active state in resting T cells and, therefore, stochastic fluctuation in positive signals or a drop in negative control pathways may be sufficient in such T cells that have adapted their networks to low signaling strengths.

The graded signaling defects in the ZAP70-mutated mice are very similar to the signaling defects seen with progressive aging. Elderly naïve CD4 T cells have increasing cytoplasmic concentrations of DUSP6. This increase is due to a decline in miRNA-181a that posttranscriptionally controls the expression of DUSP6 (Li et al., 2012). DUSP6 is a cytoplasmic phosphatase that selectively regulates the phosphorylation of ERK (Muda et al., 1996; Bettini and Kersh, 2007). Phosphorylated ERK is a critical regulator of setting the TCR's threshold at which antigen recognition is translated into T cell activation. By serine phosphorylating Lck, active ERK prevents the recruitment of SHP-1 to the T cell signaling complex and therefore allows sustained signaling (Stefanova et al., 2003; Altan-Bonnet and Germain, 2005). Consequently, increased cytoplasmic concentrations of DUSP6 reduce the availability of phosphorylated ERK and increase the threshold of T lymphocytes to respond, in particular to low affinity antigens (Li et al., 2007). Overexpressing miRNA-181a or silencing DUSP6 restores T cell activation in old CD4 T cells (Li et al., 2012). Similar to DUSP6, PTPN7 controls proximal ERK phosphorylation after T cell activation and therefore the T cell receptor activation threshold (Saxena et al., 1999), however, it is currently unknown whether PTPN7 is subject to concentration changes with aging or cell differentiation as has been shown for DUSP6. How could a lesser ERK response and a heightened T cell receptor activation threshold lead to autoimmunity? It is possible that signaling networks adapt to a reduced input and that a unstable state of peripheral unresponsiveness is established which is more susceptible to spontaneous activation and interferes with tolerance maintenance similar to the ZAP70-mutated mice.

Evidence for aberrations in signaling networks has also been found in several human autoimmune diseases. Some, like the increased risk with a PTPN22 or PTPN2 variant are inherited as discussed above. Others may represent adaptations to acquired changes in signaling networks. Examples are the substitution of FcRγ for the CD3 zeta chain in SLE or the overexpression of ERK pathway members in RA (Tenbrock et al., 2007; Moulton and Tsokos, 2011).

In the previous section, we have argued that aging goes hand-in-hand with homeostatic maintenance mechanisms that eventually reshape the peripheral repertoire. This repertoire restructuring process not only impacts the selection of TCR, but is associated with profound functional changes as well (**Table 1**). Haynes and Swain have shown in mouse models that survival times for CD4 T cells increase with age due to the decreased expression of BIM and that this prolonged survival results in the acquisition of T cell defects (Tsukamoto et al., 2009). Since T cell homeostasis, in addition to the balance between pro-apoptotic and pro-survival molecules, is dependent upon TCR signaling and the homeostatic cytokines IL-7, IL-15, and IL-21, homeostatic maintenance and proliferation will eventually result in the selection of T cell clones that are optimized for their survival and growth behavior, regulated by both TCR and STAT signaling. Such selected clones should have compensated for some of the age-associated defects such as miRNA-181a loss and overexpression of DUSP6 and have recalibrated their signaling networks. In our *in silico* modeling of T cell homeostasis over lifetime, we have shown that cumulative inheritable changes in growth behavior can account for the abrupt contraction and the increased turnover that is seen in old age (Naylor et al., 2005; Johnson et al., 2012). A similar selection might also explain the age-associated increased incidence of autoimmune disease.

LYMPHOPENIA-INDUCED AUTOIMMUNITY AND HOMEOSTATIC CYTOKINES

While homeostatic proliferation and maintenance, either in form of selection for a more autoreactive TCR repertoire or for general fitness to proliferate and survive, provides a model to explain the age-associated increased frequency of autoimmunity, lymphocyte proliferation itself appears to be associated with an increased risk, probably due to tolerance-abating signals from homeostatic cytokines. In several animal models, lymphopenia significantly contributes to the occurrence of autoimmunity (Hickman and Turka, 2005); the risk has generally been related to the activity of homeostatic cytokines (King et al., 2004; Calzascia et al., 2008). The NOD mouse, which is prone to develop autoimmune diabetes mellitus, is lymphopenic and the development of disease is dependent on IL-21-mediated turnover of islet-specific T cells (King et al., 2004). Similarly, in the model described by Calzascia et al. (2008) the presence of β-islet cell-specific self-reactive CD4 T cells was not sufficient to develop diabetes, however, such autoreactive T cells conferred disease when driven into proliferation by IL-7. Also, the SKG mouse model of RA described above is lymphopenic and characterized by increased homeostatic proliferation, possibly to compensate for defective thymic generation (own unpublished observation). This increased turnover in SKG mice is directly or indirectly linked to increased activation of the ERK pathways. Data from our lab have shown that even the slightest suppression of ERK activity in the steady-state delays disease onset suggesting that homeostatic proliferation is a facilitator of autoimmune manifestations (Singh et al., 2009).

While lymphocyte replenishment is a constant challenge throughout adult life, it appears to be steady and only increases late in the eighth decade (Wallace et al., 2004; Naylor et al., 2005). Increased homeostatic proliferation, therefore, does not appear to be relevant for developing autoimmune disease during normal healthy aging. However, several autoimmune diseases exhibit evidence of accelerated immune aging, mostly concluded from age-inappropriate telomeric erosion (Goronzy and Weyand, 2003; Goronzy et al., 2006; Georgin-Lavialle et al., 2010; Hohensinner et al., 2011). It is, therefore, possible that in these patients subclinical lymphopenia already develops at an earlier age which, in turn, enhances homeostatic turnover to set the stage for overt autoimmune manifestations (Goronzy et al., 2010). The best studied example is RA where increased replicative history is not only supported by the finding of telomeric erosion (Schonland et al., 2003; Fujii et al., 2009), but also by increased dilution of TCR excision circles (Koetz et al., 2000; Ponchel et al., 2002) and by repertoire contraction in TCR β-chain diversity within the

naïve compartment (Wagner et al., 1998) and accumulation of terminally differentiated CD4 effector T cells that have lost the expression of CD28 (Schmidt et al., 1996a,b; Warrington et al., 2001). The underlying defect appears to be a dysfunctional DNA damage repair response that results in increased apoptosis and accelerated T cell loss. At least two defective pathways have been described so far. Reduced expression of telomerase in naïve RA T cells does not only lead to insufficient telomeric repair, but also to increased apoptotic susceptibility independent of telomeric lengths (Fujii et al., 2009). Second, reduced expression of ATM and other members of the ATM-MRE repair complex lead to insufficient DNA repair, chronic DNA damage responses and apoptosis (Shao et al., 2009). The increased loss may lead to the accumulation of end-differentiated cells that are relatively resistant to apoptosis (Schirmer et al., 1998; Vallejo et al., 2000). Altogether, these observations are consistent with the model that in RA increased cell loss and associated compensatory increased activity of homeostatic cytokines may be a facilitator of autoimmunity (**Figure 3**).

To directly address the question whether and how homeostatic cytokine signaling may affect tolerance mechanisms, we have examined whether exposure to homeostatic cytokines primes sensitivity to subsequent TCR stimulation (Deshpande et al., 2013). In these experiments, naïve CD4 T cells from healthy individuals were primed with increasing concentrations of IL-7, IL-15, or IL-21, followed by TCR stimulation in the absence of exogenous cytokines. Cytokine priming in general amplified TCR signals and enabled T cell activation in response to suboptimal stimulation. Of particular interest, cytokine priming of naïve CD4 T cells from HLA-DRB1*0401 healthy donors enabled proliferative responses to citrullinated vimentin and melanocyte glycoprotein gp100 peptides that have been previously shown to be relevant for the T cell responses in patients with RA (Hill et al., 2003; Law et al., 2012) and melanoma (Phan et al., 2003), respectively. The underlying mechanism is a PI3-kinase-dependent activation of RAS by the homeostatic cytokines that initiates a SOS-mediated amplification loop in ERK phosphorylation after TCR stimulation and is sufficient to overcome low-responsiveness to low affinity self-antigens (Deshpande et al., 2013). In conclusion, exposure to homeostatic cytokines transiently reduces the threshold of T cells to respond to low affinity self-antigens and could thereby initiate a program of proliferation and differentiation that leads to memory differentiation of autoreactive T cells.

STIMULATORY AND INHIBITORY NK CELL-ASSOCIATED RECEPTORS ON AGING T CELLS

One of the most striking findings in immune aging is a increased expression of regulatory cell surface receptors, mostly on end-differentiated CD8 T cells and to a lesser degree also on other CD4 and CD8 T cell subsets (**Table 2**). These receptors include killer immunoglobulin-like receptors (KIRs), killer cell lectin-like receptors (KLRs), and the immunoglobulin-like transcript (ILT/CD85) receptors, all typically associated with NK cell functions (Abedin et al., 2005; Cavanagh et al., 2011). Their expression is generally correlated with the loss of the classical costimulatory molecules CD27 and CD28 (Namekawa et al., 2000; Snyder et al., 2002; Ince et al., 2004; van Bergen et al., 2004). Although varying in fine specificity, KIRs and CD85/ILTs all recognize MHC class I molecules

Molecular Mechanisms of Accelerated Immune Aging in Rheumatoid Arthritis

Mechanism	Consequence
Telomerase deficiency	Accelerated telomeric erosion
Telomerase deficiency	Apoptosis susceptibility
Deficiency of the DNA damage sensing kinase ATM	Suboptimal repair of DNA double-strand breaks
Decline of p53	Inactive cell cycle checkpoint
Activation of DNA-PKcs	Activation of stress kinase signaling, JNK-dependent apoptosis

FIGURE 3 | Molecular mechanism of accelerated immune aging. Rheumatoid arthritis is an autoimmune disease that is characterized by accelerated immune aging. The principle defects reside in impaired DNA repair responses.

Table 2 | Mechanisms altering T cell receptor responsiveness with age.

Loss of CD28
Acquisition of co-stimulatory receptors (KIR2DS/3DS, CD94/NKG2C, NKG2D)
De novo expression of co-inhibitory receptors (KIR, KLR, ILT2, PD1)
Rewiring of signaling cascades due to chronic cytokine stimulation
Rewiring of signaling cascades due to kinase or adaptor expressions (seen in RA and SLE, not yet shown in aging)
Rewiring of signaling cascades due to increased phosphatase expression (e.g., DUSP6)

(Boyington et al., 2001; Trowsdale, 2001; Vilches and Parham, 2002). The C-type lectin receptors are more diverse in their ligand specificities. CD94/NKGs recognize MHC class I molecules (Lopez-Botet and Bellon, 1999), NKG2D ligands include a number of cellular stress-inducible molecules (Gonzalez et al., 2008) while KLRG1 binds to cadherin (Ito et al., 2006). These receptor-ligand pairs are fundamentally different from the traditional costimulatory or inhibitory receptors that fine-tune antigen-induced T cell activation. The ligands are constitutively expressed on cell types that are not specialized in antigen presentation. The expression of these regulatory receptors on T cells with aging therefore confers regulatory control to tissue-residing cells that usually do not participate in immune responses.

The majority of these molecules have one or several ITIM signaling domains and function to recruit cytoplasmic phosphatases. Similar to their role in NK cells, they can dampen activation signals and are therefore thought to account for defective T cell responses in the elderly, a model reminiscent of T cell exhaustion conferred by PD1 (Wherry, 2011). However, TCR-induced activation of effector function such as cytotoxicity or cytokine secretion is hardly suppressed in aged T cells and is much less affected than proliferation (Henel et al., 2006; Henson et al., 2009). We have proposed that recruitment of phosphatase activity to the signaling complex may be late or incomplete; therefore, only inhibiting selected functions, in particular proliferation (Henel et al., 2006). In this model, expression of inhibitory receptors could be beneficial for the aging immune system. While T cells are still competent to respond to antigen recognition, clonal expansion is restricted protecting the host from undue skewing of the repertoire.

Increased frequencies of CD28-negative CD4 and CD8 T cells expressing NK cell-associated receptors are a common finding in autoimmune diseases, in particular rheumatoid arthritis (Yen et al., 2001; Nakajima et al., 2003; Snyder et al., 2003; Qin et al., 2011; Boita et al., 2012). While most of the receptors are inhibitory, some of them have stimulatory function, notably NKG2D, KIR2DL4, and the short isoforms of KIRs as well as CD94/NKG2C. Like in NK cells, the expression of different isoforms appears to be stochastic. This has been best shown for KIRs where expression on T cells and NK cells is entirely and exclusively dependent on promoter demethylation (Santourlidis et al., 2002; Chan et al., 2003; Li et al., 2008, 2009). We have analyzed the KIR expression pattern on the progeny of an *in vivo* expanded T cell clone identified by the identical T cell receptor (Snyder et al., 2002). The data were most consistent with the model that acquisition of different KIR isoform on each cell is successive and cumulative generating increasingly complex patterns. Expression of stimulatory receptors on T cells could therefore overcome tolerance or anergy. NKG2D has been implicated in several autoimmune diseases including celiac disease, type 1 diabetes mellitus, Crohn's disease, and rheumatoid arthritis (Gonzalez et al., 2006; Van Belle and von Herrath, 2009). Dependent on the co-expression of its adaptor molecule DAP10, NKG2D can activate the PI3K-AKT pathway and thereby bypass costimulatory deficits (Billadeau et al., 2003). Stimulatory KIR molecules (e.g., KIR2DS1, KIR2DS2, etc.) require DAP12 to be fully functional (Wu et al., 2000). We have isolated KIR2DS2-DAP12-positive CD4 T cells from RA patients (Snyder et al., 2004a). Stimulation of KIR2DS2 in these T cells led to full activation without the need for TCR stimulation. Co-expression of a stimulatory KIR and DAP12 is infrequent, however, even KIR2DS2 in the absence of DAP12 can be expressed on the cell surface and then provides a costimulatory signal to TCR triggering (Snyder et al., 2004a,b). Stimulatory KIRs have been described as disease risk genes for RA and psoriasis, supporting a role for these receptors in autoimmunity (Yen et al., 2001; Martin et al., 2002). The KIR receptor KIR2DL4, while containing an ITIM motif in its cytoplasmic tail, can also provide stimulatory signals, but mostly at the level of the endosome (Rajagopalan and Long, 2012). When KIR2DL4 binds its ligand, HLA-G, the complex is internalized and activates DNA-PKcs and downstream PI3K (Rajagopalan, 2010; Rajagopalan et al., 2010).

In summary, NK cell-associated stimulatory receptors when expressed on aged T cells provide positive signals that are partially or even fully stimulatory and then activate the T cell even in the absence of antigen. Notably, relevant ligands are not restricted to APCs. As such, they can provide signals to T cells without the temporal and spatial control afforded by traditional costimulators.

SIGNALING PATHWAYS INDUCED BY CHRONIC DNA DAMAGE RESPONSES

Telomeric erosion is an accepted index of aging and is found in several cell lineages including naïve and memory T cells (Hodes et al., 2002; Colmegna et al., 2008). Generally considered as a corollary of increased proliferation, changes in telomerase expression and failing DNA repair mechanisms appear to be at least equally contributory (Reed et al., 2004; Fujii et al., 2009; Effros, 2011; Hohensinner et al., 2011). Replicative attrition of telomeric ends activates DNA damage response pathways which can arrest cell division (Ciccia and Elledge, 2010; Hiom, 2010) but also skew the homeostasis of the cytoplasmic and nuclear signaling networks in the absence of extrinsic stimuli (Rube et al., 2011; Armanios, 2013). Telomeric erosion has been found in many autoimmune diseases where it may not only be a consequence of the inflammatory disease, but also contribute to disease development and manifestation. This concept has been best studied in patients with rheumatoid arthritis who have evidence of accelerated immune aging by about 20 years (Goronzy et al., 2010). In these patients, cells express reduced telomerase activity which causes telomeric erosion in all hematopoietic lineages ranging from stem cells to mature naïve and memory

T cells (Fujii et al., 2009). Increased DNA damage is not limited to the telomeres, RA patients have also evidence of DNA double-strand breaks compare to age-matched healthy controls as determined by comet assay (Shao et al., 2009). DNA damage is more prevalent in memory T cells where it gradually increases with age. In contrast, naïve T cells in healthy individuals have little DNA damage up to the age of 70 years when it starts to increase (Shao et al., 2009). At any age and in both T cell subsets, DNA damage is increased in RA patients. The underlying mechanism is a reduced expression and function of the DNA damage sensing kinase ATM and other members of the ATM-MRE pathway.

Chronic DNA damage responses play a major role in regulating intrinsic cell activation and in particular the production of inflammatory mediators following cellular senescence (Rodier et al., 2009). Rodier and Campisi (2011) have described the senescence-associated secretory phenotype (SASP) in senescent fibroblasts which may be applicable to other cell types as well. SASP is dependent on DNA damage response signaling involving the DNA repair molecules ATM, NBS1, and CHK2. p53 activation is a negative regulator of SASP, but can be overcome in the context of DNA damage responses by p38/NFκB (Freund et al., 2011; Gudkov et al., 2011). SASP-mediated production of proinflammatory cytokines is sensitive to the suppressive action of glucocorticoids, which, however, cannot revert the senescence-associated growth arrest (Laberge et al., 2012).

A second pathway through which chronic DNA damage responses reprogram cells toward proinflammatory effector functions is the activation of DNA-PKcs. T cells from patients with RA have increased DNA-PKcs activity, possibly as a consequence of ATM deficiency and associated increased DNA damage (Shao et al., 2009, 2010). DNA-PKcs influences intracellular signaling pathways through at least two mechanisms. It activates the inflammasome and increases NFκB activity; and it activates the stress kinase JNK pathway (Rajagopalan et al., 2010; Shao et al., 2010). Both pathways can contribute to the production of inflammatory cytokines.

CONCLUSION

The pathomechanisms of autoimmunity are as multifaceted as the manifestations of immune aging. Although both processes appear to be contradictory at first sight, there are many parallels. In particular, impaired immune effectiveness, a hallmark of immune aging, mirrors many of the conditions in animal models that render mice susceptible to develop an autoimmune disease. Moreover, it is a well-known clinical experience that patients with many inheritable immune deficiency diseases are prone to also develop an autoimmune disease. It is not the responsiveness of the immune system, but its lack of stability that predisposes for tolerance failure. The aging immune system, through its attempt to endure and to compensate for age-associated defects is acquiring an unstable state. An increased risk for autoimmunity may be the price we have to pay to preserve some immune function into older age.

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Human T cell aging and the impact of persistent viral infections

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Aging is associated with a dysregulation of the immune response, loosely termed "immunosenescence." Each part of the immune system is influenced to some extent by the aging process. However, adaptive immunity seems more extensively affected and among all participating cells it is the T cells that are most altered. There is a large body of experimental work devoted to the investigation of age-associated differences in T cell phenotypes and functions in young and old individuals, but few longitudinal studies in humans actually delineating changes at the level of the individual. In most studies, the number and proportion of late-differentiated T cells, especially CD8+ T cells, is reported to be higher in the elderly than in the young. Limited longitudinal studies suggest that accumulation of these cells is a dynamic process and does indeed represent an age-associated change. Accumulations of such late-stage cells may contribute to the enhanced systemic pro-inflammatory milieu commonly seen in older people. We do not know exactly what causes these observed changes, but an understanding of the possible causes is now beginning to emerge. A favored hypothesis is that these events are at least partly due to the effects of the maintenance of essential immune surveillance against persistent viral infections, notably Cytomegalovirus (CMV), which may exhaust the immune system over time. It is still a matter of debate as to whether these changes are compensatory and beneficial or pathological and detrimental to the proper functioning of the immune system and whether they impact longevity. Here, we will review present knowledge of T cell changes with aging and their relation to chronic viral and possibly other persistent infections.

Keywords: aging, immunosenescence, T cells, CMV, chronic stimulation

INTRODUCTION

Aging affects all physiological systems, of which one of the most important interacting with and regulating many of the others is the immune system. This is composed of multiple cell types with specifically defined roles within a complex network of interactions. Immunologists and gerontologists have been interested in studying the effects of aging on the immune system for many years (1). Early work suggested that aging changed only adaptive immunity, but as analytical techniques became more refined, the innate arm was also found to be subject to age-related alterations. Nevertheless, T cells still seem to be most severely affected by aging (2). These changes may lead to clinical consequences such as the increased incidence of infections and most probably of cancers, cardiovascular diseases, and neurodegeneration in the elderly. However, despite a considerable amount of experimental work and knowledge we still do not know what causes these changes with age. Although changes in the innate immune response have been recently described (3–6) we will concentrate on changes in the human adaptive immune response. This review will describe changes in human T cell phenotypes, functions, and interactions as well as the possible causes of these changes, focusing on the effects of persistent viral infection.

IMMUNOSENESCENCE

The aging of the immune system is a dynamic process which may at least partly reflect adaptation of the response to the evolving pathogen milieu (7, 8). Not all compartments of the immune response are aging in the same way, at the same speed or the same direction (9, 10). There was an assumption that all parts and functions of the immune system were decreasing with age, but currently the realization that compensatory increases may be developing over time is gaining ground. Even previously identified "senescent" T cells considered as anergic, have been shown to be able to maintain functions important for mounting effective immune responses (11, 12). Based on these findings, the question is whether these changes are exclusively detrimental or can be viewed as an adaptation mechanism to the exposures experienced by the aging organism over the lifetime (8). Better knowledge of these changes in human aging will also help to design treatments aimed at restoring appropriate immunity by identifying which components to manipulate.

The changes occurring in the immune system with aging are collectively designated "immunosenescence." This appellation describes all the changes that occur in all parts of the immune response with aging, but strictly should be limited only to those shown to be truly deleterious. Of all the age-associated changes

identified, those actually known to be deleterious are certainly in the minority. At the same time, these changes are accompanied by development of a low grade inflammatory status in many elderly people (13). Such "inflammaging" is more clearly associated than cellular changes with syndromes of aging including sarcopenia, cardiovascular disease, neurodegeneration, and physical frailty. It would perhaps be more accurate to call the global phenomenon occurring in the immune system the "Immunopause," like many changes in the aging endocrine system, with less overtly negative implications.

The most noticeable changes are observed in the adaptive immune system and especially in T cells. T cells are the backbone of the adaptive immune response. The results of these changes are relatively well documented in the elderly. They are likely to be associated with an increased susceptibility to infections but indirectly possibly also to cancers, autoimmune disorders and chronic inflammatory diseases. T cell status is monitored primarily by surface marker phenotyping identifying the distribution of differentiation stages which we extrapolate to predict functional consequences, some leading to the above-mentioned diseases associated with aging. Several lines of experimental evidence suggest that aging is associated with an increase in memory cells, quantitatively more so in the CD8+ compartment but also in the CD4+ compartment (7, 14). Concomitantly, there is a decrease of the naïve T cells probably mainly because of the involution of the thymus with age (15, 16) and the continuous consumption of existing immunological resources (17) by new and persistent infections.

How can we phenotypically classify T cells? There are several surface markers which can be used. The most important are the receptors CCR7, CD28, and CD27 and the phosphatase CD45, which play important roles in T cell activation, proliferation, cytokine production, and lymph node homing. *In fine*, the disappearance of these markers from the cell surface may denotes functional alterations in these T cell properties (7). The use of these markers of differentiation/exhaustion/memory inflation (18) enables us to identify T cell differentiation stages from recent thymic emigrant naïve T cells to late-stage TEMRA cells. These surface markers can be used to distinguish T cell populations the majority of which will be naïve (N: CCR7+, CD27+++, CD28+++, CD45RA+), central memory cells (CM: CCR7+, CD27++, CD28++, CD45RA−), effector memory cells (EM: CCR7−, CD27±, CD28±, CD45RA−), and terminally differentiated memory cells re-expressing CD45RA (TEMRA: CCR7−, CD27−, CD28−, CD45RA+). This is currently the most widely accepted phenotyping model for CD8+ T cells but can be applied to some extent to CD4+ T cells as well. The memory cells protect the host from subsequent infections by the same pathogens. They survive and turn-over homeostatically, driven by IL-7 or IL-15 (19). Because thymic involution beginning early in life severely curtails the egress of fresh supplies of naïve cells to the periphery, the diversity and integrity of the T cell repertoire must be preserved by the homeostatic maintenance of naïve and memory cells (20).

What does the picture look like in elderly individuals? The current paradigm is that the more we age (i) the more differentiated/memory T cells are accumulating because of the host's immune exposure history (EM and TEMRA cells); (ii) the number of naïve T cells is decreasing because of thymic involution and exposure to specific antigen in the periphery; (iii) as there is a physical limit in the body to the number of circulating cells the accumulation of memory cells unbalances the naïve/memory ratio because clonally expanded memory cells are present in larger numbers than the naïve cells from which they were derived (1, 21) (**Figure 1**). There is a strong correlation between the chronological age and the frequency and absolute numbers of TEMRA CD8+ T cells in most human populations. Moreover, many of these TEMRA cells can be identified as dysfunctional in terms of cytokine response to stimulation and mediation of cytotoxic activity, as well as inability to proliferate. Hence, the accumulated population of late-stage memory cells includes those that might be senescent in the sense originally used by cell biologists, i.e., replicative senescence of fibroblasts, defined as permanent cell cycle arrest after a finite number of cell divisions (1, 22). The term "immunosenescence" is often confounded with this more restricted meaning of replicative senescence, especially because the lack of expression of the costimulatory receptor CD28 by late-stage CD8+ T cells is associated with essentially post-mitotic cells. Hence the notion that CD8+CD28− negative cells were senescent came to be common because of the role of CD28 in proliferation and functional commitment, in maintaining telomerase function, in p38 pathway activation, in modulating apoptosis and for adequate cell metabolism (23–25). By analogy to the replicatively senescent cells of Hayflick's fibroblast cultures, those T cells which are not able to proliferate further were also considered as senescent. However, since these early observations were made, it has become evident that these differentiated T cells can under some circumstances regain proliferative capacity, such as after manipulation of KLRG1 (26, 27). The most likely explanation to account for the observed accumulation of late-stage memory cells in older humans is that while many are fully

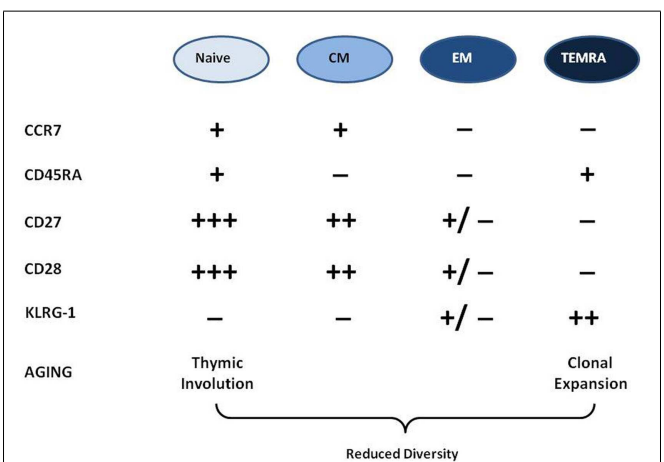

FIGURE 1 | Phenotypic characterization of T cells and their changes in aging. There are four major differentiation stages of T cells characterized by the presence or absence of different surface markers in this popular model. With aging the naïve cell compartment is shrinking while the memory is expanding. There is also a loss of diversity with aging.

functional, this functionality no longer requires further clonal expansion of those antigen-specific cells. On the other hand, the accumulation of very large amounts of CD8+ TEMRA cells may result in overcrowding (shrinkage of the "immunological space" for other cells) because at least some of them are genuinely senescent.

THE CONTRIBUTION OF LONGITUDINAL STUDIES

Swedish longitudinal studies were the first to investigate immune predictors of survival and death (28). Therefore, most of our findings concerning the accumulation of late-stage differentiated CD8+ T cells and whether this results in any negative consequences come from studies such as the OCTO and NONA longitudinal studies (29). These defined an "Immune Risk Profile" (IRP) as a cluster of parameters characterized by an inverted CD4/CD8 ratio due to an accumulation of CD8+CD28− T cells, associated with poor proliferative responses to T cell mitogens, but including low B cell counts and seropositivity for CMV. The IRP was defined a metric predictive of 2, 4 and 6 year mortality in people 85 years old at baseline. These findings largely contributed to our understanding of the cellular and molecular mechanisms underlying the decreased immune response with age (30). They characterized for the first time by TCR clonotype mapping the expansion of specific CD8+ T cell clones already described in mice and humans (31, 32). This decreased diversity of the total CD8 repertoire paralleled that of CD4 with age (33). Not only is there a decreased diversity, but these studies confirmed that the CD8 clones were strongly associated with CMV seropositivity (34), however in a much larger number of elderly and even older than in all other previous studies (30). It is now clear that persistent/latent CMV infection *per se* is associated with the accumulation of large populations of late-stage differentiated CD8+ T cells characterized by lack of expression of CD28 and acquired expression of the negative signaling receptor CD57, accompanied by decreased functionality (35). Moreover, in these Swedish studies it was also shown that the best predictor for survival was the inverted CD4/CD8 ratio (36) absent in "successful aging," as epitomized by centenarians (37). The role of CMV in this respect was puzzling and to some extent remains so.

A seminal study by Simanek et al. (38) showed for the first time in a population of >10,000 adults that CMV positivity together with higher levels of the inflammatory marker CRP was associated with significantly poorer survival compared to those who did not have CMV seropositivity and/or CRP increased level. This remained the case in this NHANES cohort after correcting for multiple confounding factors. This suggests that CMV seropositivity is associated with poorer survival in people with increased background inflammation levels, borne out by such deaths being mostly cardiovascular. However, causation was not demonstrated and involvement of alterations in the immune system not shown (39). It also needs to be borne in mind that CMV infection *per se* has not been associated with increased background inflammation in all studies (40). Nonetheless, other studies also support the notion that CMV-seropositive elderly individuals have a higher propensity to suffer from other age-associated diseases with an inflammatory component, such as cardiovascular disease and cancer (14, 39, 41). Moreover, the IRP could rarely be found in other healthy populations or even in very ill elderly (42). So the question arises whether these changes are related to aging or to the chronic CMV infection or do they exist as an innocent bystander amplification?

WHAT MIGHT THEN CAUSE THESE CHANGES IN ADAPTIVE IMMUNITY WITH AGE?

Recently a new paradigm emerged, mainly following these longitudinal studies, to explain the changes with age in T cell phenotypes and functions characterized by more and more differentiated CD4+ and CD8+ T cells: CMV seropositivity determines the increase of CD8+ TEMRA cells with a large proportion recognizing CMV antigens (43). It is of note that EBV-specific peripheral T cells are mainly effector memory phenotype, not so differentiated as for CMV. In parallel, EBV has not been strongly associated with immunosenescence and with mortality. Therefore, circumstantial evidence points to a unique effect of CMV on the accumulation of CD8+ TEMRA cells, presumably due to chronic antigenic stimulations by the persisting virus. However, this does not exclude the possibility that other viruses, or other completely different antigen sources, could have similar effects, for example, in the absence of CMV infection.

To better understand the eventual contribution of the different viruses we should clarify what persistent and chronic viral infections mean (44). Contrarily to acute viral infections, persistent infections have a longer duration mainly because the viral source is not cleared by the immune system and usually resides inside certain cell types (e.g., immune cells, neuronal cells, and epithelial cells). Persistent infections may involve stages of both silent and productive infection without rapidly killing or even producing excessive damage of the host cells. Varicella-zoster virus, measles virus, HIV-1, HHS-6, HHS-7, HSV-1, HSV-2, and human cytomegalovirus (CMV) are examples of viruses that cause typical persistent infections. Latent infection is characterized by the lack of demonstrable infectious virus between episodes of recurrent disease. A latent infection is a phase in certain viruses' life cycles in which after initial infection, virus production ceases (HSVs, VZV). Chronic infection is characterized by the continued presence of infectious virus following the primary infection and may include chronic or recurrent disease. A chronic infection is a type of persistent infection that may be eventually cleared such as HBV, while latent infections such as EBV, CMV, and other herpes viruses persist for the lifetime of the host. Although persistent in nature their natural histories are completely different (44). It is not known why CMV has such a unique effect on immunity, or whether it remains latent in older people. Certainly, CMV reactivates periodically in adults, usually asymptomatically and therefore difficult to identify and study. Little is known about whether reactivation is more frequent on the elderly, and if so what the consequences are, if any.

Thus CMV has evolved to avoid elimination by the hosts' immune effector mechanisms and to persist mostly, presumably, in a non-replicative latent state (because CMV viremia is rare in healthy people). There is evidence to suggest that this latency is nevertheless a highly dynamic condition during which episodes of viral gene desilencing, which can be viewed as incomplete reactivations, cause intermittent antigenic activity that stimulates CD8

clonal expansion (8, 45, 46). All the other persistent virus have a different tissue specificity, reactivation inducers and amounts in the circulation as antigen resulting in a very different immune modulating effect compared to CMV (44, 47). We describe and summarize these differences in **Figure 2**.

CHRONIC ANTIGENIC STIMULATION

The effect of chronic infection caused by CMV will be specifically discussed although various other chronic viral infections such as EBV, hepatitis B virus (HBV), human papilloma virus (HPV), or HIV (48) can also be considered as sources of chronic stress (**Figure 2**). It is well known that these viruses can reactivate when immunity is suppressed, suggesting that constant immune surveillance is required to prevent their reactivation (49, 50). They induce responses of virus-specific CD8+ T cells which can be detected long after the virus is controlled (51). Among these viruses HIV, inducing a distinct and well characterized viral disease at any age, was shown to be constantly replicating and causing an antigen-dependent clonal expansion of the memory T cells somewhat resembling what is found in the aging immune system (52). This led to a generalized concept of premature immunosenescence in HIV patients independent of their age (53). It is of note that basal viral load is constantly present in these individuals (44). Thus HBV (54), HIV (52), and CMV (55) are likely to drive the accumulation of memory T cells at any age, but not EBV and VZV due to their different life cycle and pathophysiology (47, 56, 57).

In this regard, HIV is very interesting and became important for the elderly since the success of antiretroviral therapy (ART). HIV was considered as a premature immunosenescence driver either for CD4+ or CD8+ T cells which showed certain characteristic surface markers and functions as found in elderly HIV uninfected people (53, 58). Many subjects suffering from AIDS now survive to become elderly and thus represent those with a different chronic viral infection from CMV. A persistent systemic inflammation characterizes the clinically latent or asymptomatic stage of the disease under good viral load control by ART (59, 60). Thus, there is continuous antigen stimulation, ongoing inflammatory process and CD4+ T cell loss. The chronological aging of HIV subjects is thus a good model to contrast with the known effects of CMV for discovering what is really due to a specific virus or to general virus-specific processes or to the aging process itself (53).

	CMV	HIV	HBV	EBV	VZV	HSV-1
Expansion (Tetramer)	+++	+	+	+	–	–
Viral Load	+/ –	+	+/ –	+/ –	+/ –	+/ –
Reactivation	?	++	?	?	+/ –	+/ –
Phenotype of Specificity	TEMRA	EM TEMRA	EM	EM	CM EM	EM
Immunological Aging	+++	++++	+	++	–	–
Clinical Impact Young	Moderate	Severe	Mild	Moderate	Mild	Mild
Clinical Impact Elderly	Moderate	Severe	Mild	Moderate	Severe	Mild

FIGURE 2 | Impact of different chronic viral infections on the immune system and clinical consequences in young and elderly subjects. The immunological and clinical impact of these viral infections is different in young and elderly and even among elderly; however they present a persistent antigenic stimulation throughout life.

In addition to viruses, other stimulating agents may be present. This is perhaps the case for the continuous emergence of cancer antigens independent of the overt presence of clinical cancer (61). Indeed, many cancers such as advanced renal carcinoma, head and neck cancers, and melanomas are associated with the presence of late-differentiated CD8+ T cells (62, 63). Moreover, bacterial chronic/latent infections may also contribute; a good example might be Helicobacter pylori. Furthermore, one important source of continuous antigenic stimulation is the gut. It is conceivable that in aging the epithelial surface integrity is disrupted and becomes leaky to antigens coming directly from nutrients or from the gut microbiome, as is the case in HIV infection (64). These antigens can also drive T cells to late differentiation or exhaustion. Furthermore, CD28− CD8+ T cells are found in healthy elderly and in many disease states either in young or elderly subjects. This suggests that the origin of these cells is related to the disease more than the chronological age, and could be caused by chronic antigenic stimulation such as may occur in Alzheimer's Disease (AD) or rheumatoid arthritis (65–67). In fact, we hypothesize that the increase of late-differentiated CD8+ and CD4+ T cells in AD is related to the constant presence of amyloid beta peptide. Therefore, the effects of these chronic antigenic stimulations may be quite different from that observed during chronic CMV infection. Thus, why CMV is so special?

CHRONIC CMV STIMULATION

The fraction of the population infected with CMV depends on socio-economic status, with a prevalence is between 60 and 70% in industrialized countries, while in emerging countries it is almost 100%. Many studies were also performed in younger groups and children to confirm the persistence of this infection. It is of note that CMV may intermittently reactivate during the lifetime and must be maintained in a quiescent state to allow the remaining immune system to function. That is why the paradigm that constant antigenic stimulation by CMV induces immunosenescence was suggested. Nevertheless, it is of particular importance in this context that even young children infected with CMV have similar increased memory CD8+ profiles, decreased diversity in the naïve pool either and this led to the concept that CMV infection results in immune senescence at an earlier age (17, 68, 69). This would suggest that the so-called T cell senescence is not only age-dependent but CMV-dependent and what is seen in aging is the same as is observed in young or middle-aged subjects except that more time has passed since the initial infection. These findings underscore the general concept developed above that chronic antigenic stress is responsible for age-associated changes to the distribution of T cell differentiation phenotypes independent of chronological age, but increasing as age increases due to more extensive pathogen exposures throughout life.

The price paid by the adaptive immune system to maintain CMV in a latent phase is very high in terms of resource dedication (70, 71). In elderly subjects as many as 50% of CD8+ and 30% of CD4+ T cells can be CMV-specific (72) at the expense of

the naïve and other more highly diversified memory T cells. The question is whether the remaining T cells are functional or not and this warrants further investigation. If they are not devoted to CMV their changes should be related to the aging process only, although "bystander" effects of the CMV-specific cells are conceivable too.

Current evidence suggests that CMV infection is a pre-eminent agent driving the differentiation of the CD8+ T cell compartment with aging (73–75) as has also been shown in mice (45, 76, 77). As mentioned above, most of these CMV-specific CD8+ T cells are of TEMRA phenotype with decreased CD28, CD27, CCR7, CD62L, and increased CD57 and re-expressed CD45RA surface markers indicating some putative functional alteration (52). These include decreased proliferative capacity (78, 79) but maintenance of markers of lineage commitment, memory, and cell–cell adhesion. However, even though some may be senescent, for example in terms of smaller proportions responding to specific antigen by producing IFN-γ, the absolute number of these cells is so greatly increased that the overall reactivity is enhanced, not decreased (80). This is also likely to contribute to the development of age-associated low grade inflammation and the consequent age-associated diseases (13, 81, 82). It is of note that this phenomenon is also seen in HIV-infected patients (53). Perhaps, as with the well-defined replicatively senescent cells these TEMRA CD8+ T cells may be largely pro-inflammatory producing a large amount of IL-6, TNFα, and IL-1β (83). However, more evidence has recently emerged that CD28-negative T cells are not necessarily (all) terminally differentiated or senescent because using specific costimuli such as 4-1BBL, OX40L, and cytokine supplementation with IL-2 or IL-15 revealed a retained proliferative capacity (11, 12). As they proliferate under IL-15 this implies that they are also sensitive to homeostatic control *in vivo* and cannot be considered as truly replicatively senescent (12, 84, 85). Furthermore, these TEMRA cells express high levels of Bcl-2 and are more apoptosis-resistant than naïve CD8+ T cells (34). This means that they will continue to accumulate over a longer period of time (i.e., during aging). The survival of these cells is energy-consuming. It is clear that in the competition for nutrients and factors to maintain these "functionally" different pro-inflammatory CD8+ T cells, they will largely outcompete the other T cell subpopulations. They have a survival advantage, with probably a very strong metabolic advantage too. Thus, their maintenance from a metabolic/energetic point of view is very costly and potentially harmful for the survival and function of other T cell subpopulations. This is further corroborated by the fact that CMV largely relies on the host's mTOR pathway to maintain its viral reactivation potency from its latent form (86, 87). Thus, the presence of these metabolically very active CD8+ T cells may contribute to reactivation of CMV and thus to their further expansion, creating a vicious circle increasing with age. Consistent with this, recent evidence highlights and confirms the suppressive effect of mTOR toward CMV infection (87, 88). Thus, not only are these cells accumulating, but their maintenance in the system costs a lot of energy, thus depleting reserves, further impairing the composition and function of the remaining immune system.

In summary, apart from the robust Swedish longitudinal studies involving CMV infection/seropositivity in the so-called IRP, there is very little data to establish whether and how CMV positivity can further influence and shape the immune response in elderly subjects. There is no doubt that CMV is a contributory factor but certainly not the whole story (73, 74). Further longitudinal and functional studies are badly needed to establish the relationship between CMV and aging and mainly the true immunological and clinical effects of this relationship.

WHAT IS THE SIGNIFICANCE OF THESE CHANGES IN THE PERSPECTIVE OF AGING?

All these experimental data suggest that the dysregulation of the immune response or "immunopause" with aging may be due to a chronic antigen-driven process, especially but most likely not exclusively due to chronic CMV infection. The involvement of other factors as well as CMV must of course be recognized, if only because not all individuals are infected with CMV. Thus, there is a significant decrease in naïve T cells independent of CMV positivity even if CMV may further contribute to their decrease (55). CMV-negative individuals show less differentiated (CM and EM profile mainly) CD8+ T cells than CMV-positive elderly individuals (EM and TEMRA mainly) but this is just a question of proportion and both types are increased and have different functioning in elderly compared to young subjects. These CD8+ T cell phenotypic changes are also present in young CMV-positive individuals (66, 68), but we have no longitudinal data on the survival and T cell shifts in these young individuals throughout time. Additionally, the CD4+ compartment is also altered with aging (68, 72) but here CMV plays as much less prominent role than for CD8+ T cell differentiation.

CLINICAL CONSEQUENCES

The clinical consequences of the altered immune response with age are quite well-defined even if for most of them we have only circumstantial evidence. The increased infections, mainly new infections, indicate that naïve T cells' functionality and/or diversity is altered also with aging and probably not only because of expanded CMV-specific T cells. It has been difficult to demonstrate that elderly have more active CMV infections than young subjects (89). Our own unpublished study showed that in elderly subjects suffering from pneumonia, we could not find acute CMV infection. This means that either the CMV-specific T cells are very efficient or that aging does not influence their functionality even if their numbers are increasing. It is of note that our actual clinical measure of CMV reactivation is far from being accurate.

An important clinical consequence of this increased CMV-specific CD8+ T cell clonally expanded pool with the concomitant decrease of the naïve T cell diversity is the decreased vaccine response in elderly (90). Thus, individuals having a large number of CD28− CD8+ T cells produce much less protective antibody following influenza vaccination than those having less of these cells (91). There were no studies on the CMV-specificity of these cells; nevertheless, regarding earlier studies it was speculated that CMV-induced CMV-specific CD8+ T cells decrease the response to influenza vaccine (92). However, in a different study, Saurwein-Teissl et al. (93) could not demonstrate any specificity of these CD8+ T cells for CMV, EBV or influenza viruses. The negative

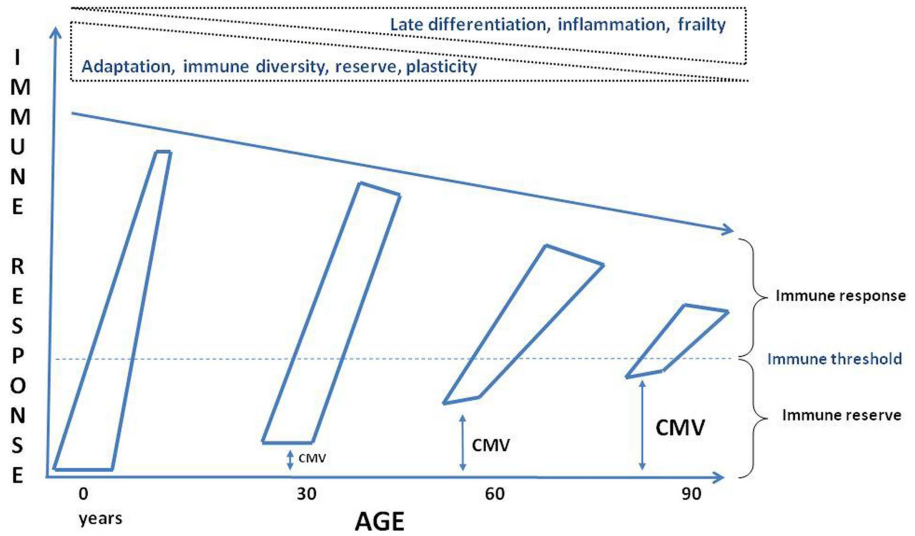

FIGURE 3 | Schematic conceptualization of the effects of aging on the immune system. Through the progression of time the immune reserves are decreasing, the baseline is approaching to the threshold for inducing an efficient response, the immune response is less vigorous and maintained due to a complete reshaping of the immune cells by the immune history, such as persistent viral infection, putatively CMV. ↔: Represents possible CMV infection effects on the immune reserve and the threshold.

impact of CMV infection on the outcome of influenza vaccination in elderly people has been explicitly tested more recently and is seen in many studies [e.g., (94, 95)], but not all (96). However, the latter study in nursing home elderly only considered the antibody response to one of the three viral strains in that seasonal vaccine and found no difference in titers between CMV-infected and CMV-negative. As the WHO criteria for responsiveness specific reactions to two or more of the three strains, had this study examined the other two, a difference may have emerged. It is unclear why this was not reported.

Furthermore, CMV-infected individuals having expanded memory T cell populations have been shown to have a higher risk for coronary heart disease due to vascular inflammation (97, 98) and even for type 2 diabetes (99). Thus, CMV infection can be involved indirectly in the development of chronic inflammatory diseases which are increased in aging. Or vice versa the existence of these inflammatory diseases renders the elderly more susceptible to CMV reactivation. Nevertheless, as already mentioned, CMV positivity is associated with higher all cause of mortality than CMV seronegativity.

CONCLUSION

It is well known that aging is associated with increased susceptibility to many diseases due to dysregulated immune responses (**Figure 3**). However, we still do not know what the causes of this dysregulated immune response with aging really are. Here, it was argued that chronic antigenic stimulation, especially but not only due to CMV infection, plays a crucial role. However, dissecting the effects on immune alterations in elderly individuals with respect to age, low grade-inflammation, disease and CMV seropositivity remains a big challenge. We are currently approaching this challenge by assessing individual variations in responses to CMV, namely antibody titer, specificity, and neutralizing activity and determination of the specific CMV cell reservoirs (e.g., monocytes) rather than just "infected vs. not infected" (100). This approach appears to us more likely to yield informative data in populations where almost all subjects are infected with the virus, for instance elderly individuals even in industrialized countries and essentially everyone in developing countries. Furthermore, longitudinal studies are needed including young and elderly healthy individuals to dissect the effects of age vs. CMV infection. It is also warranted that comparable studies alone or in combination with other persistent viruses to be carried out to exactly determine their effects on the aging immune system. Finally, we should also consider that CMV infection is not uniquely a deleterious process but a constant trigger to maintain "alertness" in the aged immune system to be able to respond correctly to known antigens and overcoming the immunopause by the immunoadaptation triggered and maintained by persistent viral infection (**Figure 3**). Future experimental studies will help us to design efficient and intelligent interventions such as a vaccination or other means (gene silencing, antiviral agents, tissue-specific cell deletion, anti-inflammatory agents) to reinforce homeostasis and maintain a holistically adapted immune response to aging.

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The avalanche is coming … and just now it's starting to snow

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Previous vaccine development has been driven mainly by policies and concerns around childhood, with the aim of preventing premature death amongst young children from infectious disease and stopping them from acting as carriers. Whilst this is important, the face of the world has changed recently and the problem has now to be extended to include the protection of a vulnerable aging population. Here we present the case for a need to develop a prophylactic regimen for older individuals which features vaccination at the center of a portfolio of treatments.

Advances in medical, social, and economic conditions have resulted in human population growth and an ever-extending life expectancy such that for the first time in our history the world population will soon have more people over the age of 65 than under the age of 5. The development of antibiotic therapy, progresses in vaccine technology combined with mass vaccination programs and government schemes to improve economic and social well-being mean that the majority of individuals over the age of 65 are currently physically more active than their counterparts a few decades ago (Michel et al., 2008). Moreover these individuals now travel more than either their parents or grandparents. The world is now so closely networked that any pathogen may spread across the globe within hours, as was observed with the recent H5N1 and H1N1 pandemics (Lang and Aspinall, 2012). In addition to the continuing trend toward increased life expectancy and the shifting demographics, the incidence and prevalence of chronic diseases are also expected to reach unprecedented levels as evidenced from a recent study on individuals over 80 years of age, which showed that many are living with an increasing number of comorbidities (Collerton et al., 2009).

INFLUENZA AND AN AGING POPULATION

Influenza is a disease of viral origin commonly associated with a rapid onset of symptoms which may include fever, chills, fatigue, headache, joint pain, and nasal congestion. But the illness may be asymptomatic in many individuals facilitating the spread of the virus. In older individuals the most common presenting symptom may be a cough. The incidence of fever at presentation in older individuals is much less common than in younger individuals (McElhaney, 2005). Global estimates are that influenza infections cause between 250,000 and 350,000 deaths per year but that there are between 3 and 5 million severe cases per year (http://www.who.int/mediacentre/factsheets/fs211/en/). Influenza is commonly thought to be a disease of limited duration, but in older individuals it may lead to a period of prolonged bed rest. Because long periods of bed rest are associated with a loss of aerobic capacity (1% per week whilst bedridden), a loss of muscle capacity (5% per week), and a loss of bone density (~1% per week) this may leave a previously healthy older individual as a weak frail person dependent on assistance from others for their normal activities of daily living (McElhaney, 2005). Moreover a common complication of influenza is secondary bacterial infection which greatly increases the likelihood of complications such as pneumonia and contributes to a poorer prognosis (Rothberg et al., 2008).

Influenza is mostly transmitted by aerosol, so infection is very dependent on social interaction. A diameter of ~1 m around an individual is the area within which they can become infected by the airborne route (Lofgren, 2011). A potential second route of infection is through self-infection arising because many individuals frequently bring their hands in close contact with their faces. In humans face touching can occur up to almost 40 times per hour (Dimond and Harries, 1984). This action as a potential route of infection becomes important when one considers the survival of infectious organisms on inanimate surfaces such as handrails, door handles, or lift buttons. One recent study reviewed the survival of several infectious agents on seemingly dry inanimate surfaces and suggested that influenza could persist for 1–2 days on such surfaces (Kramer et al., 2006). From this it would appear that one of the greatest risks for becoming infected occurs during periods of close association with a number of individuals some of whom may be infected and the greatest likelihood of this occurring could be during periods of travel (Field et al., 2010). One distinct change between older individuals now compared with previous generations is that older individuals represent a substantial proportion of national and international travelers and might be at higher risk for some travel-associated diseases than younger individuals (Gautret et al., 2012).

INFLUENZA VACCINE FOR OLDER INDIVIDUALS; PROBLEMS, AND PERSPECTIVES

In many countries vaccination with the trivalent influenza vaccine (TIV) is recommended for all adults over the age of 60–65 to combat transmission (Michel et al., 2009). This vaccine contains three strains of inactivated influenza virus (influenza A H3N2 and H1N1 and influenza B) and whilst this immunization strategy has probably contributed to saving many lives, the exact magnitude of its benefits is still hotly debated (Lang et al., 2011). In terms of protection, studies have shown that TIV led to protection in more than 70% of younger

individuals but was ineffective in over half of the elderly population (Hannoun et al., 2004; Monto et al., 2009; Lang et al., 2011). Greater resolution of this issue in the past has been attempted by holding randomized placebo controlled trials (Govaert et al., 1994), but this approach cannot be considered further, so the efficacy of the vaccine, especially in the elderly, has to be mainly derived from observational studies using data from research databases or health care utilization data systems (Lang et al., 2011).

The poor performance of the TIV in providing protection in older individuals has been known for some time, but to date there has been no satisfactory resolution of the issue. Several studies have been undertaken on possible reasons for this problem and these have produced a number of studies on age-associated changes in immunity which have identified problems with Langerhans cells (Shaw et al., 2011), dendritic cells (Panda et al., 2010), T cell function (Saurwein-Teissl et al., 2002), B cell function (Sasaki et al., 2011), T and B cell repertoires (Yager et al., 2008; Ademokun et al., 2011), innate immunity (Shaw et al., 2010), natural killer cells (Chidrawar et al., 2006), in addition to how all of these are affected by the nutritional status of the individual (Langkamp-Henken et al., 2006).

One of the major challenges for vaccine development in this population is associated with identifying measureable surrogate markers which are acceptable readouts of immune protection (Lang et al., 2011). Currently the widely accepted concept of protection is associated with post-vaccination antibody levels; a serum antibody hemagglutination inhibition (HI) titer ≥40 UI/L is the level associated with >50% reduction of the risk of developing influenza infection after exposure (Hannoun et al., 2004). But this correlate of protection was determined in healthy young individuals and not in older people with intercurrent comorbidities. In older adults other immune parameters actively contribute to protection so that HI titer alone may not guarantee immunity or predict susceptibility (Lang et al., 2011). This has led some authors to consider that no single marker should be considered as a surrogate of protection especially in a complex multicomponent system such as the immune system and that protection reflects the sum of various immune responses, including antibody and cell-mediated responses. So in this instance, poor responses to vaccine may result through the accumulation of multiple immune deficits (Lang and Aspinall, 2012).

A PORTFOLIO APPROACH

In a complex system such as the immune system with its multiple component overlaps, predicting individual responsiveness to influenza vaccination using a single method able to distinguish between a healthy and an immunosenescent state, would be very challenging. Especially as the ability of the immune system to respond to vaccination is further impinged upon by the impact of non-immune factors such as nutritional and metabolic status, and comorbidities from which adults increasingly suffer from as they age (Collerton et al., 2009).

The presence of these multiple issues associated with vaccine ineffectiveness underlines the degree of diversity which exists amongst older individuals, which in turn is somewhat expected since aging is neither regulated nor programed. Although each individual ages differently and in a way, which in part is dependent on lifestyle choices and environmental factors, there are common threads (Murabito et al., 2012). This may provide a route to a better understanding of method of protection following immunization in the future.

Since there are currently no tests associated with defining an individual's ability or inability to produce a protective response to vaccination, a more valuable approach would be to measure several aspects of the immune response following vaccination within an individual and compare these not only with known benchmark levels but also to confront these with validated biological and clinical outcomes. This would entail some degree of classification of older individuals into specific trends of aging on the basis of defined characteristics or their absence. In the past efforts have been made to achieve this and define an immune risk phenotype which includes T cell phenotypes, subset numbers, function, CMV status, and cognitive impairment (Wikby et al., 2005). Others have suggested that T cell receptor excision circles (TREC) levels and may be of interest in this classification (Mitchell et al., 2010). Once a characteristic is known to be outside the normal range it would be considered to be in "functional deficit" and the collection of deficits within the immune system which accrue with age would enable us to produce an accumulation of deficits score which could be correlated with clinical outcomes (Lang and Aspinall, 2012). These could assist us to classify individuals into clusters with common features. So, for example, a portfolio of treatments for immunosenescent individuals in the cluster characterized by low TREC levels who were also CMV positive could include therapy to rejuvenate output from primary lymphoid organs such as the use of interleukin 7 (Aspinall et al., 2007) whilst at the same time treating the herpes virus infection with a guanosine analog such as valaciclovir prior to vaccination. Use of such antivirals has provided some interesting results in mouse models. Treatment of elderly mice infected with CMV with valaciclovir, led to reduced influenza viral loads when they were subsequently challenged with influenza (Beswick et al., 2013). Alternatively individuals who are CMV negative and have both normal for age thymic output and T cell function but functionally underperforming dendritic cells may be recommended to receive the vaccine with additional adjuvant.

Such a move from the existing immunization menu toward a greater degree of personalized medicine could contribute to accelerating the development of new vaccines with higher efficacy and of specific combined therapeutic approaches than those currently available.

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How to define biomarkers of human T cell aging and immunocompetence?

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In 2012, 13–22% of the population in Europe, the US, and China were older than 60 years of age. In 2050, demographic models predict that 27–34% of the population in these countries will be older than 60 years of age. Worldwide, more than two billion persons will be age 60 years or over by 2050 (United Nations Department of Economic and Social Affairs Population Division, 2012). This will pose an enormous medical and socioeconomic challenge to our future society. One of the most recognized consequences of aging is a decline in immune function, which limits the protective effect of vaccinations and renders elderly people more susceptible to certain infectious diseases and to newly emerging infections, such as influenza (Gavazzi and Krause, 2002; Goronzy and Weyand, 2013). A higher prevalence of urinary tract infections, lower respiratory tract infections, skin and soft tissue infections, infective endocarditis, bacterial meningitis, tuberculosis, and herpes zoster has been observed in the elderly. Yet, infections in the elderly are not only more frequent but also more severe and have distinct features with respect to clinical presentation (Gavazzi and Krause, 2002). Accordingly, pneumonia, influenza, and septicemia are ranked among the 10 major causes of deaths in people aged 65 years and over in developed countries.

Although considerable progress has been made to identify the cellular changes and molecular mechanisms underlying T cell aging, we still lack biomarkers of T cell aging that have been validated in large populations and that correlate with functional immune responsiveness. This opinion article will therefore focus on how aging affects the number, phenotype, and function of human naïve and memory T cells, and how to identify and validate potential biomarkers of T cell aging. The availability of a valid set of biomarkers will be of utmost importance to improve medical and preventive treatments in the elderly and to evaluate potential therapies that aim to rejuvenate the aged immune system.

THE IMPACT OF AGING ON HUMAN NAÏVE T CELLS

Of the many different types of immune cells, the decline of T cell number and function appears to be a key feature of human immune cell aging. This is due to a decreased capacity of aged hematopoietic stem cells to generate committed lymphoid progenitors and an age-related atrophy of the maturation organ for T cells, the thymus (Linton and Dorshkind, 2004; Geiger et al., 2013). At the age of 50 years, 90% of functional thymic tissue has been lost. This leads to a dramatic shortage of naïve T cells in the peripheral blood (PB), lymph nodes, and bone marrow (BM) (Fagnoni et al., 2000; Lazuardi et al., 2005; Herndler-Brandstetter et al., 2012). Further evidence that supports the key role of the thymus to maintain naïve T cell number and function derives from a study demonstrating premature T cell aging in patients thymectomized during early childhood (Sauce et al., 2009). A decline of naïve T cells in the PB and BM has also been observed in individuals with persistent viral infections, in particular with human cytomegalovirus (HCMV) infection (Almanzar et al., 2005; Herndler-Brandstetter et al., 2012). This indicates that the expansion and accumulation of high numbers of HCMV-specific effector-memory T cells (T_{EM}) leads to an exhaustion of the naïve T cell pool (Sylwester et al., 2005). Another possibility could be that HCMV infection itself contributes to thymic atrophy, as has been reported for murine CMV (MCMV) infection (Price et al., 1993). In summary, the rate of naïve T cell decline during aging is determined by two key factors: the pace of thymic atrophy and certain persistent infections.

The aging process also changes the *quality* of naïve T cells. There are two subsets of naïve ($CD62L^+CD45RA^+$) $CD4^+$ T cells in the PB of humans, defined by the presence or absence of CD31 (PECAM-1) (Kimmig et al., 2002). $CD31^+$ naïve $CD4^+$ T cells may resemble recent thymic emigrants, with a high content of T cell receptor excision circles (TRECs), while $CD31^-$ naïve $CD4^+$ T cells have a lower TREC content and a restricted T cell repertoire (TCR). The loss of CD31 expression has been attributed to TCR-mediated peripheral post-thymic homeostatic proliferation. Accordingly, $CD31^+$ naïve $CD4^+$ T cell numbers decline during aging in the PB. Yet, aged naïve $CD4^+$ T cells respond with an unimpaired IL-2 production upon stimulation with neoantigen and major signaling defects, such as the lack of calcium influx observed in aged mouse naïve T cells, are not seen in humans (Gomez et al., 2004; Tsukamoto et al., 2009). However, human naïve $CD4^+$ T cells from elderly individuals have a reduced TCR-mediated signaling capacity of the ERK pathway due to an age-related decline in miR-181a (Li et al., 2012). Although $CD31^+$ naïve $CD4^+$ T cells were already described in 2002, no follow-up studies have validated CD31 as a functional biomarker of immunocompetence, e.g., protection after immunizations with neoantigens in elderly persons or decreased risk of influenza-associated hospitalization.

Similar to human naïve $CD4^+$ T cells, aged human naïve ($CD62L^+CD45RA^+$) $CD8^+$ T cells display a dramatically restricted TCR repertoire, shortened telomeres and express IL-6Rα and IL-7Rα at a lower frequency (Pfister et al., 2006; Alves et al., 2007; Herndler-Brandstetter et al., 2011). The narrowing of the TCR repertoire by

homeostatic proliferation may be explained by the preferential selection of naïve T cells for high TCR:pMHC avidity, as recently shown in a mouse model. This high avidity naïve $CD8^+$ T cells underwent faster rates of homeostatic proliferation and preferentially acquired a "memory-like" phenotype (Rudd et al., 2011). This study accentuates an important problem that has not received great attention. How reliable are surface markers, such as CD62L, CD45RA, CCR7, CD27, and CD28 in identifying aged human naïve T cells? Not only are memory T cells able to re-express CD45RA and CCR7 (Wills et al., 1999; van Leeuwen et al., 2005), but, and more importantly, naive T cells that undergo extended homeostatic proliferation acquire a memory-like phenotype. This has been demonstrated for murine naïve $CD8^+$ T cells (Murali-Krishna and Ahmed, 2000). In humans, data are very limited. However, a non-regulatory $CD62L^+CD45RO^+CD25^{dim}$ $CD8^+$ T cell population has been identified in healthy, HCMV-seronegative elderly persons who characteristically still had a good humoral response following influenza vaccination (Schwaiger et al., 2003; Herndler-Brandstetter et al., 2005). This novel memory-like $CD8^+$ T cell population had a relatively diverse TCR repertoire, long telomeres, and produced large amounts of IL-2, and may therefore encompass homeostatically expanded naïve T cells (Herndler-Brandstetter et al., 2008).

In conclusion, naïve T cell numbers decline during aging, aged naïve T cells have a restricted TCR diversity and shortened telomeres but seem to retain some of their functional properties. Yet, naïve T cell numbers may be underestimated, as life-long homeostatic proliferation of aged naïve T cells may display a memory-like phenotype. Unfortunately, no large-scale studies have evaluated whether high numbers of naïve T cells with a diverse TCR repertoire and intact IL-2 production correlate with an intact immune responsiveness following vaccination with neoantigens in elderly persons or whether such elderly individuals have a decreased risk of influenza-associated hospitalization.

THE IMPACT OF AGING ON HUMAN MEMORY T CELLS

The ability to generate and maintain functional memory T cells following infection or vaccination is a hallmark of the adaptive immune system and ensures protection upon recurrent infections. In old mice, the generation of functional $CD4^+$ and $CD8^+$ T cell memory is impaired, which has been attributed to functional defects in naïve T cell stimulation and decreased effector T cell expansion (Kapasi et al., 2002; Haynes et al., 2003). In addition, the aged murine microenvironment, in particular defects in T cell migration, priming by antigen presenting cells and differentiation into follicular T helper cells, contributes to a suboptimal $CD4^+$ T cell-mediated immune response.

In humans, memory T cells seem to be less affected by the aging process compared to naïve T cells. For example, CMV-specific T cell immunity is maintained in immunosenescent rhesus macaques and overt CMV disease is rare in the elderly (Rafailidis et al., 2008; Cicin-Sain et al., 2011). However, herpes zoster, which is caused by reactivation of the Varicella zoster virus that causes chickenpox in children, occurs more frequently in the elderly. Following routine vaccinations in healthy and frail elderly persons, decreased IgG antibody concentrations, delayed peak antibody titers, and a more rapid decline in antibody titers were observed compared to young adults (Weinberger et al., 2008). A decreased tumor necrosis factor (TNF)-α synthesis by macrophages also restricts cutaneous immunosurveillance by memory $CD4^+$ T cells during aging, which may thereby contribute to the increased susceptibility to cutaneous infections and malignancies in older humans (Agius et al., 2009). In summary, these studies indicate that memory T cells, as well as their interaction with B cells and antigen presenting cells, are less efficient in old age.

In humans, three major classes of memory T cells can be distinguished based on their phenotypic and functional characteristics: central-memory T cells (T_{CM}) with a $CD45RO^+CD28^+CD62L^+$ phenotype, T_{EM} with a $CD45RO^+CD28^+CD62L^-$ phenotype, and highly differentiated T_{EM} ($CD28^-$ T cells) with a $CD45RO^{\pm}CD28^-CD62L^-$ phenotype. During human aging, the number of T_{EM} and $CD28^-$ T cells increases in the PB and BM (Almanzar et al., 2005; Kovaiou et al., 2005; Herndler-Brandstetter et al., 2012). The accumulation of effector-memory $CD28^-CD8^+$ T cells, which have a highly restricted TCR repertoire, shortened telomeres, and decreased antigen-induced proliferation, has been included in a set of parameters defining the immune risk phenotype and correlates with a lack of antibody production after influenza vaccination in elderly persons (Olsson et al., 2000; Saurwein-Teissl et al., 2002). The loss of the co-stimulatory molecule CD28 and the consequent age-dependent accumulation of $CD28^-CD8^+$ T cells can be attributed to two mechanisms: repeated antigenic stimulation and IL-15-mediated homeostatic proliferation (Valenzuela and Effros, 2002; Almanzar et al., 2005; Chiu et al., 2006). Although there is some confusion in the literature about how to accurately describe this $CD28^-CD8^+$ T cell population, the term "highly differentiated" may be most suitable. Other descriptions, such as dysfunctional or senescent are misleading and do not reflect the properties of $CD28^-CD8^+$ T cells, as these cells are not anergic, are susceptible to apoptotic cell death, proliferate upon proper stimulation and are highly cytotoxic (Chiu et al., 2006; Waller et al., 2007; Brunner et al., 2012). As large numbers of highly differentiated $CD28^-CD8^+$ T cells accumulate during aging, the targeted depletion of these cells has been proposed to generate more "space" for naïve and T_{CM} survival. However, $CD28^-CD8^+$ T cells may be important for tissue-mediated immunity and due to their lack of lymph node homing markers, these cells are likely to occupy different niches than naïve and T_{CM} (Remmerswaal et al., 2012). Moreover, a sudden drop of T cell numbers due to depletion of $CD28^-CD8^+$ T cells may lead to massive peripheral naïve T cell proliferation.

In conclusion, although several human memory T cell subsets have been defined, we still lack information about their origin and maintenance, their functional and migratory properties. The identification of microenvironmental niches of specific memory T cell subsets, in particular of $CD28^-$ T cells, would enable the search of novel markers to distinguish homeostasis- from repeated antigen-driven memory T cells.

CONCLUDING REMARKS

Age-related changes within the human T cell pool have almost exclusively been studied in cells derived from the PB. However, the PB contains only two percent of the total body T cell pool (Di Rosa and Pabst, 2005). Very limited data are available how aging affects

naïve and memory T cells in lymphoid and extra-lymphoid organs (Lazuardi et al., 2005; Herndler-Brandstetter et al., 2012; Sathaliyawala et al., 2013). In particular, as memory T cells in non-lymphoid tissues have been shown to provide enhanced local immunity during infection (Gebhardt et al., 2009). A prerequisite for defining biomarkers of human immune aging and testing strategies to reverse or delay immunological aging is to analyze naïve and memory T cell populations in different organs and validate their phenotypic and functional characteristics. The impact of aging on T cells in distinct microenvironmental niches would also help us to, e.g., identify phenotypic and functionally distinct subpopulations of $CD28^{-}CD8^{+}$ T cells that may have been generated by either chronic antigenic stimulation or life-long homeostatic proliferation. The functional analysis of aged human naïve and memory T cell subsets *in vivo*, e.g., in humanized mice, may be another promising strategy to enhance our understanding about human T cell aging (Rongvaux et al., 2013). Large-scale integrated projects that aim to define biomarkers of aging, such as the EU-funded project MARK-AGE, are also underway and may pave the way for personalized medical treatment and preventive interventions in the elderly.

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