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, $\gamma\delta$ 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_{regs} 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-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
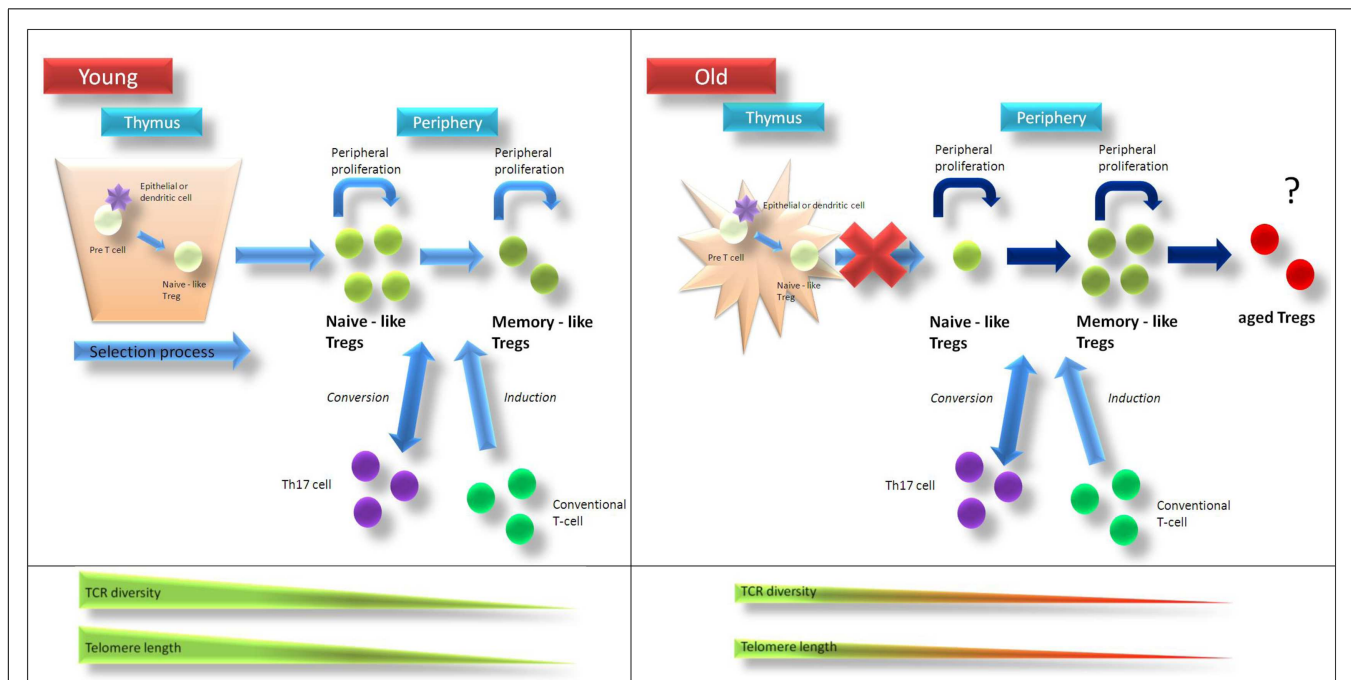

FIGURE 1 | Age-related changes of T_{reg} homeostasis. In young individuals T_{regs} are generated in the thymus and are released as "naïve-like" T_{regs} into circulation. After antigen-contact, T_{regs} develop into a "memory-like" phenotype. T_{reg} homeostasis is supported by homeostatic proliferation 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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