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Homeostasis and function of regulatory T cells in aging

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Abstract

A hallmark of aging is the progressive deterioration of immune function. Age-related immune suppression increases susceptibility to infectious diseases and cancer, significant causes of morbidity and mortality in the elderly. In particular, age-related T cell dysfunction is a major contributor to “immune-senescence”. Recently, it has become clear that the frequency of regulatory T cells (Treg) significantly increases in aged mice and humans. As Treg control the intensity of T cell responses, their accrual likely contributes to age-related immune dysfunction. This review will focus on mechanisms underlying Treg homeostasis and function in aging.

Defective T cell responses are a critical component of immune suppression in the elderly. Indeed, defects in TCR signal transduction affect effector T cell expansion and function, clonally expanded memory T cells impinge upon the breadth of the memory compartment, and aged hosts produce less naïve T cells available for de novo responses. Recent work has found that regulatory T cells (Treg), a subset of CD4+ T cells, are substantially increased in aged mice and humans [1–9]. In addition to their role in peripheral tolerance, Treg also play critical roles in controlling both acute and persistent infections (viral, bacterial, parasitic, and fungal) (reviewed in [10]). Thus, Treg may contribute substantially to inefficient T cell responses in aging.

Increased frequency of regulatory T cells in aged hosts

In young mice and humans, roughly 3–15% of the CD4+ T cells expresses FoxP3, a transcription factor that is both necessary and sufficient for Treg development [11]. Using FoxP3 as a marker, several groups have shown an increased frequency of Treg in the lymphoid organs of aged mice, but not in the circulating blood or the thymus (Table 1). This accumulation is progressive, already visible in middle-aged mice [2].

Due to the limited access of tissues, the picture is not as clear in humans (Table 1). Several studies have assessed Treg (defined as CD4+FOXP3+ or CD4+CD25+) frequency in human blood, and age-associated differences, when found, were minimal. Other studies did not observe increased Treg frequency when Treg were defined as CD25+CD45RA- [12] or CD25^{hi}CD127^{lo} [13]. The only study assessing the effect of aging on the percentage of Treg

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in human tissues also showed a substantial accumulation in the skin [7], strongly suggesting that Treg accrual also occurs in elderly humans. Moreover, these data emphasize that Treg frequency in the blood does not accurately represent their accumulation in the tissues.

Treg are comprised of two major populations: a so-called natural Treg population (nTreg) that is produced in the thymus and a second population that is induced extrathymically in the peripheral lymphoid tissues (iTreg) [14]. The gut-associated lymphoid tissue (GALT) provides a unique environment poised for generation of iTreg. Components of the GALT that promote iTreg development are TGF β and CD103⁺, retinoic acid-producing dendritic cells (DC) (reviewed in [15]). It remains unclear whether the Treg that accumulate in aging are comprised of nTreg, iTreg, or a combination of both. We have found that the majority of Treg in aged mice express high levels of the transcription factor Helios (unpublished data), described to be a specific marker of nTreg [16], although recent data have questioned its stringency as an nTreg-specific marker [17]. Furthermore, we found that aged T cells (either naïve or memory) were less prone to conversion *in vitro* [2]. Thus, these data suggest that aged Treg may be derived from nTreg, although further work is required to rule out a contribution of increased Treg conversion in aging.

Increased Treg may also be the result of clonal expansion, as described for CD8 cells [18]. Recent work has suggested that chronic viral infection can activate endogenous superantigens that drive expansion of V β 5⁺ Treg in C57BL/6 mice [19]. In elderly CMV seropositive patients, Treg and non-Treg were also enriched for V β 2⁺ cells [20]. However, Treg from aged mice have a V β profile similar to young mice, suggesting Treg accrual can occur independently of superantigen or chronic infection (unpublished data). The Treg TCR repertoire in young mice and humans has been shown to be as diverse as non-Treg [21,22], but similar studies have not yet been done in aged Treg. These will be important because changes in repertoire could impact the overall responsiveness and suppressive ability of aged Treg.

Cytokines and maintenance of Treg

Development of nTreg requires common γ c signaling as loss of CD132 results in the loss of Treg during thymic development [23–27]. IL-2R β -deficient mice also have reduced thymic Treg and while their loss of Treg is more profound than observed in IL-2-deficient mice, it is not as severe as that observed in γ c-deficient mice, suggesting that IL-2, IL-15 and perhaps other γ c signaling cytokines promote Treg development [23,28–30]. Consistently, loss of Stat5 prevents Treg development while overexpression of a constitutively active Stat5 restores Treg development in IL-2R β -deficient mice [23]. Maintenance of Treg beyond the thymus is likely also governed by γ c signaling [27]. Interestingly, Treg do not appear to require CD4 nor class II MHC for their development or peripheral survival as ample numbers of Treg are observed in CD4⁻ or class II MHC-deficient mice [31].

Given the disturbed cytokine environment in aging, mechanisms governing young Treg homeostasis may not be entirely the same as those operating to maintain aged Treg. Indeed, we and others have found that a substantial fraction of Treg in aged mice have significantly decreased expression of CD25 [1,2,32], although these CD25^{lo} Treg reside predominantly in the spleen and not the lymph nodes (LN) [1]. Substantial decline in IL-2 with age has been described, mainly based on *ex vivo* assays, in which T cell production of IL-2 was shown to be deficient in aged animals [33,34]. To our knowledge, no study has measured IL-2 levels *in vivo* in aged animals, perhaps because bio-active IL-2 is bound to heparin-sulfate [35]. Nonetheless, it is likely that factors other than IL-2 contribute to the survival of CD25^{lo} aged Treg. We found that aged Treg express increased levels of IL-7R α and IL-2R β , and while we showed that neutralization of IL-7 in aged mice did not reduce Treg [2], we cannot rule

out a potentially redundant role of IL-15. Thus, the use of neutralizing antibodies to all three cytokines (or the γ c) will be needed to critically evaluate the roles of γ c cytokines in Treg survival in aged mice.

Expression of the pro-apoptotic molecule Bim is progressively downregulated in aging and contributes to Treg accrual

Our data point towards increased peripheral survival as the main mechanism involved in Treg accrual in aging, as Treg in aged mice survive significantly better than Treg from young mice, while in vivo Treg proliferation was similar in young and aged mice, and thymic Treg was decreased in aged mice (Figure 1A) [2].

A link between γ c cytokines and cell survival is their ability to manipulate expression and function of Bcl-2 family members (reviewed in [36]). γ c cytokines promote cell survival by increasing expression of Bcl-2 [37] and by decreasing expression of the pro-apoptotic molecule Bim [38]. Mechanistically, we found that Treg have a progressive and significant decrease in their expression of Bim with age. Further, genetic loss of Bim resulted in a rapid accrual of Treg (Treg frequency is similar between 6-month old Bim-deficient and 24 month-old WT mice) [2]. Thus, this normally decreased expression of Bim within Treg provides them with a survival advantage that promotes their accumulation.

There are multiple, non-mutually exclusive explanations for decreased expression of Bim in aged Treg. First, the accumulated Treg in aged mice may be Treg that were produced as part of an effector response, so-called “memory” Treg. We have found that effector memory T cells (which bear a similar phenotype to Treg) have lower levels of Bim compared to central memory T cells [39]. Second, levels of the anti-apoptotic molecules Bcl-2 and Mcl-1 were also decreased in aged Treg [2]. We recently found that the level of Bcl-2 “determines” the level of Bim that CD8⁺ T cells can tolerate and survive [39]. Thus, the loss of Bim by Treg may be a selective event driven by a primary loss of an anti-apoptotic family member. Third, we found that exogenous administration of IL-2 promoted the accrual of Treg with low levels of Bim [2], suggesting that γ c cytokines control the accrual of long-lived Treg. Fourth, it is possible that epigenetic mechanisms lead to methylation of the Bim promoter that decreases Bim expression in aged Treg, as such mechanism is involved in survival of EBV-infected B cells [40]. Together, our data suggest that the accrual of Treg is due to the progressive down modulation of Bim, but further studies are required to determine the underlying mechanism(s).

Expression of markers associated with Treg-mediated suppression

FoxP3 is not only critical for Treg development, but also for Treg function [41]. Treg control T cell responses by targeting effector T cells (Teff) and DC [42], using both contact dependent (CTLA-4, CD39, perforin, etc.) and contact independent (IL-10, TGF- β , etc.) mechanisms. FoxP3 expression per cell is maintained in aged Treg (Table 2), suggesting that Treg function is maintained with age. Indeed, most studies have found that several markers associated with contact-dependent suppression were preserved in aged Treg (Table 2). Few studies have examined levels of contact independent mediators in aging, but we found that Treg IL-10 production is preserved in aged mice [1]. Paradoxically, increased levels of IL-10, TGF- β have been correlated with healthy human aging [43], although the source of these cytokines (Treg vs. non-Treg) is unclear. Overall, it appears that aged Treg express as much, if not more, suppressive markers as young Treg, with the exception of CD25 (at least in the spleen).

Regardless of the mechanism Treg use to suppress, their ability to traffic to or be retained within sites of inflammation is also important. The integrin CD103 may facilitate Treg homing to nonlymphoid tissues. Interestingly, the percentage of splenic CD103⁺ Treg increases with age [1]. Treg in the skin uniformly express CD103, although they may acquire CD103 expression only after arrival [44]. Nevertheless, similar to its proposed role in retaining T cells in the intestinal epithelium, CD103 could increase Treg retention in the skin, especially in humans [7], and this could be an underlying mechanism for increased incidence of skin cancers and infections with aging [45].

Functionality of aged Treg

In general, *in vitro* studies assessing the capacity of human peripheral blood Treg have reported similar, or increased suppressive capacity of aged Treg versus young Treg. *In vitro* CD8 effector function (i.e. cytotoxicity, perforin expression, and IFN- γ production) was similarly decreased by CD25⁺ Treg from young and elderly subjects. On the other hand, CD4 effector function (IL-2 or IFN- γ production) was more suppressed by aged Treg than young Treg, but their effect on proliferation was the same [20,46,47]. The same degree of suppression of human Treg on non-Treg activated in the presence of antigen-presenting cells (APCs) was found irrespective of the source of APCs (autologous [20,48] or allogeneic young APCs [6]).

In mice, aged and young CD25⁺ Treg similarly suppressed APC-driven activation of non-Treg *in vitro* [5,9,32]. In contrast, when Treg were purified from the LN on the basis of FoxP3 expression, not CD25, aged Treg reduced the proliferation of CD4⁺ non-Treg roughly 3-fold better than their young counterparts [1]. The differences observed in these studies can be attributed to technical factors (Treg separation by microbead versus fluorescence-activated cell sorting), the markers used to define Treg (CD25 versus FoxP3 expression), or the source of Treg (LN Treg express more CTLA-4 and CD25 than splenic or blood Treg). Importantly, the suppressive function that aged Treg exert on young effector cells may not reflect the suppressive potential of aged Treg on aged effector cells.

Despite the significance of the question, few studies have so far investigated the *in vivo* functionality of aged Treg. One study showed an age-dependent defective tumor clearance, which correlated with Treg elevation [4]. Importantly, CD25-depletion led to tumor clearance, suggesting that aged Treg limit anti-tumor immunity [4]. Similarly, using the *Leishmania major* infection model, which is exquisitely sensitive to the Treg/Teff ratio [49], we showed that depletion of CD25⁺ T cells reduced lesion size in aged infected mice [1]. Transfer of aged CD25⁺ Treg also controlled delayed type hypersensitivity responses albeit less than their young counterparts [9]. Combined, these studies showed that depletion of CD25⁺ Treg could significantly enhance protective memory T cell responses. Although the suppressor mechanism(s) used by Treg remain unclear (Figure 1), one group showed that CD25-depletion increased co-stimulatory molecule expression on myeloid DC, suggesting that Treg may suppress by limiting APC function [3]. Importantly, to our knowledge, *in vivo* depletion of FoxP3⁺ Treg in aged mice, instead of depletion of CD25⁺ Treg, has not yet been done. These studies are technically feasible since the development of FoxP3-DTR mice, and will be crucial to our understanding of Treg functionality in aged mice.

Summary and future directions

Age-related immune suppression is a growing problem commensurate with the dramatic increase in the aged human population. Novel therapies to boost or reverse immune aging are needed to offset the resulting economic and health burden. Increased Treg proportion in aged mice and humans likely contributes to age-related immune-suppression. However, several tantalizing questions remain unanswered: what maintains elevated numbers of Treg

in aged hosts? How do aged Treg suppress immunity? Can partial Treg depletion be employed to enhance vaccine or anti-tumoral responses and/or elimination of chronic infection? Experimental answers to these questions will hopefully lead to new therapeutic strategies to enhance the quality (and quantity) of life for the world's aged population.

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Abbreviations

FoxP3	forkhead box protein 3
Treg	regulatory T cells
nTreg	natural regulatory T cells
iTreg	induced regulatory T cells
Teff	effector T cells
GALT	gut-associated lymphoid tissue
CTLA-4	cytotoxic T lymphocyte-associated protein-4
GITR	glucocorticoid-induced tumor necrosis factor receptor
CMV	cytomegalovirus
EBV	epstein-barr virus
PD-1	programmed death 1
ICOS	inducible costimulator
APC	antigen-presenting cell
DC	dendritic cell
LAG-3	lymphocyte activation gene-3
LN	lymph nodes

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- of special interest
- of outstanding interest

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Highlights

- Regulatory T cells (Treg) accumulate with age in mice and humans.
- The pro-apoptotic molecule Bim is progressively downregulated in Treg with aging.
- Treg accrual may negatively impact immune responsiveness in aged mice and humans.

Figure 1. Increased survival contributes to accrual of functional Treg with age

Treg frequency progressively increases with age, although overall thymic output declines dramatically with age, due to thymic involution while output of Treg from the thymus is not enriched as mice age. In contrast, the progressive down-regulation of Bim contributes to increased Treg survival and accrual with age. Peripheral proliferation, as assessed by *in vivo* BrdU incorporation, showed no difference in Treg proliferation between aged and young mice [2]. While *in vitro* conversion of naïve or memory T cells to Treg was not different between young and old mice [2], the contribution of peripheral conversion of naïve or effector T cells to Treg accrual *in vivo* remains unclear. Treg from aged mice and humans express similar or higher levels of multiple inhibitory molecules, and may act directly on naïve/effector T cells or on dendritic cells (DC) to restrain T cell responses. Treg depletion *in vivo* significantly enhances protective memory T cells in aged mice.

Table 1

CD4+ FoxP3+Treg proportion and absolute numbers in aged versus young hosts

Human	Frequency	Absolute number
Peripheral Blood	<ul style="list-style-type: none"> Increased [1, 6] Not increased [13, 47] 	<ul style="list-style-type: none"> Not increased [6]
Skin	<ul style="list-style-type: none"> Increased [7] 	<ul style="list-style-type: none"> N/A
Mouse	Frequency	Absolute number
Peripheral Blood	<ul style="list-style-type: none"> Increased [9] ** Not increased [1, 3] 	<ul style="list-style-type: none"> Not increased [3]
Spleen	<ul style="list-style-type: none"> Increased [1–5, 8] 	<ul style="list-style-type: none"> Increased [2–4]
LN	<ul style="list-style-type: none"> Increased [1–4] 	<ul style="list-style-type: none"> Increased in undescribed LNs [4], mediastinal LNs [3], mLN [2] Not increased in pLN [2]
Thymus	<ul style="list-style-type: none"> Not increased [2, 3] 	<ul style="list-style-type: none"> Decreased [2, 3]
Lung	<ul style="list-style-type: none"> Not increased [3] 	<ul style="list-style-type: none"> Not increased [3]

* Defined and determined the Treg frequency as the percentage of CD25+ in CD4+ cells, but also confirmed the expression of FOXP3 in CD4+ CD25+ Treg.

N/A = Not assessed

Table 2Relative expression profile of different markers in aged splenic CD4⁺ FoxP3⁺ Treg versus young Treg

Treg-associated markers	Percentage positive (%)	MFI	Absolute number	References
FOXP3	=	=	N/A	[1, 3, 5]
CTLA-4	=	=	N/A	[1, 5]
GITR	=	+	N/A	[1, 5]
PD-1	+	N/A	++	[1, 8, 50]
ICOS	N/A	N/A	++	[50]
LAG-3	N/A	N/A	+	[50]
CD103	++	N/A	N/A	[1]

= Similar value between aged and young mice

+ 1.5 to 2 folds increase in aged mice

++ >2 folds increase in aged mice N/A: not analyzed MFI: Mean Fluorescence Intensity