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IL-6 and ICOS Antagonize Bim and Promote Regulatory T Cell Accrual with Age

Jana Raynor,* Rebekah Karns,† Maha Almanan,* Kun-Po Li,* Senad Divanovic,*‡ Claire A. Chougnet,*‡ and David A. Hildeman*‡

Regulatory T cells (Tregs), a subset of CD4⁺ T cells, dramatically accumulate with age in humans and mice and contribute to age-related immune suppression. Recently, we showed that a majority of accumulating Tregs in aged mice expressed low levels of CD25, and their accrual is associated with declining levels of IL-2 in aged mice. In this study, we further investigated the origin of CD25⁺ Tregs in aged mice. First, aged Tregs had high expression of neuropilin-1 and Helios, and had a broad Vβ repertoire. Next, we analyzed the gene expression profile of Tregs, naive T cells, and memory T cells in aged mice. We found that the gene expression profile of aged CD25⁺ Tregs were more related to young CD25⁻ Tregs than to either naive or memory T cells. Further, the gene expression profile of aged Tregs was consistent with recently described “effector” Tregs (eTregs). Additional analysis revealed that nearly all Tregs in aged mice were of an effector phenotype (CD44⁺CD62L⁻) and could be further characterized by high levels of ICOS and CD69. ICOS contributed to Treg maintenance in aged mice, because in vivo Ab blockade of ICOSL led to a loss of eTregs, and this loss was rescued in Bim-deficient mice. Further, serum levels of IL-6 increased with age and contributed to elevated expression of ICOS on aged Tregs. Finally, Treg accrual was significantly blunted in aged IL-6–deficient mice. Together, our data show a role for IL-6 in promoting eTreg accrual with age likely through maintenance of ICOS expression.

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The immune system undergoes significant, progressive changes with age that contribute to a dramatic decline in the efficacy of immune responses in the elderly, leading to increased incidences of infections and cancers and decreased vaccine efficacy (1, 2). This suppressed immune phenotype observed in the elderly has been termed “immunosenescence,” and it is driven by defects in both the innate and adaptive immune systems (3, 4). Within the adaptive immune system, T cells exhibit intrinsic defects in TCR signaling, which reduces their ability to proliferate in response to Ag stimulation (5–8). T cells also exhibit defects at the population level, as aged mice have reduced naive T cells because of thymic involution and a constrained repertoire caused by clonal expansion of memory T cells (9–13). Finally, we and others have shown that Foxp3⁺ regulatory T cells (Tregs), a subset of CD4⁺ T cells, significantly accumulate with age and also contribute to age-related immunosenescence (14–18).

Several factors contribute to Treg homeostasis, including production in the thymus, survival, and conversion in the periphery. IL-2 has been described as a major Treg survival factor, because Tregs are decreased significantly in IL-2–deficient mice (19, 20). In addition to IL-2, other common γ-chain cytokines, such as IL-15, contribute redundantly to Treg survival, because CD122⁻ or CD132-deficient mice have a greater loss of Tregs compared with IL-2–deficient mice (19, 21–23). Nonetheless, it is clear that such cytokine signaling promotes Treg homeostasis by antagonizing the proapoptotic activity of Bim (24, 25). However, IL-2 levels decrease with age, favoring the accrual of Tregs that have dramatically reduced levels of Bim and are less dependent on IL-2 for survival (25). Further, combined neutralization of IL-2/15 in vivo led to significant but not complete reduction of Tregs in aged mice (25), suggesting other factors contribute to Treg accrual and homeostasis with age.

In addition to thymic production, Tregs can also be derived from peripheral conversion of naive CD4⁺ T cells via multiple mechanisms (26). Although these converted Tregs normally predominate in the gut tissues, they can populate secondary lymphoid organs sufficient to control autoimmunity under conditions where thymic production is absent (27). Using one in vitro model of Treg conversion, we have shown that, if anything, Treg conversion is reduced in aged mice (28). The lack of distinguishing markers had hampered the ex vivo identification of peripherally converted Tregs, until recent gene expression profiles identified neuropilin-1 (Nrp-1) and Helios as markers of thymically derived Tregs (29–31). However, it remains unclear whether the in vivo accumulation of Tregs in aged mice reflects an expanded peripheral Treg pool or a persisting thymic Treg pool.

Other cytokine-independent mechanisms can also contribute to Treg maintenance, because costimulatory receptors CD28 and ICOS have been shown to affect Treg homeostasis (32, 33). Recent work has defined two subsets of Tregs that differ in their homeostatic requirements: “central” Tregs (cTregs; CD44⁺CD62L⁻)
appear to be more dependent on IL-2 signaling, whereas “effector” Tregs (eTregs; CD44hi CD62Llo) appear to be more dependent on ICOS signaling for their maintenance (34). With age, it is unclear whether the accumulating Bimlo Treg population that is less dependent on IL-2 is reflective of an increase in the eTreg subset.

Aging is also associated with altered systemic cytokine production, and whereas some cytokines such as IL-2 decline (25), others such as IL-6 increase with age (35). Increased inflammatory cytokines are reflective of an overall increase in inflammation that occurs with age, which has been termed “immunaging” (36). It is unclear how this increased inflammatory environment may affect Treg homeostasis with age. However, in young mice, LPS has been shown to promote ICOS expression and expansion of eTregs (34). Increased IL-6 may promote Treg maintenance because IL-6 has been shown to promote the survival of naive T cells and decrease Bim expression within activated T cells (37, 38). Further, one study using IL-6 transgenic mice showed that increasing the levels of IL-6 in vivo can enhance the numbers of Tregs (39). Conversely, IL-6 has been shown to inhibit Treg differentiation in vitro by promoting Th17 lineage commitment along with TGF-β signaling (40, 41). Because Th17 cells are also increased with age (42, 43), the role of IL-6 in promoting IL-6 in promoting Treg accrual remains unclear.

In this study, we further characterized Tregs in aged mice and determined the role of IL-6 and ICOS in their homeostasis. We found that Tregs in aged mice have a predominately effector phenotype and that ICOS is critical for their maintenance, likely by inhibiting Bim-mediated death. In addition, we found that IL-6 contributed to Treg accrual in aged mice and promoted expression of ICOS on Tregs. Thus, although IL-6 clearly promotes inflammation, our data suggest a novel role of IL-6 to counterbalance this inflammation by elevating Tregs.

Materials and Methods

Mice and Ab treatments

Young C57BL/6 mice were purchased from Taconic Farms (Germantown, NY) or were received from the National Institutes of Aging colony located at the Chalk River Laboratories (Wilmingtong, MA). Old C57BL/6 mice were aged in-house or were received from the National Institutes of Aging colony. Foxp3-ires-DR-IRES-DTR-GFP knock-in C57BL/6 mice (44) and Foxp3-IRES-YFP/Cre mice (45) were a generous gift from Dr. A. Rudensky and were aged in-house. Bim-deficient (Bim knockout [KO]) mice were a kind gift from Dr. P. Bouillet and were backcrossed to C57BL/6 mice for 20 generations (Walter and Eliza Hall Institute, Melbourne, Australia). Bim+ mice on the C57BL/6 background were generated in collaboration with Dr. P. Bouillet and were then crossed to Foxp3-IRES-YFP/Cre mice, as previously described (25). IL-6-deficient (IL-6KO) mice on the C57BL/6 background were originally purchased from The Jackson Laboratory (Bar Harbor, ME) (B6.129S2-Igsflm1Sor/J) and maintained and aged in-house. Mice were housed under specific pathogen-free conditions in the Division of Veterinary Services at Cincinnati Children’s Hospital Research Foundation. All animal protocols were approved by the Institutional Animal Care and Use Committee.

Young (2–4 mo) and old (18 mo) C57BL/6 mice were injected i.p. with 7.5 mg/kg anti-ICOSL (HK5.3; BioXcell, West Lebanon, NH) or with rat IgG2A isotype control (2A3; BioXcell) on days 0, 3, 6, and 9 and sacrificed on day 12.

Flow cytometry

Spleen, peripheral lymph nodes (inguinal, axillary, and brachial), and mesenteric lymph nodes were harvested and crushed through 100-μm filters (BD Falcon) to generate single-cell suspensions. A total of 1 × 10⁶ cells were surface stained with a combination of the following Abs: anti-CD4, -CD44, -CD62L, -ICOS, -CCR7, -CD69, -CD25, -Nrp-1 (all from eBioscience, San Diego, CA), and Vp1-17 (BD Biosciences, San Diego, CA). Cells were intracellularly stained for BM (Cell Signaling Technology, Danvers, MA), Bcl-2 (generated in-house), Ki67 (eBioscience), Helios (eBioscience), and Foxp3 (eBioscience) using the eBioscience Foxp3 staining kit and protocol. For surface staining of CCR7, cells were incubated at 37°C for 1 h before adding the anti-CCR7 Ab. Data were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed using FACSDiva software (BD Biosciences). Histogram overlays were generated using FlowJo software (FlowJo, Ashland, OR); the smoothing effect was applied to the histograms, and the y-axis is representing the data normalized to the mode.

In vivo cytokine capture assay and ELISAs

IL-6 and TNF-α in vivo cytokine capture assay was performed as previously described (46–48). In brief, young (2–4 mo) and old (18 mo) C57BL/6 mice were injected i.v. with 10 μg biotinylated anti-IL-6 (MP5-32C11; eBioscience) and anti–TNF-α (TN3; eBioscience) capture Abs; mice were bled 24 h later and serum was collected. A luminescent ELISA was performed using anti-IL-6 (MP5-20F3; eBioscience) or anti–TNF-α (G281-2626; BD Biosciences) as the coating Ab.

Serum IL-1β was measured via Multiplex Assay using Luminex (Milipore, Billerica, MA) according to the manufacturer’s instructions. Serum endotoxin (LPS) was determined using the QCL-1000 Limulus amebocyte lysate (LAL) end-point assay (Lonza, Allendale, NJ), as previously described (49).

Next-generation sequencing

Spleen cells from young (3–5 mo, n = 3, pooled) and old (18 mo, n = 3, pooled) Foxp3-ires-DR-IRES-DTR-GFP mice were enriched for CD4+ T cells using the negative selection MACS CD4+ T cell Isolation kit II (Miltenyi Biotec, San Diego, CA). Cells were stained with anti-CD4, -CD44, -CD62L, and -CD25 Abs, and the following populations were sorted by a FACSAria (BD Biosciences): CD4+ Foxp3GFP+ CD45hi (CD45lo Treg), CD4+ Foxp3GFP+ CD25hi (CD25lo Treg), CD4+ Foxp3GFP+ CD25lo (CD25hi Treg), CD4+ Foxp3GFP+ CD25hi (CD25lo Treg), CD4+ Foxp3GFP+ CD45hi CD25lo (naive CD4+), CD4+ Foxp3GFP+ CD45hi CD25hi (memory CD4+). More than 85% purity was obtained (data not shown). RNA was isolated from the sorted cells using an RNeasy Minikit (Qiagen, Valencia, CA), and amplified with the Ovation RNA-Seq System (NuGEN Technologies, San Carlos, CA). The cDNA library was generated using Illumina NGS library preparation and sequenced on the Illumina HiSeq 2000 with single-end 100-bp reads (Illumina, San Diego, CA) in the Cincinnati Children’s Hospital RNA sequencing core.

RNA sequencing analysis was performed entirely in GeneSpring NGS software (Agilent Technologies, Santa Clara, CA). Sequences were aligned to the mouse reference genome (mm9) with annotations produced by the Ensembl project. Aligned gene read counts were quantified and used to compute reads per kilobase per million for each transcript within each sample. Reads per kilobase per million were normalized using the DESeq algorithm, with read counts thresholded to 1, and the baseline was set to the median of all samples. The data were further filtered, requiring each transcript to have ≥10 reads in at least one of the eight samples (n = 13,940 genes).

We identified differentially expressed genes with a fold change test, using a cutoff of 2.0. In addition, gene lists were built using rank ordering, selecting the top and bottom 500 genes expressed, based on cell type and age. To identify sample clustering based on top and bottom expressed genes based on the age effect, we performed a principal component analysis on the top and bottom 500 genes of old and young Tregs (n = 4144 genes). Four principal components were generated adequate to account for 80% of variability, and samples were clustered based on principal component scores calculated from expression values.

The RNA sequencing data have been deposited to the National Center for Biotechnology Information Sequence Read Archive (http://trace.ncbi.nlm.nih.gov/Traces/sra/), accession number SRP058464.

Results

Aged Tregs express high levels of neuropilin-1 and Helios and have broad TCR Vβ usage

Although our and others’ previous data showed that Tregs accumulate with age (14–18, 25, 28), the origin of these cells remained unclear. One possibility was that the accrued Tregs represent peripherally derived or converted Tregs. To test this, we examined their expression of markers that have been reported to distinguish thymus-derived from peripheral-derived Tregs, Nrp-1 and Helios (29–31). Notably, Tregs from aged mice expressed high levels of both Nrp-1 and Helios (Fig. 1A), suggesting a thymic origin for these cells. Another possibility was that they were oligoclonally expanded cells, similar to CD8+ T cells, perhaps in response to endogenous superantigen (49, 50). However, flow-cytometric
analysis of their TCR Vβ chains showed that aged Tregs have a similar TCR Vβ usage compared with Tregs from young mice (Fig. 1B). Thus, Tregs in aged mice represent a relatively diverse pool of cells expressing markers denoting a thymic origin.

**Gene expression profiling reveals an eTreg phenotype in aged mice**

Our prior data showed that a substantial fraction of Tregs that accumulate with age express low levels of CD25 (25). To further characterize these cells, we sort-purified CD25lo and CD25hi Tregs (as well as naive and memory CD4+ T cells) from young and old Foxp3-GFP reporter mice (44), and subjected the isolated RNA from these cells to high-throughput sequencing. The CD25lo Tregs, from both young and old mice, had a gene expression profile different from memory CD4+ T cells and expressed genes associated with Tregs (i.e., Foxp3, Cita4, Tnfrsf18 [GITR], Il10, Iltge [CD103]; Fig. 2A). To independently determine the relationships between these populations, we performed a principle component analysis. This analysis showed a tighter clustering of CD25lo with CD25hi Tregs than with naive or memory CD4+ T cells (Fig. 2B). Thus, principle component analysis shows that Tregs from aged mice are more like Tregs from young mice than they are old memory cells. However, both old CD25lo and CD25hi Tregs cluster closer together than young CD25lo and CD25hi Tregs (Fig. 2B). In terms of their gene expression, both CD25lo and CD25hi Tregs from aged mice had enhanced expression of genes associated with recently described eTregs (Il10, Icos, Prdm1 [Blimp-1], Ebi3, Ccr6; Fig. 2A) (34, 51, 52).

**Prior work showed that high expression of CD44 and low expression of CD62L marked an eTreg population (34). Further, these eTregs were less dependent on IL-2 (34), similar to what we previously reported for CD25lo Tregs (25). Therefore, we longitudinally characterized the “effector” (CD44hi CD62Llo) versus “central” (CD44lo CD62Lhi) phenotype of Tregs in young (3 mo), middle-aged (12 mo), and old (>18 mo) mice. Strikingly, by middle age, most Tregs had acquired an effector phenotype (>85%), which increased only slightly in old mice (Fig. 3A, 3B). The increase in eTreg frequency with age occurs mainly within the lymphoid tissues and not the nonlymphoid tissues, which are comprised predominately of eTregs even in young mice (Supplemental Fig. 3A). Further, the overall increase in numbers of Tregs in aged mice was largely due to the accrual of eTregs (Fig. 3C).

To further characterize eTregs in aged mice, we assessed their expression of ICOS, CD69, CCR7, and CD25, and identified by our RNA sequencing analysis as changing in aged Tregs. As expected, both ICOS and CD69 are increased in eTregs with age, and this is evident already by middle age (Fig. 3D, 3E). The progressive increase in ICOS expression with age is not Treg-specific, because CD4+ Foxp3+ T cells (both memory and naive) also have an increase in ICOS expression; however, the fold increase is less compared with Tregs (1.4-fold in eTregs vs. 2.17-fold in memory CD4+ T cells (both memory and naive) also have an increase in ICOS expression; however, the fold increase is less compared with Tregs (1.4-fold in eTregs vs. 2.17-fold in memory CD4+ T cells, Fig. 2B). Thus, principle component analysis shows that Tregs from aged mice are more like Tregs from young mice than they are old memory cells. However, both old CD25lo and CD25hi Tregs cluster closer together than young CD25lo and CD25hi Tregs (Fig. 2B). In terms of their gene expression, both CD25lo and CD25hi Tregs from aged mice had enhanced expression of genes associated with recently described eTregs (Il10, Icos, Prdm1 [Blimp-1], Ebi3, Ccr6; Fig. 2A) (34, 51, 52).

**eTregs preferentially accumulate with age**

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**Deletion of Bim promotes eTreg accrual**

We have previously shown that Bim is a critical negative regulator of Treg homeostasis (25, 28). Intracellular flow-cytometric analysis showed that cTregs have higher expression of Bim relative to eTregs (Fig. 4A). Given that cTregs maintain higher expression of Bim, even in aged mice, it is possible that the high levels of Bim within cTregs may drive their decline via apoptosis. To test this, we examined Treg subsets in aged mice with Treg-specific deletion (Foxp3-Cre Bimfl/fl) or germline deletion of Bim (BimKO). The deletion of Bim did not rescue the loss of cTregs with age (Fig. 4B), even though Bim was effectively deleted in both eTregs and cTregs (Supplemental Fig. 2). In fact, the accrual of eTregs was accelerated in mice with either a germline or Treg-specific loss of Bim. Combined, these data show that eTreg, but not cTreg, accrual with age is limited by Bim-mediated death.
Aged IL-6KO mice have reduced Tregs

Previous work showed that inflammatory stimuli such as LPS altered expression of ICOS and CCR7 on Tregs, resulting in a more eTreg phenotype (34). Because LPS is known to drive inflammatory cytokine expression, we first determined the levels of inflammatory cytokines in aged mice. Similar to prior reports, we found that aged mice have a 3-fold increase in serum IL-6; however, no change in the inflammatory cytokines TNF-α and IL-1β or in serum LPS was observed (Fig. 5A). To assess whether the accumulation of IL-6 with age contributes to Treg accrual, we aged IL-6KO mice to 18 mo. As expected, young IL-6KO mice had no difference in Treg frequencies or numbers compared with wild-type (WT) mice (Fig. 5B). However, aged IL-6KO mice had significantly reduced frequencies and numbers of Tregs (Fig. 5B). This reduction was mainly within the lymphoid tissues (spleen, peripheral lymph nodes, mesenteric lymph nodes), and not within some nonlymphoid tissues (liver, intestinal intraepithelial lymphocytes) (Supplemental Fig. 3B). Given the role of Bim in limiting Treg accrual, we assessed the effect of IL-6 on Bim expression in Tregs with age. We found that the diminution of Bim expression with age is impeded in IL-6–deficient mice (Fig. 5C). Importantly, aged IL-6KO mice had similar frequencies of eTregs compared with WT mice, and the eTregs in aged IL-6KO mice had higher Bim expression (data not shown). Together, these data show that IL-6 represses Bim and promotes Treg accrual with age.

IL-6 promotes ICOS expression on Tregs

TCR signaling promotes the expression of ICOS (53); however, it is unclear whether cytokine signaling can induce ICOS expression. Given the increase in both serum IL-6 and Treg ICOS expression with age, we asked whether IL-6 promotes ICOS expression on Tregs. Although IL-6 alone does not have a significant effect on ICOS expression ex vivo, IL-6 did have an additive effect in combination with TCR signaling (Fig. 6A). Further, the absence of IL-6 resulted in decreased ICOS expression on aged eTregs and cTregs in vivo (Fig. 6B). Thus, IL-6 enhances TCR-driven ICOS expression on Tregs and contributes to the increase in ICOS expression with age.

ICOS promotes eTreg maintenance in old mice

Given that ICOS/ICOSL interactions are critical for eTreg homeostasis in young mice (32, 34), we examined the role of ICOS in eTreg homeostasis in old mice. Notably, ICOS signals predominately through a PI3K/Akt/FOXO pathway, which is known to affect Bim expression in T cells (54, 55). In Tregs, high expression of ICOS correlates with lower expression of Bim, in both young and old Tregs (Fig. 6C). To test whether ICOS/ICOSL interactions are critical for Treg maintenance in old mice and whether such interactions affect the expression of Bim, we neutralized ICOSL in young and old mice. Neutralization of ICOSL resulted in a significant decrease in the number of eTregs in young and old mice (Fig. 6D), whereas the numbers of cTregs were not affected regardless of age. The fold loss of eTregs was greater in old mice compared with young mice (2.3-fold versus 1.4-fold, respectively), resulting in a substantially skewed eTreg/cTreg ratio in aged mice treated with anti-ICOSL (Fig. 6E). Importantly, the effects of anti-ICOSL on Treg homeostasis were not due to decreased proliferation, because the frequency of Ki-67+ cells was not changed after anti-ICOSL treatment (Fig. 6F). Further, the numbers of dendritic cells were not changed after anti-ICOSL treatment (data not shown). Instead, anti-ICOSL treatment led to a slight, albeit statistically significant, increase in expression of Bim in the eTregs (Fig. 6G). Given the critical role for Bim in mediating apoptosis of Tregs, it is possible that further increases in Bim due to ICOSL blockade results in cell death, making it difficult to detect the potential magnitude of Bim induction. To test whether the loss of eTregs was driven by Bim-mediated death, we treated BimKO mice with anti-ICOSL. Importantly, the loss of eTregs was rescued in the absence of Bim (Fig. 6H). Together, these data show that aged eTregs are more dependent on ICOS for survival and that ICOS enhances Treg survival by antagonizing Bim.
Discussion

Our and others’ data show that Tregs accumulate with age and contribute to suppressed immune responses (14–18, 28). Further, there is a growing appreciation that the Treg population is heterogeneous, comprised of subsets that have differences in transcriptional regulation, tissue localization, and functionality (34, 52, 56, 57). To date, it is unclear what subsets contribute to Treg accrual with age. We previously reported that a population of CD25lo Tregs preferentially accrue with age (25), and in this article we show that these cells resemble the recently defined eTregs (Foxp3+ CD44hi CD62Llo), both at the transcriptional and the protein levels. The eTregs that accumulate with age have increased expression of ICOS and CD69. ICOS promotes the maintenance of aged eTregs, likely by reducing Bim-mediated death. Further, we show that the inflammatory environment in aged mice, namely, IL-6, is required to maintain optimal ICOS expression and Treg accrual. This study elucidates a novel pathway of Treg accrual and maintenance with age, mediated by an IL-6–ICOS–Bim axis.

There are two sources of Tregs that may contribute to the accumulated Treg pool in aged hosts, thymically derived Tregs and Tregs that are converted in the periphery from conventional CD4+ T cells. One study has suggested that Treg accrual with age is absent when peripheral induction of Tregs is impaired (58). However, this study looked at Treg frequencies only at 8–12 mo of age, a time point when only modestly increased Treg frequencies are seen, and the reported frequencies of Tregs in their WT mice were substantially elevated compared with what is historically seen (58). In this study, we used Nrp-1 and Helios expression to differentiate between thymic and peripherally derived Tregs, and showed that aged Tregs are enriched for Nrp-1+ Helios+ cells, suggesting aged Tregs are of thymic origin. Although the specificity of these molecules as markers for thymic Tregs remains controversial, it is clear that peripherally derived Tregs in the gut are negative for expression of Nrp-1 and Helios (29, 59–61). Given the reduction in Tregs in the thymus with age (28), the accrual of thymus-derived Tregs with age is likely due to increased survival. Indeed, we showed that Bim-deficient mice have accelerated Treg accrual that begins after cells have left the recent thymic emigrant compartment (28), and importantly these cells remain Nrp-1+ Helios+ (data not shown). Thus, within the secondary lymphoid organs, the Tregs that accumulate appear to be largely thymus derived.

We and others have defined Treg subsets as CD25lo and CD25hi (16, 25); however, with age these populations become more homogeneous at the protein and transcript level, and thus CD25 is likely not the best marker to differentiate aged Treg subsets. Instead, we assessed Treg subsets as eTregs and cTregs, as recently described (34), and showed that it is the eTregs that accumulate with age. Further, the eTreg have increased expression of ICOS with age and are partially dependent on ICOS for their maintenance. Mechanistically, TCR signaling promotes ICOS expression (34). Further, LPS-induced inflammation promoted increased ICOS expression (34). However, how ICOS is controlled in aged

FIGURE 3. Tregs that accumulate with age have an eTreg phenotype. Splenocytes from young (3 mo, n = 4), middle-aged (12 mo, n = 4), and old (≥18 mo, n = 4) WT mice were stained with Abs against CD4, CD44, CD62L, ICOS, CCR7, CD69, CD25, and Foxp3, and analyzed by flow cytometry. (A) The dot plots show representative frequencies of CD4+ Foxp3+ cells that are memory (CD44hi CD62Llo) and naive (CD44lo CD62Lhi), and the frequencies of CD4+ Foxp3+ cells that are eTregs (CD44hi CD62Llo) and cTregs (CD44lo CD62Lhi). (B) Data show the frequency of Foxp3+ that are eTregs (gray) or cTregs (black) (± SE). The statistics are comparing the eTreg populations. (C) Data show the total number of cells that are Foxp3+ (total Treg), eTregs, and cTregs in young (black), middle-aged (gray), and old (white) mice (± SE). (D) The representative histograms show the expression of ICOS, CCR7, CD69, and CD25 on eTregs (gray) and cTregs (black). The numbers are the MFI (ICOS and CCR7) or the frequency of eTregs and cTregs (CD69 and CD25). (E) The bar graphs show the average MFI of ICOS and CCR7 expression on eTregs and cTregs in young (black), middle-aged (gray), and old (white) mice, as well as the frequency of cells that are CD69+ (± SE). Data are representative of at least three independent experiments. *p ≤ 0.05, **p ≤ 0.01 (Student t test).
Treg is unclear. Our data show that the proinflammatory cytokine IL-6 can enhance ICOS expression in the context of TCR signaling; however, whether IL-6 promotes ICOS expression on aged Tregs through direct or indirect mechanisms remains unclear. IL-6 can directly signal in Tregs, and young and old Tregs express similar levels of IL-6R (data not shown), although IL-6–induced STAT3 phosphorylation is slightly impaired in old Tregs compared with young Tregs (43, 53). ICOS expression is also upregulated downstream of TCR/CD28 via NFATc2 and ERK signaling (53). IL-6 can induce ERK activation (62), and thus may enhance TCR/CD28-driven ICOS expression on Tregs. We cannot exclude indirect pathway(s) by which IL-6 promotes ICOS expression on Tregs, because IL-6 can affect dendritic cells and macrophage maturation (63, 64). Indeed, dendritic cells can promote Treg homeostasis (65). Thus, elevated IL-6 may promote enhanced Ag presentation and CD80/CD86 expression, prolonging TCR/CD28 signaling. Future work will determine whether Tregs or other non-Tregs need to express IL-6R to promote Treg accrual.

ICOS signaling promotes eTreg homeostasis (34); however, the mechanisms still remain unclear. Smigiel et al. (34) showed that blocking ICOSL selected against Bcl-2lo Tregs without affecting proliferation, suggesting that the loss in Tregs was due to cell death. Mechanistically, ICOS signaling may promote survival by inhibiting Bim-mediated death through activation of the PI3K/Akt pathway (54), which is known to limit Bim expression through modulating FOXO3 transcription factor activation (66, 67). Indeed, Bim is a critical negative regulator of T cell survival and homeostasis, and we have shown that the levels of Bcl-2 determine the levels of Bim a T cell can tolerate (68). Consistently, we show that blocking ICOSL results in increased Bim expression, and the absence of Bim rescues the loss of eTregs. Thus, with age, enhanced ICOS expression on eTregs likely limits Bim expression via activation of the PI3K/Akt/FOXO pathway, promoting eTreg survival.

The cTreg population decreases with age, and this loss may be driven by multiple nonmutually exclusive mechanisms. First, Treg production declines with age because of thymic involution (12, 28). The majority of Tregs in the thymus are cTregs (34); thus, thymic production is likely a major source of this population. Second, cTregs can become eTregs, a process driven by TCR signaling (34). Indeed, we found that transfer of CD25hi Tregs (likely mostly cTregs) converted to CD25lo Tregs (likely mostly eTregs) after adoptive transfer (25). Further, both eTregs and cTregs in old...
mice have an increased frequency of CD69+ cells, a marker of recent T cell activation, supporting a model of cTreg activation and conversion. Lastly, we showed that serum IL-2 levels declined with age (25), which may select against cTregs because these cells are more dependent on IL-2 for maintenance (34). IL-2 promotes Treg survival by combating Bim-mediated death (24, 25). However, deletion of Bim did not rescue the loss of cTregs with age. These data argue against increased Bim-mediated cell death as driving the loss of cTregs with age; however, we cannot exclude the role for another proapoptotic, such as Puma. Alternatively, it is possible that the potential Treg conversion of cTregs to eTregs is dominant to the death process.

Our data showing that IL-6 promotes Treg accrual is seemingly contradictory to the literature showing that IL-6, along with TGF-β, inhibits Tregs while promoting Th17 differentiation (40, 41, 69). This role of IL-6 was established with in vitro cultures using undifferentiated naive CD4+ T cells (40, 41). In vivo, models of limiting IL-6 promote increased Tregs in the context of inflammation (70–72), supporting an inhibitory role of IL-6 on peripheral Treg induction. However, the effect of IL-6 signaling on thymically derived Tregs is less clear. A recent study has shown that IL-6 can induce a “reprogramming” of peripheral Tregs through downregulating the transcription factor Eos (73). Eos+ Tregs maintained normal Foxp3 expression but exhibited both proinflammatory and anti-inflammatory properties, depending on the tissue localization of the Tregs and the inflammatory environment (73). Aged Tregs do not exhibit decreased Eos expression (data not shown); thus, elevated IL-6 with age is not likely driving Treg “reprogramming.” In addition, increased in vivo IL-6 levels can promote increased Treg numbers, because IL-6 transgenic mice have elevated thymus-derived Treg numbers and these Tregs are functional, whereas induction of peripheral derived Tregs was inhibited (39). Thus, the role of IL-6 on Treg differentiation and homeostasis is multifactorial and likely cell context dependent.

Recently, the PTEN-mTORC2 axis has been implicated in regulating Treg homeostasis and functionality (74, 75). Interestingly, PTEN-deficient Tregs have an eTreg phenotype similar to aged Tregs, with elevated CD44, ICOS, and CD69 expression (75). At the transcriptional level, aged Tregs do not have decreased PTEN expression (data not shown); however, it is unclear whether PTEN signaling changes with age and contributes to eTreg homeostasis. Understanding eTreg homeostasis is of broader relevance, because humans have a population of ICOS+ IL-10+ Tregs that resemble eTreg in mice (76). Thus, future studies investigating the heterogeneity within the eTreg subset with age are of clinical relevance, because they may further elucidate Treg homeostasis and functionality with age and may provide potential therapeutic targets for manipulating Treg numbers and function in the elderly.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


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**FIGURE 6.** ICOS contributes to eTreg maintenance in aged mice. Splenocytes from young (2–4 mo) and old (≥18 mo) mice were stained with Abs against CD4, CD44, CD62L, Bim, ICOS, and Foxp3, and analyzed by flow cytometry. (A) Splenocytes from young WT mice (2 mo, n = 4) were cultured for 24 h with IL-6 (5 ng/ml), anti-CD3/CD28 (3 μg/ml), or both. Data show the ICOS MFI on total CD4+ Foxp3+ cells from WT mice. (B) Data show the ICOS MFI on eTregs and cTregs isolated from the spleens of young (n = 5–8/group) WT and IL-6KO mice (± SE). (C) Representative dots plots show the expression levels of Bim against ICOS on total CD4+ Foxp3+ cells from WT mice. (D–G) Young (n = 5) and old (n = 5) WT mice were treated with isotype control (black) or anti-ICOSL blocking Ab (white) for 12 d and splenocytes were analyzed by flow cytometry. Data show the total number of CD4+ Foxp3+ cells that are eTregs or cTregs in young and old mice (D), the ratio of eTregs to cTregs in young and old mice (E), the frequency of eTregs that are Ki67+ (F), and the average Bim MFI in eTregs and cTregs (± SE) (G). Young (3 mo, n = 4–5 mice/group) WT and BimKO mice were treated with isotype control (black) or anti-ICOSL blocking Ab (white) for 12 d. Data show the total number of CD4+ Foxp3+ that are eTregs or cTregs (± SE). *p ≤ 0.05, **p ≤ 0.01 (Student t test).


