Anti-apoptotic Mcl-1 is critical for the survival and niche-filling capacity of Foxp3+ regulatory T cells

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Abstract

Foxp3+ regulatory T (Treg) cells are a crucial immunosuppressive population of CD4+ T cells, yet the homeostatic processes and survival programs that maintain the Treg cell pool are poorly understood. Here we report that peripheral Treg cells markedly alter their proliferative and apoptotic rates to rapidly restore numerical deficit through an interleukin 2–dependent and costimulation-dependent process. By contrast, excess Treg cells are removed by attrition, dependent on the Bim-initiated Bak- and Bax-dependent intrinsic apoptotic pathway. The antiapoptotic proteins Bcl-xL and Bcl-2 were dispensable for survival of Treg cells, whereas Mcl-1
was critical for survival of \( T_{\text{reg}} \) cells, and the loss of this antiapoptotic protein caused fatal autoimmunity. Together, these data define the active processes by which \( T_{\text{reg}} \) cells maintain homeostasis via critical survival pathways.

The expression of the transcription factor Foxp3 in T cells results in radical transcriptional rewiring\(^1\) and the consequent functional differentiation of these cells into \( T_{\text{reg}} \) cells. The most profound effect is the switch from a proimmunity potential to a protolerance function that is essential for preventing fatal systemic autoimmunity\(^2\). In addition to this archetypal characteristic, the transcriptional rewiring also alters the basic cellular properties of \( T_{\text{reg}} \) cells, including differential reliance on cytokines\(^3,4\) and T cell receptor (TCR) signaling\(^5\) for homeostasis as well as an unusual anergic and apoptotic behavior \textit{in vitro}. The size of the \( T_{\text{reg}} \) cell population is critical for immunological balance; even relatively minor modulation alters immunity\(^6\). Abnormally low numbers of \( T_{\text{reg}} \) cells have been observed in multiple autoimmune and inflammatory conditions\(^7\), whereas high numbers of \( T_{\text{reg}} \) cells in the aged are thought to contribute to the partial immunosuppressed state\(^8,9\). In addition, \( T_{\text{reg}} \) cells are postulated to impede effective immunity against cancer\(^10\), with expansion of \( T_{\text{reg}} \) cells after radiotherapy reported to limit lymphoma clearance\(^11\). Given the growing interest in manipulating \( T_{\text{reg}} \) cell numbers in clinical settings, there is a pressing need to understand the cellular and molecular biology of \( T_{\text{reg}} \) cell homeostasis.

Apoptotic cell death is a major regulator of hematopoietic cell homeostasis\(^12\). In vertebrates, two distinct, but ultimately converging, pathways control apoptosis. The ‘extrinsic’ or ‘death receptor’ pathway is initiated by ligation of cell-surface death domain–containing members of the TNF-receptor family. The ‘intrinsic’ or ‘mitochondrial’ pathway is initiated by cellular stressors that alter the balance between proapoptotic and antiapoptotic members of the Bcl-2 family of proteins, culminating in the activation of Bax and Bak, and subsequent release of apoptogenic factors from mitochondria\(^13\). The two pathways converge upon the activation of ‘executioner’ caspases that demolish the cell. In the intrinsic apoptosis pathway, activation of Bax or Bak is the ‘point of no return’ and thus requires tight regulation. The prosurvival proteins Bcl-2, Bcl-xL, Mcl-1, A1 and Bcl-w restrain the activation of Bax and Bak, whereas proapoptotic proteins containing only the Bcl-2 homology domain BH3 (the so-called BH3-only proteins: Bim, Puma, Bid, Bmf, Bad, Noxa, Bik and Hrk) inhibit the prosurvival members and are essential for initiation of apoptosis signaling\(^14\). The relative importance of each of these proteins varies among different cell types or cytotoxic stimuli and therefore has to be evaluated on a case-by-case basis.

In this study we took advantage of the location of \( Foxp3 \) on the X chromosome and the effect of X inactivation on a diphtheria toxin (DT) receptor knock-in construct to study the dynamics of \( T_{\text{reg}} \) cell responses to homeostatic perturbation in a highly controlled manner. We found that modulation of the \( T_{\text{reg}} \) cell population created a TCR costimulation-dependent and IL-2–dependent feedback loop, which increased proliferation of \( T_{\text{reg}} \) cells and diminished apoptosis to drive rapid restoration of \( T_{\text{reg}} \) cell numbers. Furthermore, we found that the Bak- and Bax-dependent intrinsic apoptotic pathway naturally limited the \( T_{\text{reg}} \) cell population, with \( T_{\text{reg}} \) cell accumulation observed in absence of Bak and Bax. Prosurvival Bcl-2 family member Mcl-1 safeguarded the survival of \( T_{\text{reg}} \) cells; deletion of
Mcl-1 caused a rapid loss of T<sub>reg</sub> cells and onset of fatal autoimmunity. Mcl-1 expression is regulated by interleukin 2 (IL-2), which increased Mcl1 transcription during the T<sub>reg</sub> expansion phase after <i>in vivo</i> depletion. Finally, the BH3-only protein, Bim, is the primary antagonist of Mcl-1 in T<sub>reg</sub> cells, as conditional deletion of Bim led to accumulation of excess T<sub>reg</sub> cells, as observed with loss of Bak and Bax.

**RESULTS**

**T<sub>reg</sub> cells exhibit IL-2–dependent niche-filling behavior**

To determine the homeostatic characteristics of T<sub>reg</sub> cells, we compared the proliferative behavior of Foxp3<sup>+</sup> T<sub>reg</sub> cells in 5-bromodeoxyuridine (BrdU) labeling experiments. In contrast to prior <i>in vitro</i> studies that characterized T<sub>reg</sub> cells as semianergic, quiescent cells, we found that T<sub>reg</sub> cells proliferated at a substantially faster rate than conventional T cells (CD4<sup>+</sup> or CD8<sup>+</sup>) <i>in vivo</i>, with ~50% of the population having undergone proliferation within a 10-d window in unmanipulated hosts during homeostatic conditions (Supplementary Fig. 1). As these results suggest a highly dynamic yet stable population, we developed a 50% depletion system to examine responses to such perturbations in the T<sub>reg</sub> cell niche using two different Foxp3 constructs. The first was Thy1 (Thy1.1 variant) and the second was human HBEGF (diphtheria toxin receptor; DTR), each knocked into the Foxp3 locus on the X chromosome. Female mice heterozygous for the Foxp3<sub>Thy1.1</sub> and Foxp3<sub>DTR</sub> alleles (Foxp3<sub>Thy1.1/DTR</sub> mice) have two distinct populations of T<sub>reg</sub> cells due to random X inactivation of Foxp3 alleles. Half express the marker Thy1.1, and the other half express DTR. Non-T<sub>reg</sub> cells express neither marker. Upon injection of DT, the DTR<sup>+</sup> T<sub>reg</sub> cells will be rapidly eliminated and the response of the DTR<sup>-</sup>Thy1.1<sup>+</sup> compartment to this 50% drop in total T<sub>reg</sub> cell numbers can be tracked. An additional advantage of this system is that the use of Thy1.1 to mark untouched T<sub>reg</sub> cells circumvents the difficulties in measuring apoptosis caused by the cleavage of Foxp3 by activated caspases (Supplementary Fig. 2). DT addition efficiently eliminated DTR<sup>+</sup> T<sub>reg</sub> cells, but the overall proportion of Foxp3<sup>+</sup> cells was rapidly restored by the expansion of DTR<sup>-</sup>Thy1.1<sup>+</sup> T<sub>reg</sub> cells (Fig. 1a). The six fold increase in the number of Thy1.1<sup>+</sup> T<sub>reg</sub> cells by day 5 caused an initial overshoot of ~200% in total T<sub>reg</sub> cells, followed by a slow decline to basal levels (Fig. 1a and Supplementary Fig. 3). During the niche-filling process, proliferation rate of Thy1.1<sup>+</sup> T<sub>reg</sub> cells increased, with the percentage of cells expressing the cell cycle protein Ki67 increasing from ~20% to ~70% (Fig. 1b). At the same time, the apoptosis rate of Thy1.1<sup>+</sup> T<sub>reg</sub> cells decreased from ~40% to ~20% active caspase-3<sup>+</sup> (Fig. 1c). We found no evidence of any substantial contribution to the peripheral homeostasis of T<sub>reg</sub> cells by recent thymic emigrants (tracked using a Rag2-GFP transgene or comparison to thymectomized mice; Supplementary Fig. 3h–j) or peripheral conversion of conventional T cells into T<sub>reg</sub> cells, as this mechanism would have resulted in equal contribution by DTR<sup>+</sup> and Thy1.1<sup>+</sup> T<sub>reg</sub> cells (the injected DT is cleared within hours). Therefore, we conclude that the expansion of existing T<sub>reg</sub> cells must be the major driver of niche-filling in the 50% depletion system.

We observed an increase in effector-memory CD44<sup>hi</sup>CD62L<sup>lo</sup>CD4<sup>+</sup> T cells after partial depletion of T<sub>reg</sub> cells (Supplementary Fig. 3); therefore, we determined whether T<sub>reg</sub> cell niche-filling depended upon activation of T cells. Costimulatory blockade with a soluble...
CTLA4-immunoglobulin fusion protein (CTLA4-Ig) to prevent activation of T cells negated restoration of T<sub>reg</sub> cell numbers after partial depletion (Fig. 1d), consistent with a requirement for activation of conventional T cells (although this experiment does not rule out a T<sub>reg</sub> cell–intrinsic requirement for costimulation). To test the role of dendritic cells (DCs) in this process, Itgax-Cre (CD11c-Cre) transgenic mice were crossed to Rosa26<sup>-stop</sup>-DTR (stop sequence flanked by LoxP-sites) knock-in mice to allow depletion of DCs via injection of DT. Depletion of dendritic cells (DCs) in <i>Foxp3<sup>DTR/+</sup></i> CD11c-Cre Rosa26<sup>-stop</sup>-DTR mice simultaneous with partial depletion of T<sub>reg</sub> cells did not alter niche-filling kinetics (Supplementary Fig. 4), indicating that residual DCs or an alternate costimulation source is sufficient. Synchronous with T<sub>reg</sub> cell niche-filling was an increase in plasma IL-2 levels and downregulation of the high-affinity subunit of the IL-2 receptor, CD25, by T<sub>reg</sub> cells (Fig. 1e). The primary shift in CD25 expression was from CD25<sup>hi</sup> to CD25<sup>int</sup>, a change associated with increased proliferation (Supplementary Fig. 4), identifying IL-2 as a potential mediator of feedback from activation of conventional T cells to T<sub>reg</sub> cells. To determine the source of IL-2 in this context, we generated mice with a bacterial artificial chromosome (BAC) transgenic Thy1 (Thy1.1 variant) reporter of IL-2 production (unpublished data, RJ Luther and CT Weaver and crossed it onto the <i>Foxp3</i><sup>DTR/+</sup> background to track IL-2 production during 50% T<sub>reg</sub> cell depletion. The numbers of IL-2–expressing conventional CD4<sup>+</sup> T cells paralleled T<sub>reg</sub> cell expansion kinetics (Fig. 1f). Accordingly, antibody-mediated IL-2 blockade in <i>Foxp3<sup>Thy1.1/DTR</sup></i> mice during the 50% T<sub>reg</sub> cell depletion impaired niche-filling by partially inhibiting the greater proliferation rate and by completely blocking the decrease in apoptosis in the remaining T<sub>reg</sub> cells (Fig. 1g–i). By contrast, short-term neutralization of IL-2 had little effect in the mice not treated with DT (data not shown), which may suggest that the low to undetectable baseline amounts of IL-2 have little effect on the default proliferative or apoptotic characteristics of T<sub>reg</sub> cells, whereas the greater amount of IL-2 after perturbation drives substantial changes. The partial nature of the anti–IL-2 treatment may be due to the inability of the blocking antibody to completely inhibit this paracrine factor or may indicate that other factors can drive proliferation of T<sub>reg</sub> cells during homeostatic expansion (Fig. 1h). Nevertheless, IL-2 was directly responsible for the altered apoptotic rate in T<sub>reg</sub> cells (Fig. 1i), inhibition of which substantially blunts T<sub>reg</sub> cell homeostatic expansion (Fig. 1g). Collectively, these data demonstrate that T<sub>reg</sub> cells actively maintained homeostasis by swiftly responding to partial insufficiency via a feedback loop involving activation of conventional T cells, greater IL-2 production and altered T<sub>reg</sub> cell proliferation and apoptosis.

Peripheral T<sub>reg</sub> cell number is constrained by apoptosis

The cellular dynamics of T<sub>reg</sub> cells after ablation highlighted the potential importance of T<sub>reg</sub> cell apoptosis during two phases, namely during the expansion phase, when apoptosis was decreased, and during the contraction phase, when apoptosis mediated the decline in numbers. Conversely, proliferation rates when there was a surplus of T<sub>reg</sub> cells did not drop below baseline at homeostasis (Fig. 1b), and we found no evidence for ‘deconversion’ of excess T<sub>reg</sub> cells into conventional T cells when we used a fate-mapping tracker in <i>Foxp3<sup>Cre/DTR</sup></i> Rosa26<sup>-stop</sup>-YFP (stop sequence flanked by LoxP-sites) mice (Supplementary Fig. 3k). Despite this (indirect) evidence for modulation of apoptosis being crucial for homeostasis of T<sub>reg</sub> cells, defects in the ‘death receptor’ pathway have not been
associated with increases in T<sub>reg</sub> cell numbers<sup>15,16</sup>. By contrast, the marked expansion of T<sub>reg</sub> cells observed in mice lacking the proapoptotic proteins Bax and Bak, or those lacking the BH3-only protein Bim, implicates the intrinsic pathway of apoptosis in regulating numbers of T<sub>reg</sub> cells<sup>4,17</sup>, although these studies could not distinguish greater conversion into the T<sub>reg</sub> cell lineage because of defective thymocyte deletion from elevated peripheral homeostasis. To analyze the impact of the intrinsic pathway of apoptosis specifically on peripheral T<sub>reg</sub> cell homeostasis, we generated Foxp3<sup>Cre</sup>Bak<sup>1−/−</sup>Bax<sup>fl/fl</sup> mice. Owing to the redundancy of Bak and Bax<sup>18</sup>, this resulted in a T<sub>reg</sub> cell–specific knockout of the entire intrinsic apoptotic pathway. These mice exhibited normal thymic development of T<sub>reg</sub> cells (Supplementary Fig. 5), but peripheral accumulation of Foxp3<sup>+</sup>T<sub>reg</sub> cells to twice the normal numbers (Fig. 2). This accumulation was not due to excess proliferation (turnover was lower; Fig. 2a). These data indicate that the intrinsic pathway of apoptosis is a critical regulator of peripheral T<sub>reg</sub> cell homeostasis.

**T<sub>reg</sub> cells require Mcl-1 for survival**

Bak and Bax activation is tightly regulated by prosurvival members of the Bcl-2 family, of which Bcl-2, Bcl-x<sub>L</sub> and Mcl-1 have been reported to be expressed in T<sub>reg</sub> cells<sup>1</sup>. The leading prosurvival candidate for maintaining T<sub>reg</sub> cell survival was Bcl-2, because of the dynamic expression of Bcl-2 observed in T<sub>reg</sub> cells<sup>19–21</sup> and the T<sub>reg</sub> cell accumulation that occurs in mice with forced Bcl-2 overexpression<sup>21</sup>. However, lethally irradiated mice reconstituted with a 50:50 mixture of C57BL/6.Ly5.1:Bcl2<sup>−/−</sup> hematopoietic precursors exhibited a normal proportion of T<sub>reg</sub> cells derived from the Bcl2-deficient compartment, demonstrating that Bcl-2 is dispensable for their survival (Fig. 3a,b). We could not analyze the second candidate, Bcl-x<sub>L</sub>, using conventional knockouts (and hematopoietic reconstitution), as it is required for cell survival at the CD4<sup>+</sup>CD8<sup>+</sup> thymocyte stage<sup>22,23</sup>. We therefore created mice with T<sub>reg</sub> cell–specific ablation of Bcl-x<sub>L</sub> by crossing a Foxp3<sup>Cre</sup> strain<sup>24</sup> with Bcl2l1<sup>fl</sup> mice<sup>25</sup>. The resulting Foxp3<sup>Cre</sup>Bcl2l1<sup>fl/fl</sup> mice had normal numbers of T<sub>reg</sub> cells in both the thymus and periphery with no obvious immunological or pathological phenotype, demonstrating no role for this anti-apoptotic protein in homeostasis of T<sub>reg</sub> cells (Fig. 3c,d).

To investigate a potential role of Mcl-1 in regulating T<sub>reg</sub> cell apoptosis, we used a huCD4 reporter of Mcl-1 expression. The Mcl1<sup>β</sup> allele we used was designed to bring a human CD4 reporter in-frame after Cre recombinase–mediated excision<sup>26</sup>. When we crossed Mcl1<sup>β+/+</sup> mice to Cd127<sup>Cre</sup> knock-in mice<sup>27</sup>, expression of Cre recombinase in early lymphoid progenitors resulted in recombination of the Mcl1<sup>β</sup> allele in the entire lineage, allowing for tracking of Mcl1 transcription in all T cell subsets using the huCD4 reporter, while maintaining their survival with the wild-type Mcl1 allele (that is, the genotype becomes Mcl1<sup>huCD4+/+</sup> in the T cell lineage). This strategy allows the relative quantification of Mcl-1 expression in all T cell subsets with a greater dynamic range than previous profiling<sup>28</sup>. During thymic development, Mcl-1 reporter expression peaked at the DP stage, before an ~80% decrease in conventional CD4<sup>+</sup>CD8<sup>−</sup> thymocytes (Fig. 4a). In contrast to conventional CD4<sup>+</sup>CD8<sup>−</sup> cells, Foxp3<sup>+</sup>CD4<sup>+</sup>CD8<sup>−</sup> cells maintained elevated Mcl-1 reporter expression (Fig. 4a), and likewise peripheral T<sub>reg</sub> cells expressed the Mcl-1 reporter at ~50% higher levels than conventional T cells (Fig. 4b,c). To assess the function of Mcl-1 in T<sub>reg</sub> cells,
while circumventing the impact of its loss on the early thymocyte stage\textsuperscript{29}, we created mice with T\textsubscript{reg} cell–specific deletion of Mcl1. In contrast to the redundancy of Bcl-2 and Bcl-x\textsubscript{L} in survival of T\textsubscript{reg} cells, we found that Mcl-1 was essential. Foxp3\textsuperscript{Cre}\textsuperscript{Mcl1fl/fl} mice succumbed to a fatal immunopathology, surviving to only 4–8 weeks of age (Fig. 4d,e).

Pathology was associated with immunological dysregulation, inflammatory infiltrate, hyper-IgE (100 times normal levels), elevated amounts of antibodies to dsDNA, abnormally high proliferation of CD8\textsuperscript{+} T cells, greater activation of CD4\textsuperscript{+} T cells and spontaneous differentiation into T\textsubscript{H}1, T\textsubscript{H}2 and T\textsubscript{H}17 effector cells (Fig. 4f–i, Supplementary Fig. 6 and data not shown), all hallmarks of the Foxp3-deficient scurfy phenotype.

In young Foxp3\textsuperscript{Cre}\textsuperscript{Mcl1fl/fl} mice, thymic T\textsubscript{reg} cell development was relatively undisturbed and initially there was only a ~60% decrease in peripheral T\textsubscript{reg} cells (Supplementary Fig. 6). However, unlike the Foxp3\textsuperscript{Thy1.1/DTR} model, this T\textsubscript{reg} cell deficit could not be corrected by peripheral expansion in Foxp3\textsuperscript{Cre}\textsuperscript{Mcl1fl/fl} mice, with an additional decrease in T\textsubscript{reg} cell numbers observed (Fig. 5a). Loss of Mcl-1–deficient T\textsubscript{reg} cells was even more extreme in a competitive environment (Supplementary Fig. 7). Nevertheless, the deficit-sensing mechanism appeared intact, as the remaining T\textsubscript{reg} cells in Foxp3\textsuperscript{Cre}\textsuperscript{Mcl1fl/fl} mice demonstrated a compensatory increase in proliferation (Fig. 5b). We observed no evidence of an outgrowth of T\textsubscript{reg} cells with intact Mcl1, nor Mcl1 excision in Foxp3\textsuperscript{+} cells, using the huCD4 reporter expressed only when Mcl1 is excised (Fig. 5c). To measure the kinetics of T\textsubscript{reg} cell loss after Mcl1 ablation, we generated mixed hematopoietic chimeras with 50% Ly5.1 congenically labeled bone-marrow and 50% Ly5.2 labeled bone-marrow bearing a tamoxifen-inducible Cre\textsubscript{ERT2} and loxP-flanked (floxed) alleles of either Mcl-1 or Bcl2l11. After reconstitution, we induced inducible knockout in a competitive context by oral gavage with tamoxifen. Punctual deletion of Mcl1, but not Bcl2l11, in this system caused T\textsubscript{reg} cell numbers to collapse within 2 d (Fig. 5d,e), revealing an acute necessity for Mcl-1 in survival of T\textsubscript{reg} cells.

### Regulation of Mcl-1 in T\textsubscript{reg} cells by Bim and IL-2

The importance of Mcl-1 expression for T\textsubscript{reg} cell survival suggested that regulation of Mcl-1 may be important in setting the homeostatic balance of T\textsubscript{reg} cells. Several of the proapoptotic BH3-only members of the Bcl-2 family can overcome the prosurvival function of Mcl-1 and thereby initiate apoptosis\textsuperscript{30}; however, elevated numbers of T\textsubscript{reg} cells have been observed only in Bim-deficient mice\textsuperscript{4,17,19,21}. In these mice with germ-line deletion of Bcl2l11, this effect has been ascribed to additional T cells entering the T\textsubscript{reg} cell lineage because of defective negative selection\textsuperscript{17}, and secondary effects owing to low-grade inflammation\textsuperscript{31}. To circumvent these issues, we generated mice bearing a floxed Bcl2l11 allele and crossed them with Foxp3\textsuperscript{Cre} mice to create a T\textsubscript{reg} cell–specific deletion of Bcl2l11, where Bim is lost only after T\textsubscript{reg} cell development. The Foxp3\textsuperscript{Cre}\textsuperscript{Bcl2l11fl/fl} mice exhibited normal thymic differentiation, with substantial peripheral expansion of Foxp3\textsuperscript{+} T\textsubscript{reg} cells (Fig. 6a,b). Notably, the scale of peripheral T\textsubscript{reg} cell expansion expansion in Foxp3\textsuperscript{Cre}\textsuperscript{Bcl2l11fl/fl} mice was not as great as that observed in Foxp3\textsuperscript{Cre} Bak\textsuperscript{−/−} Bax\textsuperscript{fl/fl} mice (Fig. 2a,b), indicating that, although Bim is the primary initiator of homeostatic T\textsubscript{reg} cell apoptosis, additional BH3-only proteins (for example, Puma\textsuperscript{32}) may have additional roles. In addition to the negative regulation of Mcl-1 by Bim, the IL-2–dependent decrease in
apoptosis during niche-filling (Fig. 1i) suggested that IL-2 might act as a positive regulator of Mcl-1. In vitro we observed that stimulation of T_reg cells with IL-2 increased the amount of Mcl-1 protein (Fig. 6c). We therefore crossed the Cd127CreMcl1huCD4/+ reporter system (described above) to the Foxp3DTR/+ partial depletion system, to determine whether the greater availability of IL-2 during expansion of T_reg cells (Fig. 1) was linked to in vivo changes in expression of Mcl-1. We observed a rapid increase in Mcl-1 reporter expression in T_reg cells after partial depletion (Fig. 6d), coinciding with the IL-2–dependent decrease in apoptosis. To directly test the ability of IL-2 to induce expression of Mcl-1 in vivo, we injected Mcl-1 reporter mice (Cd127CreMcl1huCD4/+ with complexes of IL-2 and antibody to IL-2 (IL-2–anti-IL-2), which (as previously reported33) caused a rapid expansion of Foxp3+ T_reg cells, with a twofold increase in the peripheral blood by day 2 (Fig. 6e). Accompanying this increase in Foxp3+ T_reg cells was an increase in huCD4 expression (reporter for Mcl-1 transcription) in T_reg cells, whereas conventional CD4+ and CD8+ T cells exhibited no change in huCD4 expression (Fig. 6f). Together, these results demonstrate that IL-2 regulates expression of Mcl-1 in vitro and in vivo, indicating a direct pathway in T_reg cells from greater availability of IL-2 availability to greater expression of Mcl-1 (and perhaps lower Bim expression; data not shown), less apoptosis and subsequent peripheral expansion (Supplementary Fig. 8).

DISCUSSION

Far from being the semianergic lineage first described in in vitro experiments, Foxp3+ T_reg cells were highly dynamic and responsive in our experiments in vivo. Although entry into the Foxp3+ lineage is a gated event34, peripheral proliferation and apoptosis are the primary determinants of compartment size, with ~50% of the population having undergone proliferation every 10 d under homeostatic conditions. Several explanations present themselves for the necessity of a high turnover of the T_reg cell pool. First, chronic proliferation caused by TCR stimulation via self-antigen stimulation35 may necessitate compensatory apoptosis; second, a proapoptotic effect of Foxp3 expression leads to greater basal apoptosis levels in T_reg cells36, which may require compensatory proliferation; or third, high turnover itself may be required for sufficient regulatory function37. Assessment of T_reg cell responsiveness to perturbations from this basal state requires both swift contraction and a mechanism to leave the remaining cells unaffected. The natural chimeric state of female Foxp3Thy1.1/DTR mice used here fulfills both of these conditions, unlike models which rely on partial antibody-mediated depletion38 or escape of abnormal DT-resistant T_reg cell clones39. This approach revealed a much more rapid and dynamic T_reg cell response to perturbation than that demonstrated in previous models38,39, characterized by promptly increased proliferation with concomitant decrease in apoptosis. Furthermore, this system revealed an ‘overfilling’ effect followed by the slower attrition of T_reg cells via apoptosis to re-establish homeostatic levels. The contrast between rapid expansion in the face of T_reg cell deficiency and gradual contraction during T_reg cell excess is commensurate with the more severe physiological consequences of suboptimal immune suppression. In the range of induced variation studied here, modulation of the proliferation to apoptosis balance was sufficient to drive homeostatic correction, with any substantial involvement of thymic production, peripheral conversion or ‘de-conversion’ all excluded through experiments.
under these conditions. While these processes are capable of rapidly restoring T_{reg} cell numbers, an implication of this heavy reliance on remaining T_{reg} cell expansion (as opposed to production of new T_{reg} cells) is that repeated episodes of partial T_{reg} cell deficiency may narrow the diversity of the TCR repertoire in the restored T_{reg} cell pool. The extent of any TCR repertoire restriction is unknown, but may be substantial in aged individuals due to periodic T_{reg} cell contraction and expansion, in addition to the stochastic loss of diversity due to the high baseline rates of T_{reg} cell proliferation and apoptosis.

Dissection of the molecular mediators of T_{reg} cell homeostatic responsiveness revealed how well-known participants in T_{reg} cell biology modify previously unappreciated survival pathways. We found a direct correlation between modulation of T_{reg} cell numbers and production of IL-2 by conventional T cells in a costimulation-dependent manner. Elevated expression of IL-2 by conventional T cells occurs before the attainment of a typical activated cell-surface profile, a phenomenon that may be related to the lower threshold of TCR signaling required for cytokine production\(^{40}\) and which may serve to shorten the time lag of T_{reg} cell expansion in response to depletion. Consistent with previous reports identifying the importance of IL-2 for T_{reg} cells\(^{35}\), blockade of IL-2 blunted the homeostatic rebound after 50% T_{reg} cell depletion, playing an essential role in the transient decrease in T_{reg} cell apoptosis and a significant role in the boost to proliferation. The reliance of the T_{reg} cell homeostatic feedback circuit on an inducible cytokine lies in stark contrast to the homeostatic feedback loops of B cells and non-regulatory T cells mediated by the cytokines, BAFF\(^{41}\) and IL-7 (ref. 42), respectively, which are not made by activated T cells but constitutively produced by stromal cells\(^{43,44}\). Notably, a feature of such static consumption-based homeostatic systems is that only numerical, rather than functional, sufficiency is selected for. In the T_{reg} cell homeostatic system described here, by contrast, the dynamic production of IL-2 in response to Foxp3\(^{+}\) regulatory T cell numbers makes the niche dependent on regulatory T cell function (that is, restraining improper T cell activation) as opposed to merely numerical sufficiency. It is predicted that such a homeostatic model will prove to show greater robustness when challenged by variation in the efficiency of T_{reg} cell suppression, as demonstrated by the increase in T_{reg} cell numbers in several models of impaired function\(^{45,46}\).

A previously unappreciated key feature of the T_{reg} cell homeostatic feedback loops described here is the central role for apoptosis in regulating T_{reg} cell numbers. Expansion of T_{reg} cells during numerical deficit was accompanied by an IL-2–dependent suspension of apoptosis, while contraction during surplus involved apoptotic processes. Accordingly, T_{reg} cell–specific ablation of the intrinsic apoptosis pathway provoked the accumulation of surplus T_{reg} cells. Our approach of systematic assessing pro-survival members of the intrinsic apoptosis pathway revealed that Bcl-2 and Bcl-x\(\_L\) were redundant, in contrast to prior supposition\(^{17,19–21}\), while Mcl-1 was essential for T_{reg} cell survival. Furthermore, Mcl-1 appears to represent a rheostat for controlling the T_{reg} cell homeostatic niche, with positive regulation via IL-2 and antagonism by Bim during homeostatic perturbation. This role of the Mcl-1 and Bim axis in driving the return of T_{reg} cells to homeostatic levels represents a potential intervention point for therapeutic manipulation.
Online Methods

Mice

*Bak*−/− (ref. 18), *Bax*^fl/fl^ (ref. 47), *Bcl-x*(*Bcl2l1*/*Bcl2l1*)^fl/fl^ (ref. 25), *Bim*(*Bcl2l11*)^fl/fl^ (ref. X), CD11c-Cre-Tg (ref. 48), CD127-Cre-Tg (ref. 27), *Foxp3*GFP (ref. 49), *Foxp3*YFPCre (ref. 24), *Foxp3*Thy1.1 (ref. 50), IL-2.BAC-Thy1.1 mice (manuscript in preparation, RJ Luther and CT Weaver), *Mcl1*^fl/fl^, *Rag2*−/− (ref. 51), Rag2GFP-Tg (ref. 52), *Rosa26*β-stop^YFP^ (ref. 53), *Rosa26*β-stop^DTR^ (ref. 54) and *Rosa26*Cre-ERT2 (ref. 55) mice were all generated on, or backcrossed to, the C57BL/6 background. *Foxp3*^DTR^ mice were backcrossed to the C57BL/6.Ly5.1 background. Experimental mice were housed under specific pathogen-free conditions. Disease development was monitored by frequent observation and post-mortem analysis. Cohorts of mice for the survival test were removed from the study at death or when veterinary advice indicated likely death within 48 h. All experiments were approved by the University of Leuven animal ethics committee or the WEHI animal ethics committee. Histological examination was performed by Histology Consultation Services and pathology reports were generated by BioGenetics.

Design of animal experiments

All endpoint experiments used 3 or more mice of the specified genotypes and age-range to enable statistical comparisons. Mice were assigned to groups based on a semi-randomization process that included a post-randomization reassignment to ensure equal age-sex distribution within groups. Investigators performing experiments were only aware of mouse ID numbers, not genotype. Genotypes were revealed only upon analysis.

*Foxp3*^DTR/Thy1.1^ heterozygous females, 6–8 weeks of age, were injected intraperitoneally with a dose of 50 μg/kg of DT (Sigma-Aldrich) diluted in saline on days 0 and 1 (for CD11c depletion, on days 0, 1, 2 and 3) of the experiment. A daily dose of 50 μg of IL-2–neutralizing antibody (S4B6) or IgG2a isotype-matched control antibody (eBR2a, eBioscience) was administered intraperitoneally starting on the day of the first DT injection. CTLA4-Ig (abatacept, Bristol-Myers Squibb) was injected intraperitoneally (25 mg/kg) on days 0, 2 and 4.

*Cd127*^Cre^ *Mcl1*^wt/fl-huCD4^ female mice, 6–8 weeks of age, were injected intraperitoneally with IL-2 complex at a dose of 16.5 μg/mouse per day. IL-2 complex was generated by mixing mouse recombinant IL-2 (eBioscience) with anti-mouse IL-2 antibody (JES6-1A12, eBioscience) at a 1:10 ratio, and was injected intraperitoneally at a dose of 16.5 μg/mouse per day, as per the published protocol.

Bone-marrow chimeric experiments were performed using recipient C57BL/6 *Rag2*−/− female mice sub-lethally irradiated with 9.5Gy at 8–12 weeks of age and reconstituted within 24 h using an intravenous injection with a total of 2 × 10^6^ hematopoietic cells from bone marrow donors. Chimeric mice were analyzed at or after 6 weeks after reconstitution. For inducible deletion of *Bcl-x* or *Mcl1*, chimeric mice were given two doses of 200 mg/kg of tamoxifen (Sigma, T5648) via oral gavage on days 0 and 1.
BrdU exposure was initiated in C57BL/6 male mice, 6–8 weeks of age, via an intraperitoneal injection of 100 μg/100 μl BrdU (Sigma). A subset of mice were additionally given continuous exposure to 8 mg/ml BrdU in the drinking water, changed daily.

Flow cytometry

Leukocytes from peripheral blood, thymus, spleen or lymph nodes were analyzed using the following antibodies: anti-BrdU-APC (1/50) (B44, BD), anti-CD25-PECy7(1/300) (PC61.5), anti-CD25-PE (1/300) (BD), anti-CD4-APC-H7 (1/250) (GK1.5, BD), anti-CD4-PerCP (1/200) (GK1.5, BioLegend), anti-huCD4-PE (1/50) (OKT4, BD), anti-CD4-PE (1/200) (GK1.5), anti-CD4-FITC (1/200) (GK1.5), anti-huCD4-PE-Cy7 (1/25) (RPA-T4), anti-TCR-beta-PE-Cy7 (1/100) (H57.59.1, BioLegend), anti-CD44-PerCP-Cy5.5 (1/500) (IM7), CD62L-PE-Cy7 (1/500) (MEL-14), anti-CD8-PerCP-Cy5.5 (1/300) (53-6.7), anti-CD8-APC-eFluor780 (1/300) (53-6.7), anti-CD8-APC (1/200) (GK1.5), anti-CD8-APC (1/200) (GK1.5), anti-CD8-PECy7 (1/100) (BVD6-24G2) (all eBioscience unless indicated otherwise). Intracellular staining for Ki67 and Foxp3 was performed after fixation and permeabilization using the reagents from the eBiosciences Foxp3 staining kit. Intracellular staining for BrdU was performed after Foxp3 staining, using the BrdU staining kit (BD). For intracellular cytokine staining, cells were stimulated for 4 h in complete RPMI in presence of Phorbol myristate acetate (50 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich), and monensin (1/1,000; BD), reagents from the BD cytofix/cytoperm kit were used. Apoptosis was assessed using the Abcam active Caspase-3 FITC Staining Kit.

Biochemical analyses

Anti-dsDNA titers in individual plasma samples were determined by enzyme-linked immunosorbent assay (ELISA). IgE levels were measured with a mouse IgE Ready-SET-Go! ELISA assay (eBioscience). IL-2 concentrations were determined with a mouse IL-2 High Sensitivity ELISA (eBioscience). For in vitro stimulation of Treg cells with IL-2, pooled splenic and lymph node cells from Foxp3Cre mice were labeled with anti-CD4 microbeads and the CD4+ T cells enriched on an AutoMACS separator (Miltenyi Biotec). Enriched CD4+ cells were stained with anti-CD4-PerCP-Cy5.5 and anti-CD25-PE and YFP+ Treg cells or YFP− conventional T cells were purified by cell sorting on a MoFlo FACS machine (Cytomation). Treg cells were plated at 10^5 cells per well in Complete medium with 200 U/well IL-2 (Peprotech). The pan-caspase inhibitor QVD-OPH was added where indicated to prevent Treg apoptosis in the absence of IL-2. After 24 h of culture, Treg cells were recovered in lysis buffer and lysates were separated by SDS-PAGE, then transferred to PVDF membrane for probing with rabbit anti-Mcl-1 (Rockland Immunochemicals), HRPO conjugated anti-rabbit immunoglobulin (Southern Biotech) and development with ECL reagents (GE Healthcare).
Statistics and bioinformatics

Statistical analysis was performed on all data points, excluding only technical failures. No data points were excluded on the basis of being outliers. All data points shown and used for analysis are biological replicates. Differences in animal survival rates were analyzed using a log rank test (Prism). All other statistical analyses were performed through an ANOVA with Tukey’s post-test, followed by individual unpaired two-tailed t-test comparisons between two groups, with \( P < 0.05 \) used as the threshold for statistical significance. Tests were picked in advance of data generation based on experimental design, rather than post-hoc data analysis of data distribution. Data are presented as mean ± s.d., with individual data points overlaid where appropriate.

Prediction of caspase cleavage sites was performed using CASVM\(^{58}\), using the P14-P10′ scanning window size and allowing aspartic acid and glutamic acid at P1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References


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Figure 1.
Homeostatic expansion of T\(_\text{reg}\) cells is driven by increased production of IL-2. (a) Percentages of DTR\(^+\) T\(_\text{reg}\) cells Thy1.1\(^+\) T\(_\text{reg}\) cells and total T\(_\text{reg}\) cells in Foxp3\(^{Thy1.1/DTR}\) females depleted of Foxp3\(^{DTR^+}\) T\(_\text{reg}\) cells on day 0. Blood leukocytes were assessed on indicated days (n=4,3,7,3,4,4,4,4 mice). (b,c) Proliferation rate (b; percentage Ki67\(^+\)) and apoptosis rate (c; percentage activated caspase 3\(^+\)) of Thy1.1\(^+\) T\(_\text{reg}\) cells, after DT treatment of Foxp3\(^{Thy1.1/DTR}\) females on day 0. (d) Percentages of Thy1.1\(^+\) T\(_\text{reg}\) cells in peripheral blood after DT treatment of saline-treated (n=3,3,3 mice/group) or CTLA4-Ig–treated Foxp3\(^{Thy1.1/DTR}\) female mice (n=3,3,3 mice/group). (e) Plasma IL-2 (left axis, n=27,3,3,12,14 mice/group) and surface CD25 expression on Thy1.1\(^+\) T\(_\text{reg}\) cells (right axis, n=4,3,7,3,4,4 mice/group) from days 0 to 15 for female Foxp3\(^{Thy1.1/DTR}\) mice depleted of Foxp3\(^{DTR^+}\) T\(_\text{reg}\) cells (f) Proportion of Thy1.1\(^+\) (reporter for IL-2) cells within the CD4\(^+\)Foxp3\(^-\) population after DT injection of Foxp3\(^+/DTR\).IL2-Thy1.1 mice. (g) Foxp3\(^{Thy1.1/DTR}\) mice were depleted of DTR\(^+\) T\(_\text{reg}\) cells and injected daily with an IL-2 blocking antibody (a-IL2) or an immunoglobulin isotype-matched control antibody. Splenocytes were analyzed on day 6 for the percentages of DTR\(^+\) and Thy1.1\(^+\) T\(_\text{reg}\) cells, with or without T\(_\text{reg}\) cell depletion (DT treatment) and with or without anti–IL-2 treatment (n=4,4,7,7 mice/group). (h,i) Proliferation rate (h; percentage Ki67\(^+\)) and apoptosis rate (i; activated caspase-3\(^+\)) of Thy1.1\(^+\) regulatory T cells, with or without DT treatment and with or without anti–IL-2 treatment (n=4). (a–i) Mean ± s.d., *P < 0.05, t-test. Data are representative of 3(a–f) and 2 (g–i) independent experiments.
Figure 2.
The intrinsic apoptosis pathway is required to restrain T\(_{\text{reg}}\) cell numbers to homeostatic levels. (a) Representative flow profiles (TCR\(\beta\) versus Foxp3 gated on CD4\(^+\) cells, Ki67 histograms gated on Foxp3\(^+\) CD4\(^+\) cells) for wild-type, Foxp3\(^{\text{Cre}}\)Bak\(^{-/-}\)Bax\(^{fl/fl}\) mice and control littermates at 6–8 weeks of age. Numbers in plots indicate (top) the percentage of Foxp3\(^+\)TCR\(\beta\)\(^+\) regulatory T cells and (bottom) the fraction of proliferating Foxp3\(^+\)CD4\(^+\) cells (Ki67\(^+\)). (b) Average percentages and absolute numbers (mean ± s.d.) of splenic Foxp3\(^+\) T\(_{\text{reg}}\) cells in wt, Foxp3\(^{\text{Cre}}\)Bak\(^{-/-}\)Bax\(^{fl/fl}\) mice and control littermates at 6–8 weeks of age (n=3,3,3,3 mice/group). (a–b) Data from one experiment representative of three are shown. Mean ± s.d., *P < 0.05, t-test.
Figure 3.
Regulatory T cell survival is independent of Bcl-2 and Bcl-xL. (a) C57BL/6.Ly5.1 (Ly5.1) chimeras reconstituted with a 50:50 mixture of hematopoietic precursors from wild-type Ly5.1 and either Bcl2+/- or Bcl2-/- mice, analyzed 8–12 weeks later. Gates show the percentage of Ly5.2+CD4+Foxp3+ cells recovered from the thymus or spleen. (b) Average percentages (mean ± s.d.) of Ly5.2+CD4+Foxp3+ cells recovered from the thymus and spleen of the same mixed hematopoietic chimeras reconstituted with precursors from either wild-type (Bcl2+/-) or Bcl2-/- mice (n= 4,6 mice/group). (a,b) Data from one experiment representative of three are shown. (c) Representative flow profiles of CD4 versus Foxp3 gated on CD4+ cells from Foxp3CreBcl21wt/wt and Foxp3CreBcl21betaRbetaR mice. Gates show the percentage of CD4+Foxp3+ cells recovered from the thymus or spleen. (d) Average percentages (mean ± s.d.) of CD4+Foxp3+ Treg cells in the thymus and spleen of Foxp3CreBcl21wt/wt and Foxp3CreBcl21betaRbetaR siblings at 6–8 weeks of age (n = 6,9 respectively). (c,d) Data pooled from three independent experiments are shown.
Figure 4.
Spontaneous fatal immunopathology after \( \text{T}_{\text{reg}} \) cell–specific deletion of Mcl-1. (a) HuCD4 reporter for Mcl-1 expression was measured in lymphocyte subsets in \( Cd127^{\text{Cre}}\text{Mcl1}^{\text{wt/fl}}\)-huCD4 female mice, 6–8 weeks of age. Average huCD4 reporter MFI in CD4\(^+\)CD8\(^-\) (DN), CD4\(^+\)CD8\(^+\) (DP) and single positive (SP) thymocytes, the latter subdivided into CD4\(^-\)CD8\(^+\) SP, conventional CD4\(^+\)CD8\(^-\) SP and CD4\(^+\)CD8\(^-\) Foxp3\(^+\) SP (\(n=3\) mice/group). (b) Average huCD4 reporter MFI in splenic CD19\(^+\) B cells, naïve conventional CD4\(^+\) (CD4\(^+\) nTc), activated conventional CD4\(^+\) (CD4\(^+\) actTc), Foxp3\(^+\) T\(_{\text{reg}}\) cells, naïve conventional CD8\(^+\) (CD8\(^+\) nTc) and activated conventional CD8\(^+\) T cells (CD8\(^+\) actTc) (\(n=3\) mice/group). (c) Representative histogram of huCD4 reporter MFI in naïve conventional CD4\(^+\) (CD4\(^+\) nTc), Foxp3\(^+\) T\(_{\text{reg}}\) cells and naïve conventional CD8\(^+\) (CD8\(^+\) nTc), with control huCD4 staining in wild-type Foxp3\(^+\) T\(_{\text{reg}}\) cells. (a–c) Data from one experiment representative of three. (d) Weights of male \( Foxp3^{\text{Cre}}\text{Mcl1}^{\text{wt/wt}}, \text{Foxp3}^{\text{Cre}}\text{Mcl1}^{\text{wt/fl}} \) and \( Foxp3^{\text{Cre}}\text{Mcl1}^{\text{fl/fl}} \) littermates at 6–8 weeks of age (\(n=11, 20, 16\) mice/group). (e) Survival curve for male \( Foxp3^{\text{Cre}}\text{Mcl1}^{\text{wt/wt}}, \text{Foxp3}^{\text{Cre}}\text{Mcl1}^{\text{wt/fl}} \) and \( Foxp3^{\text{Cre}}\text{Mcl1}^{\text{fl/fl}} \) littermates (\(n=16, 18, 18\) mice/group). (f) Plasma IgE levels in male \( Foxp3^{\text{Cre}}\text{Mcl1}^{\text{wt/wt}} \) and \( Foxp3^{\text{Cre}}\text{Mcl1}^{\text{fl/fl}} \) littermates at 4–8 weeks of age (\(n=6, 12\) mice/group). (g,h) Average disease score (g) and representative histology (h; scale bar, 200 \( \mu \)m) of the lungs and small intestine of male \( Foxp3^{\text{Cre}}\text{Mcl1}^{\text{wt/wt}} \) and \( Foxp3^{\text{Cre}}\text{Mcl1}^{\text{fl/fl}} \) littermates at 4–8 weeks of age (\(n=11, 9\) mice/group). (i) Average percentage of CD44\(^+\)CD62L\(_{\text{low}}\) activated cells within CD4\(^+\) and CD8\(^+\) splenic T cells in \( Foxp3^{\text{Cre}}\text{Mcl1}^{\text{wt/wt}}, \text{Foxp3}^{\text{Cre}}\text{Mcl1}^{\text{wt/fl}} \) and \( Foxp3^{\text{Cre}}\text{Mcl1}^{\text{fl/fl}} \) littermates at 6–8 weeks of age (\(n=12, 9, 11, 10, 4, d8\) mice/group). (d–i) Data pooled from 3 experiments. Mean \( \pm s.d., *P < 0.05\), t-test.
Figure 5.
Mcl-1 is required for T<sub>reg</sub> cell survival. (a) Average percentages of Foxp<sup>3+</sup> T<sub>reg</sub> cells within splenic CD4<sup>+</sup> T cells in male Foxp<sub>3</sub>Cre<sup>Mcl1<sup>wt/wt</sup></sup>, Foxp<sub>3</sub>Cre<sup>Mcl1<sup>wt/fl</sup></sup> and Foxp<sub>3</sub>Cre<sup>Mcl1<sup>fl/fl</sup></sup> littermates at 6–8 weeks of age (n=14,12,11 mice/group). (b) Average percentages of Ki67<sup>+</sup> cells within splenic Foxp<sub>3</sub> T<sub>reg</sub> cells in Foxp<sub>3</sub>Cre<sup>Mcl1<sup>wt/wt</sup></sup>, Foxp<sub>3</sub>Cre<sup>Mcl1<sup>wt/fl</sup></sup> and Foxp<sub>3</sub>Cre<sup>Mcl1<sup>fl/fl</sup></sup> littermates at 6–8 weeks of age (n=14,12,11 mice/group). (a-b) Data pooled from 3 experiments. (c) Expression of the huCD4 reporter for excision of Mcl1 in Foxp<sup>3+</sup> and Foxp<sup>3−</sup> cells in male Foxp<sub>3</sub>Cre<sup>Mcl1<sup>fl/fl</sup></sup> mice (n=3,4,3 mice/group). Data from one experiment representative of three. (d) Ly5.1 versus Foxp3 expression on lymph node cells from mixed chimeras with Mcl1<sup>fl/fl</sup> or Bcl2l11<sup>fl/fl</sup> Ly5.2 donor compartments analyzed 3 d after treatment with tamoxifen or vehicle control. Gates display the fraction of T<sub>reg</sub> cells arising from (top) wildtype Ly5.1<sup>+</sup> and (bottom) Mcl1<sup>fl/fl</sup> or Bcl2l11<sup>fl/fl</sup> Ly5.1<sup>−</sup> cells. Plots are representative of 3 experiments, each with n = 3 mice/group. (e) Ratios of Ly5.1<sup>+</sup> to Ly5.1<sup>−</sup> CD4<sup>+</sup>Foxp3<sup>+</sup> lymph node cells in mixed chimeras with the indicated Ly5.2<sup>+</sup> donor compartment 3 d after treatment with tamoxifen or vehicle (n=3,3,4,6 mice/group). Data from one experiment representative of three is shown. Mean ± s.d., *P < 0.05, t-test.
Figure 6.
Regulation of Mcl-1 in T<sub>reg</sub> cells by Bim and IL-2. (a) Representative flow cytometric profiles for CD4 vs Foxp3<sup>+</sup> gated on CD4<sup>+</sup> cells (gates indicate fraction of T<sub>reg</sub> cells within CD4<sup>+</sup> lymph node cells) and Ki67 histograms gated on Foxp3<sup>+</sup>CD4<sup>+</sup> cells (gate indicates the fraction of proliferating T<sub>reg</sub> cells) for Foxp3<sup>wt</sup>Bcl2l11<sup>fl/fl</sup> and Foxp3<sup>Cre</sup>Bcl2l11<sup>fl/fl</sup> littermates at 6–8 weeks of age. (b) Average percentages and absolute number of CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells from pooled lymph nodes of Foxp3<sup>wt</sup>Bcl2l11<sup>fl/fl</sup> and Foxp3<sup>Cre</sup>Bcl2l11<sup>fl/fl</sup> littermates (n=8,5 mice/group). Data from one experiment representative of three are shown. (c) Immunoblot analysis of Mcl-1 expression in ex vivo isolated conventional T cells (T<sub>conv</sub>) or T<sub>reg</sub> cells, or T<sub>reg</sub> cells cultured overnight with or without IL-2, in the absence or presence of QVD-OPH (a broad spectrum caspase inhibitor used to prevent apoptosis in the absence of IL-2). Data from one experiment representative of three are shown. (d) Cd127<sup>Cre</sup>Mcl1<sup>wt/fl-huCD4</sup>Foxp3<sup>wt/DTR</sup> females were depleted of Foxp3<sup>DTR+</sup> T<sub>reg</sub> cells on day 0 and huCD4 (Mcl1 reporter) expression was measured in Foxp3<sup>+</sup> T<sub>reg</sub> cells during the expansion phase. MFI was normalized to T<sub>reg</sub> cells from Cd127<sup>Cre</sup>Mcl1<sup>wt/fl-huCD4</sup>Foxp3<sup>wt</sup> mice (n=4 mice/group). Data from one experiment representative of two are shown. (e) Cd127<sup>Cre</sup>Mcl1<sup>wt/fl-huCD4</sup> mice were injected with IL-2-anti-IL-2 antibody complexes or saline and on day 2 were measured for CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub>...
cell expansion in the peripheral blood and expression of huCD4 reporter for Mcl1 expression (n=5,4 mice/group). Mean ± s.d., * P < 0.05, t-test.