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Mutual antagonism of TGF-beta and Interleukin-2 in cell survival and lineage commitment of induced regulatory T cells

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Transforming growth factor beta (TGF-β)- and Interleukin-2 (IL-2)-mediated signaling enables the generation and expansion of induced regulatory T (iTreg) cells that carry high hopes for the treatment of chronic inflammatory and autoimmune diseases. Knowledge about factors stabilizing their lineage commitment and lifespan, however, is limited. Here, we investigated the behavior of iTreg cells, derived from apoptosis-defective mouse mutants, during activated cell autonomous cell death, triggered by cytokine-deprivation, or activation-induced cell death (AICD) after restimulation of the T-cell receptor, and compared these responses with those of effector T cells. We observed that iTreg cells were much more sensitive to IL-2-deprivation but poorly susceptible to AICD. In fact, when apoptosis was compromised, T-cell receptor (TCR)-religation resulted in methylation-independent, ERK- and PI3K/mTOR-mediated loss of Foxp3 expression, impaired suppressive capacity and effector cytokine production. Although iTreg cells prevented colitis induction they rapidly lost Foxp3-GFP expression and gained ability to produce effector cytokines thereby imposing Th1 cell fate on resident effector cells. Surprisingly, iTreg cell conversion itself was limited by TGF-β-mediated *Bim/Bcl2L11*-dependent apoptosis. Hence, the very same cytokine that drives the generation of iTreg cells can trigger their demise. Our results provide novel insights in iTreg cell biology that will assist optimization of iTreg-based therapy.
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Apoptosis during lymphocyte development and the generation of regulatory T (Treg) cells are essential to establish central and maintain peripheral tolerance.[1] Treg cells are characterized by the expression of different cell surface molecules such as CD4, the interleukin (IL)-2Rα chain (CD25) but the transcription factor Foxp3 appears to be the only reliable marker.[2] Treg cells arise naturally in the thymus (nTreg) or can be induced (iTreg) in the periphery from $CD4^+Foxp3^-$ naïve T cells in response to transforming growth factor beta (TGF-β) plus IL-2[3] or retinoic acid.[4] Accordingly, the autoimmunity observed in mice lacking either cytokine is attributed to their inability to generate or maintain iTreg cells in the periphery.[5,6] Antigen encounter and expansion of naïve T lymphocytes results in the amplification of nTreg cells and the induction of iTreg cells from the $CD4^+Foxp3^-$ T cell pool by TGF-β, preventing the overshoot of an immune response.[7]

iTreg cells hold large promise for therapeutic use in the treatment of chronic inflammatory or autoimmune diseases and in the establishment of transplantation tolerance. Proof of principle has been provided in animal models as adoptive transfer of iTreg cells rescued *scurfy* mice, lacking functional Foxp3 expression, from developing fatal autoimmunity,[8] or prevented development of diabetes in a co-transfer model together with diabetogenic cells in NOD/SCID mice,[9] as well as central nervous system (CNS) abnormalities in EAE when myelin-reactive iTregs were used.[10]

Despite promising results, enthusiasm about therapeutic application was dampened by observations indicating latent commitment of iTreg cells to the Treg lineage, presumably due to weaker Foxp3 promoter methylation,[11] and the reported propensity of nTreg cells to convert into inflammatory type Th17 cells upon loss of Foxp3 expression in the presence of IL-6.[12,13] Despite that, iTreg cells remain attractive therapeutic tools. In order to achieve long-term clinical benefits, maintenance of Foxp3 expression stabilizing lineage commitment and an extended lifespan are desired. However, current knowledge about mechanisms controlling these processes in iTreg cells is limited.

Apoptotic T-cell death is triggered either via the so-called 'extrinsic pathway' by ligation of 'death receptors', members of the TNF-R family, such as TNF-R1, TRAIL-R or CD95/Apo1/

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Abbreviations: AICD, activation-induced cell death; ACAD, activated cell autonomous cell death; TGF-β, transforming growth factor beta; TCR, T-cell receptor; Bcl-2, B cell lymphoma 2; Bim/Bcl2L11, Bcl-2 interacting mediator of cell death; MFI, mean fluorescence intensity; Tcon, conventional effector T cells; iTreg, induced regulatory T cells; nTreg, natural regulatory T cells; mTOR, mammalian target of Rapamycin; PI3K, phosphoinositide-3 kinase; FoxP3, Forkhead box P3; GFP, green fluorescent protein; EAE, experimental autoimmune encephalomyelitis; IL, interleukin; IFNγ, interferon gamma; T-bet, T-BOX expressed in T cells; GATA-3, GATA-binding protein 3; RORγt, RAR-related orphan receptor gamma; PMR, percentage of methylated reference; CNS, central nervous system
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FAS. The latter receptor, for example, becomes activated upon T-cell receptor (TCR) religation-mediated induction of CD95L, a process also known as activation-induced cell death (AICD). Lack of TCR-stimulation after antigen clearance curtails cytokine production and this triggers apoptosis through the 'intrinsic' or 'Bcl-2-regulated' pathway, sometimes referred to as 'activated cell autonomous death' or ACAD.[14] For activation of the latter pathway, the proapoptotic Bcl-2-family protein of the BH3-only protein subgroup, *Bim/Bcl2L11*, has an essential role.[15] Of note, defects in either apoptosis pathway have been reported to facilitate autoimmunity in mice and humans. For example, loss of CD95/C95L signaling is causal for development of autoimmune lymphoproliferative syndrome (ALPS).[16] Also, overexpression of anti-apoptotic Bcl-2 or loss of pro-apoptotic Bim in mice facilitates the appearance of autoimmunity, and associates with disease in humans.[17] Of note, both cell death pathways can cooperate, depending on the type of antigen encountered, in the elimination of activated T cells at the end of an immune response.[15]

Hence, Treg cells and apoptosis of activated T cells are both critical to ensure immune homeostasis and more profound knowledge on how Treg numbers are regulated will help facilitate their therapeutic application. Here, we compared the susceptibility of TGF-β induced $CD4^+Foxp3^+$ iTreg cells with that of activated conventional $CD4^+$ $Foxp3^-$ T cells (Tcon) to apoptosis triggered by cytokine-deprivation or TCR-restimulation. Cell death responses were studied in the presence or absence of key-components of the intrinsic and extrinsic apoptosis-signaling pathway in relation to TCR, IL-2 and TGF-β triggered-cytokine signaling as well as Foxp3-mediated lineage commitment. In addition, we compared the therapeutic potential and stability of lineage commitment of iTreg with that of nTreg cells *in vivo* upon adoptive transfer in a T cell-driven model of inflammatory bowel disease in mice.

Results

iTreg cells are poorly susceptible to CD95-killing. We analyzed CD95 and CD95L expression as well as the susceptibility of $CD4^+$Foxp3-GFP$^+$ nTreg and naïve $CD4^+$Foxp3-GFP$^-$ T cells, isolated from the spleens from *foxp3^{gfp}* reporter mice[18] to CD95-induced apoptosis. Cells from CD95-deficient *foxp3^{gfp}lpr* mice served as a control. nTreg cells displayed significantly reduced cell survival upon CD95-ligation, when compared with freshly isolated naïve T cells, whereas cells from *lpr* mice resisted CD95-killing (Figure 1a). Of note, nTreg cells from wt mice displayed increased CD95 expression on their cell surface (Figure 1b). CD95L mRNA levels, however, were lower in nTreg cells, compared with naïve T cells (Figure 1c).

Next we compared the behavior of iTreg and activated T cells to CD95-mediated apoptosis. Cells were generated from naïve T cells purified from *foxp3^{gfp}* or *foxp3^{gfp}lpr* mice and cultured in the absence or presence of CD95L for 18 h. Interestingly, iTreg cells were highly resistant to CD95L-induced cell death when compared with nTreg or Tcon cells that died more rapidly in culture (Figure 1a).

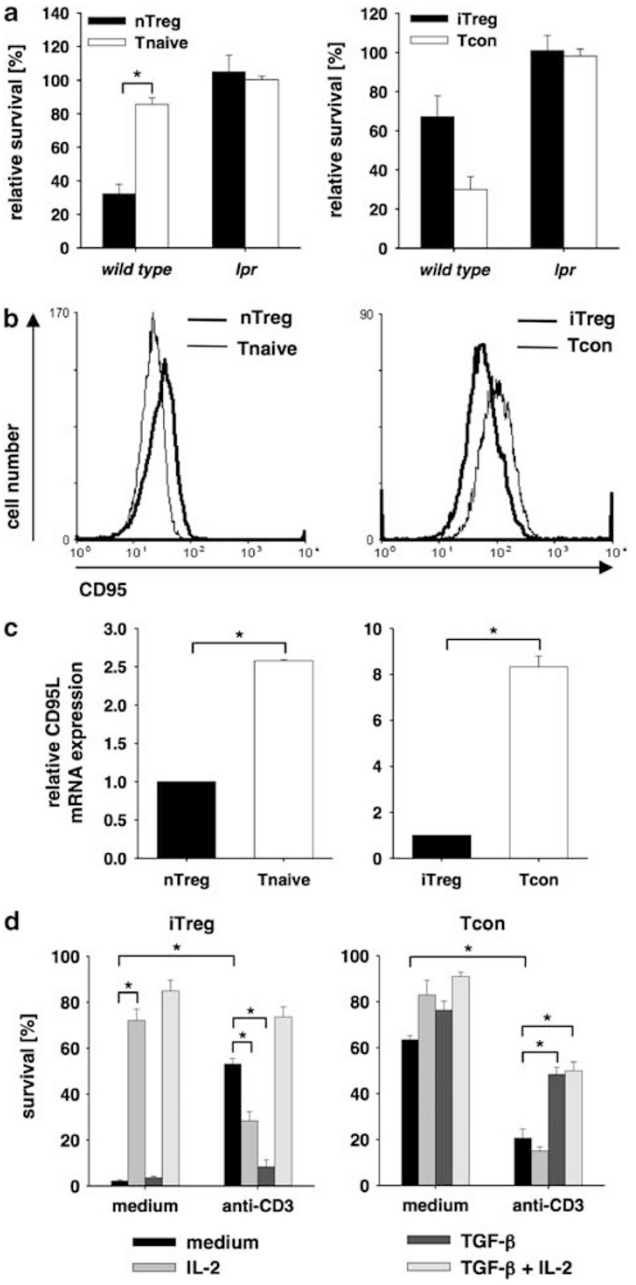


Figure 1 iTreg and Tcon cells display different responsiveness to AICD and ACAD. (**a**) nTreg and naïve T cells were isolated from the spleen of wt or CD95-deficient *lpr* mice and compared with iTreg *versus* Tcon cells generated *ex vivo* for cell survival upon CD95/CD95L ligation. Viability was analyzed by AnnexinV/7AAD staining and flow cytometry after 18 h in culture. Values were normalized to spontaneous cell death observed in culture. Bars represent means of $n=4$ (wt) or 3 (*lpr*) experiments ± S.E.M. (**b**) CD95 surface expression on nTreg *versus* naïve $CD4^+$Foxp3-GFP$^-$ T cells freshly isolated from spleen and on *in vitro* generated iTreg *versus* Tcon cells. Representative samples from three independent experiments are shown. (**c**) qRT-PCR analysis of relative CD95L mRNA levels in nTreg compared with naive $CD4^+$Foxp3-GFP$^-$ T cells and *in vitro* induced iTreg *versus* Tcon effector cells. Bars represent means ± S.E.M. of $n=3$ independent experiments performed on three different mRNA samples. (**d**) Purified iTreg and Tcon cells were cultured for 48 h, either in the absence or presence of cross-linked anti-CD3, supplemented with IL-2, TGF-β or a combination of both. Cell survival was analyzed by AnnexinV/7AAD staining and flow cytometry. Bars represent means of $n=3$ independent experiments ± S.E.M. $^*P \leq 0.05$

To assess why iTreg cells were more resistant to CD95-mediated apoptosis compared with Tcon cells we also quantified CD95 and CD95L expression in both T-cell types directly after their induction *in vitro*. Expression of CD95 protein and CD95L mRNA were significantly reduced in iTreg cells when compared with Tcon cells, providing a first explanation for their relative resistance to CD95-ligation (Figures 1b and c), whereas mRNA levels of *cFLIP*, an intracellular inhibitor of caspase-8 that is required for CD95-killing, were comparable (mean mRNA expression in iTreg *versus* Tcon 1 : 1.25 $\pm$ 0.08; $n = 3$).

Because sensitization of T cells to CD95-killing reportedly depends on activation-triggered cell cycle progression and related induction of CD95L,[19] we also assessed relative proliferation rates by quantifying mitotic phospho-histone H3 (pH3)-positive cells during expansion (Supplementary Figure S1A). Notably, mitotic activity appeared even higher in iTreg cells, excluding reduced cell cycle rates in iTreg cells as a possible explanation for the observed differences in CD95L expression and sensitivity to CD95-killing. PKH-26 label-retention corroborated these results (Supplementary Figure S1B).

TGF-β can exert opposing effects on the survival of iTreg *versus* Tcon cells. We assessed whether TGF-β present in iTreg cultures is responsible for the resistance of these cells to CD95/CD95L-mediated killing after TCR religation. iTreg and activated Tcon cells were cultured either in medium alone or restimulated with cross-linked anti-CD3 mAb. Cells were further left untreated or received fresh IL-2, TGF-β or a combination of both cytokines. iTreg cells died rapidly, undergoing activated cell autonomous cell death (ACAD), triggered by cytokine-withdrawal when cultured in medium alone, whereas comparatively little apoptosis was observed in Tcon cells (Figure 1d and Supplementary Figure S1C). Addition of IL-2 rescued iTreg cells from apoptosis, whereas Tcon cells benefited comparatively little and addition of TGF-β had no effect. Of note, adding IL-2 further induced the surface expression of CD25 on both cell types (Supplementary Figure S1D), when compared with cells investigated straight after initial expansion (MFI CD25 at day 3: iTreg 63 $\pm$ 15; Tcon 24 $\pm$ 5).

Most surprisingly, although Tcon cells died rapidly by AICD, iTreg cells responded poorly despite the lack of IL-2 (Figure 1d), coinciding with maintenance of CD25 and increased CD95 expression on iTreg cells, not observed in Tcon cells (Supplementary Figures S1D and E). Notably, at that stage, iTreg cell death was actually amplified in cultures supplemented with IL-2, an effect not observed in Tcon cells (Figure 1d). Finally, although TGF-β did not influence CD95 surface levels on Tcon cells (Supplementary Figure S1E) they were rescued from AICD. In contrast, TGF-β potently killed iTreg cells after they were restimulated via their TCR, in contrast to their reduced sensitivity to AICD (Figure 1d, Supplementary Figure S1C). Of note, levels of CD25 appeared lower on TCR-restimulated iTreg cells treated with TGF-β, an effect overcome by addition of IL-2 (Supplementary Figure S1D). Accordingly, IL-2 also delayed TGF-β-mediated apoptosis of TCR-restimulated iTreg cells but had no effect on restimulated Tcon cells. In these cells, TGF-β alone effectively increased cell survival (Figure 1d).

TGF-β treatment of restimulated iTreg cells induces BH3-only proteins. To better understand the molecular basis of these observations, mRNA levels of different Bcl-2 family members and CD95-signaling components were analyzed. Addition of TGF-β to TCR-restimulated iTreg cells resulted in mild repression of anti-apoptotic *Bcl-xL* but strong induction of pro-apoptotic *Bim* and *Bmf*, whereas expression of *Bid* as well as anti-apoptotic *Bcl-2* remained unchanged (Figures 2a–e). Consistent with our apoptosis results, presence of IL-2 abolished the TGF-β-triggered

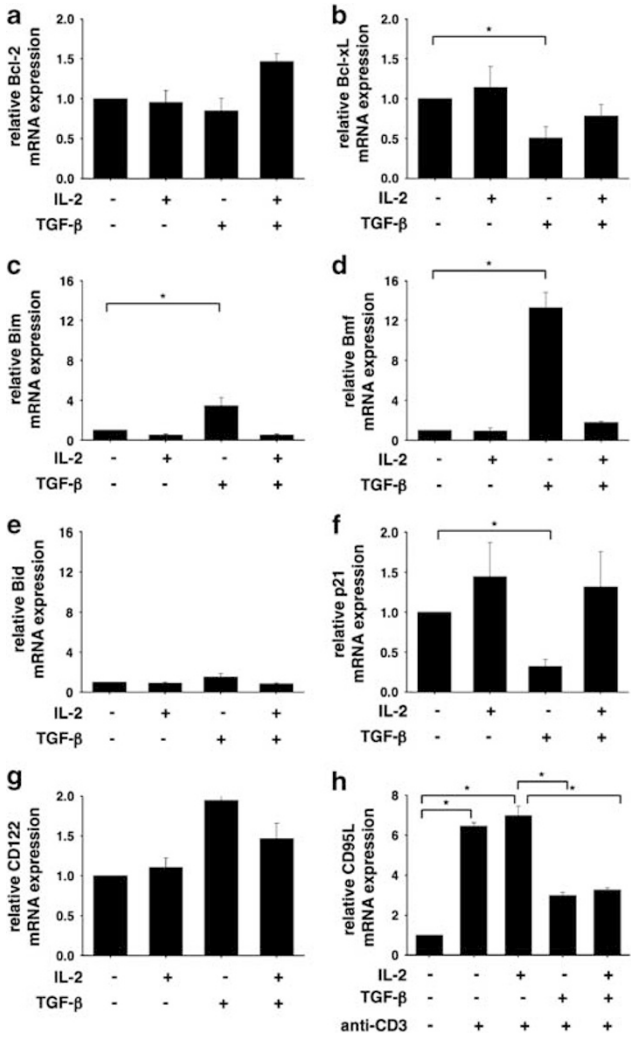


Figure 2 TGF-β induces transcription of BH3-only protein genes *Bim* and *Bmf* in restimulated iTreg cells. iTreg cells were restimulated with cross-linked anti-CD3 and cultured for 18h either without further treatment in the presence of IL-2, TGF-β, or a combination of IL-2 and TGF-β. Relative mRNA expression was analyzed by quantitative RT-PCR and normalized to the housekeeping gene β-actin. Results are depicted as fold-induction or reduction relative to anti-CD3 restimulated iTregs cultured in the absence of cytokines (**a–g**) or fresh iTreg cell before anti-CD3 restimulation (**h**). Bars represent means of $n = 3$ independent experiments performed on different mRNA/cDNA samples $\pm$ S.E.M. $^*P \leq 0.05$

upregulation of *Bim* or *Bmf* and also stabilized *Bcl-xL*, providing a mechanism how IL-2 preserves iTreg cell survival. TGF-β reportedly also mediates cell cycle arrest by induction of p21[20] and can reduce levels of the IL-2Rβ chain, CD122.[6] However, mRNA levels encoding for *IL-2Rβ* mildly increased, whereas *p21* mRNA levels were actually reduced (Figures 2f and g). Notably, TCR-restimulation also induced a strong upregulation of *CD95L* mRNA in iTreg cells that was abrogated by addition of TGF-β, but not IL-2 (Figures 2h). Thus, after TCR-restimulation of iTreg cells, only the combined signals elicited by IL-2 and TGF-β can secure their survival.

TCR-religation primes iTreg cells to CD95-dependent apoptosis. To test the relevance of above-noted changes in gene expression, iTreg and Tcon cells were induced from *foxp3^{gfp}*, *foxp3^{gfp}Bim$^{-/-}$*, *foxp3^{gfp}vav-Bcl-2* transgenic mice and cultured in the absence of cytokines or were restimulated through their TCR. Spontaneous apoptosis due to cytokine deprivation was significantly lower in Bim-deficient or Bcl-2-overexpressing cells (Figures 3a and b). Consistent with these results, IL-2 deprivation strongly induced *Bim* mRNA levels in iTreg cells, whereas levels of *CD95L* or *TGF-β* declined, perhaps due to missing TCR signals (Supplementary Figure S2A-C). As expected, survival of TCR-restimulated Tcon cells improved upon addition of an anti-CD95L mAb in all genotypes tested (Figures 3a and b). Furthermore, Tcon cells derived from *foxp3^{gfp}lpr* mice were resistant to cell death induced by TCR-religation, whereas cytokine-withdrawal triggered death occurred with similar kinetics as in wt Tcon and iTreg cells (Supplementary Figures S2D and E). Thus, TCR-religation triggers CD95-dependent apoptosis in Tcon and iTreg cells, but it occurs with strongly delayed kinetics in the latter. Notably, apoptosis of iTreg cells after CD3-restimulation was enhanced in the presence of IL-2 ($P \leq 0.05$), but prevented by anti-CD95L antibodies (compare Figures 3b and c left panels), consistent with observations that IL-2 can enhance AICD by increasing CD95L expression.[20]

To clarify how TGF-β kills iTreg cells, wt, *Bim$^{-/-}$* and *vav-Bcl-2* tg iTreg cells were restimulated with anti-CD3 mAbs and TGF-β. Loss of Bim partially and overexpression of Bcl-2 completely rescued iTreg cells from TGF-β-mediated apoptosis whereas the presence of neutralizing anti-CD95L did not further improve their survival (Figure 3d). Consistently, TCR-restimulated *lpr* iTreg cells were equally susceptible to TGF-β-induced cell death as wt cells (Supplementary Figure S2E). Thus, TGF-β triggers apoptosis of restimulated iTreg cells by activation of the Bcl-2-regulated intrinsic cell death pathway. Notably, addition of IL-2 prevented TGF-β-mediated apoptosis under these conditions, most likely by counteracting the induction of *Bim* and *Bmf* (Figures 2c, d and 3e). TGF-β rescued Tcon cells from AICD, as noted before.[21]

TCR-restimulation can cause iTreg cell conversion when apoptosis is impaired. We wondered how iTreg cells survived after TCR-restimulation in the absence of IL-2. Therefore, we compared Foxp3-GFP expression in freshly induced iTreg cells at day 3 with that from TCR-restimulated counterparts cultured in the absence or presence of IL-2 and/

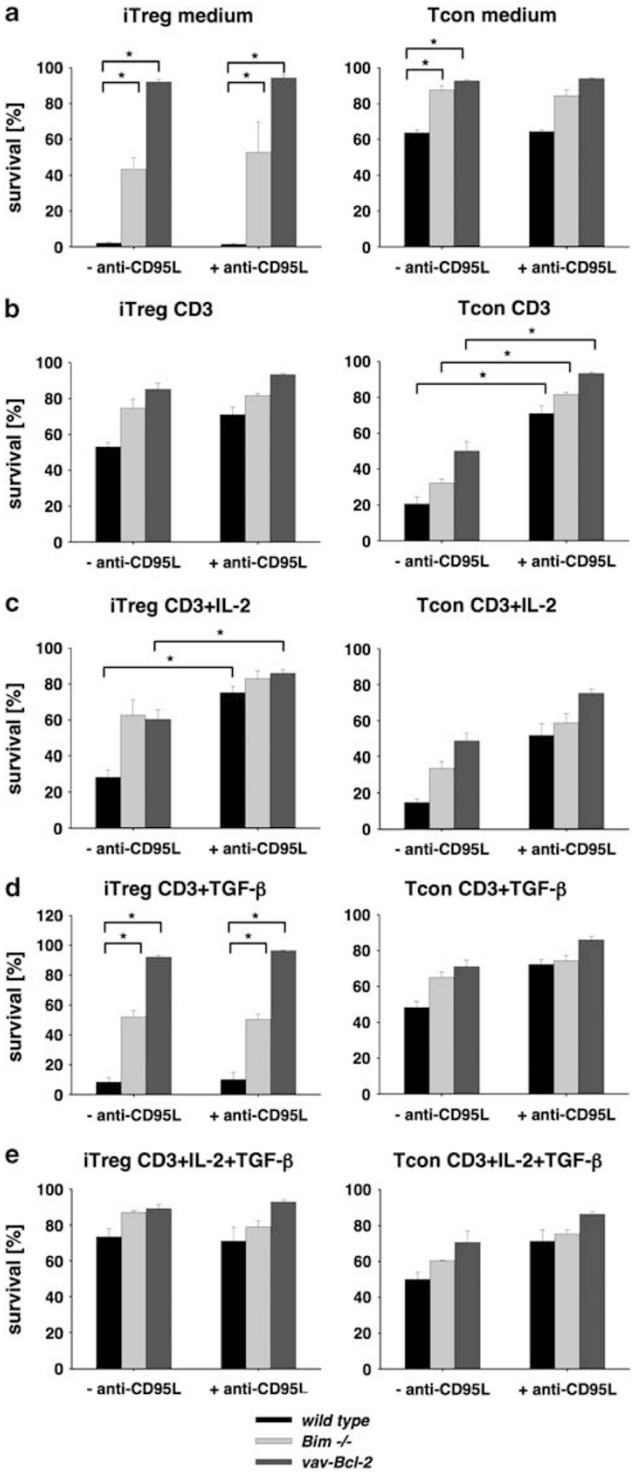


Figure 3 TGF-β modulates AICD differentially in iTreg *versus* Tcon cells. iTreg and Tcon cells were induced from *foxp3^{gfp}*, *foxp3^{gfp}Bim$^{-/-}$* and *foxp3^{gfp}vav-Bcl-2* mice and cultured either in the absence or presence of a neutralizing anti-CD95L for up to 48 h. Cells were cultured alone (**a**) or restimulated with cross-linked anti-CD3 without (**b**) or in the presence of IL-2 (**c**), TGF-β (**d**) or a combination of IL-2 and TGF-β (**e**). Cell survival was assessed by AnnexinV/7AAD staining and flow cytometry. Bars represent means of $n = 3$ independent experiments $\pm$ S.E.M. $^{*}P \leq 0.05$

or TGF-β. To avoid the gradual induction of AICD, observed at later time points after TCR-restimulation, cells were co-incubated with neutralizing anti-CD95L mAb. Foxp3-GFP expression remained stable over time in iTreg cells cultured in the presence of IL-2 or a combination of IL-2 plus TGF-β. In contrast, upon CD3-restimulation, iTreg cells rapidly lost GFP and intracellular Foxp3 expression. This was no longer prevented by addition of IL-2 but required presence of both cytokines (Figure 4a, Supplementary Figure S3). Similar results were observed using CD95-deficient *lpr* iTreg cells, excluding an influence of the neutralizing anti-CD95L mAb on Foxp3-GFP expression (Supplementary Figure S3A). Of note, Bcl-2 overexpressing iTreg cells that died much slower under these conditions, due to impaired spontaneous death in culture, also showed marked GFP-loss (Supplementary Figure S3A). This demonstrates that shutdown of Foxp3 expression depends on TCR-religation and can be uncoupled from apoptosis. As in our *in vitro* cultures, iTreg cells started to lose Foxp3 expression after adoptive transfer and TCR-religation *in vivo* (Figure 4b).

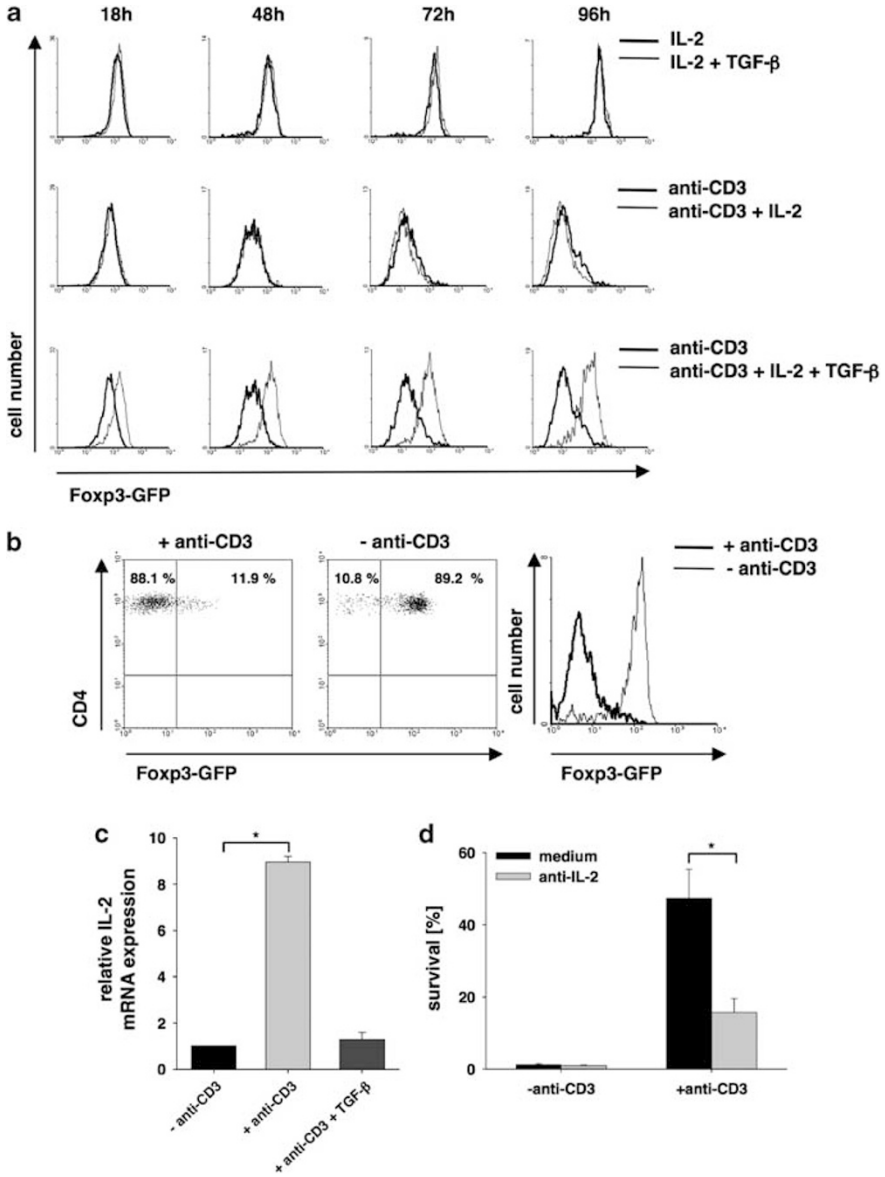


Figure 4 iTreg cells curtail Foxp3 expression and initiate IL-2 production upon anti-CD3 restimulation. (**a**) Purified iTreg cells were cultured in the presence or absence of crosslinked anti-CD3 mAb, either without cytokines, with IL-2 or IL-2 plus TGF-β. To block AICD, 10 μg/ml neutralizing anti-CD95L was added to the cultures. MFI of Foxp3-GFP was analyzed by flow cytometry. Depicted are representative overlays of histograms of Foxp3-GFP expression ($n=3$). (**b**) 5×10^5 Ly 5.2 iTreg cells were transferred *i.v.* into Ly 5.1 isogenic immunoreplete recipients, followed by injection of 20 μg anti-CD3 mAb or saline. After 3 days, animals were killed and Ly 5.2$^+$ iTreg cells traced in the spleen. A representative dot plot and histogram overlay of Ly5.2$^+$CD4$^+$TCRβ^+ iTreg cells detected in saline or anti-CD3-injected mice are shown ($n=2$). (**c**) iTreg cells were restimulated with cross-linked anti-CD3 in the absence or presence of TGF-β for 18 h. Relative IL-2 mRNA expression was monitored by qPCR and normalized to β-actin. Results are depicted as fold induction relative to untreated freshly purified iTreg cells before anti-CD3 restimulation. (**d**) iTreg cells were cultured in the absence or presence of crosslinked anti-CD3 with or without neutralizing anti-IL-2 mAb for 48 h. Cell viability was assessed by AnnexinV/7AAD staining and flow cytometry. Bars represent means of $n=3$ independent experiments $\pm$ S.E.M. $^*P\leq0.05$

We hypothesized that iTreg cells might reprogram into IL-2-producing effector cells that would explain their ability to survive without exogenous IL-2. Indeed, qRT-PCR revealed that iTreg cells started to produce *IL-2* mRNA upon TCR-restimulation, an effect curtailed by TGF-β, and antibody-mediated neutralization of IL-2 significantly reduced their survival (Figures 4c and d). Hence, we wondered whether these cells were still able to suppress and/or might express inflammatory cytokines. Therefore, we assessed the suppressive capacity of freshly induced iTreg cells in comparison with that of iTreg cells cultured for additional 4 days after CD3-restimulation. As downregulation of Foxp3 occurred gradually in CD3-restimulated iTreg cells, a small portion of these cells ($\sim$5%) was still Foxp3-GFP^{high} on day 4 (Supplementary Figure S3B). To exclude that these Foxp3-GFP^{high} iTreg cells adulterate our results we analyzed the following groups: fresh iTreg cells without restimulation, TCR-restimulated Foxp3-GFP^{high} and Foxp3-GFP^{low} cells, as well as total Foxp3-GFP^{all} iTreg cells. Fresh iTreg cells potently suppressed responder T-cell proliferation, even at an odds ratio of 16 : 1 (Figure 5a). However, upon CD3-restimulation the suppressive capacity decreased along with GFP expression. Noteworthy, CD3-restimulated Foxp3-GFP^{low} iTreg cells downregulated CD25, indicative for their reduced cytokine dependence, but maintained high levels of CTLA-4, critical for suppression (Figure 5b). When TCR-restimulation was performed in Th1 or Th2 skewing conditions, the iTreg cells that had lost GFP expression also started to produce IFNγ or IL-4 (Figure 5c), expressed mRNA encoding the T-helper lineage commitment genes T-bet or GATA-3, respectively, whereas no induction of RORγt was observed (Supplementary Figures S4A–C).

iTreg cells prevent colitis induction but are susceptible to reprogramming. We next assessed whether iTreg cells display a similar phenotype under inflammatory conditions *in vivo.* Therefore, colitis was induced in *RAG1*$^{-/-}$ mice by adoptive transfer of Ly5.1^{+}CD45RB^{high}CD4^{+}CD25^{-} Tcon cells alone (control group) or together with Ly5.2^{+}CD4^{+} CD45RB^{low}Foxp3-GFP^{+} spleen-derived nTreg or 1×10^{5} Ly5.2^{+}CD4^{+}Foxp3-GFP^{+} *in vitro* generated iTreg cells. Of note, both nTreg and iTreg cells efficiently prevented colitis induction (Figures 6a and b).

Next, we analyzed stability of lineage commitment 7 weeks after cell transfer *in vivo.* Due to expression of the Ly5.2 marker expressed exclusively on transferred nTreg and iTreg cells, loss of Foxp3-GFP was selectively traced in these populations. As observed *in vitro,* most iTreg cells in the spleen and mesenteric lymph nodes had lost Foxp3-GFP expression after adoptive cell transfer, whereas most nTreg cells still expressed Foxp3-GFP (Figure 6c). However, we also found an increased total number of GFP^{-} Ly5.2^{+}CD4^{+} T cells in mice treated with iTreg cells when compared with nTreg cell-treated animals (Supplementary

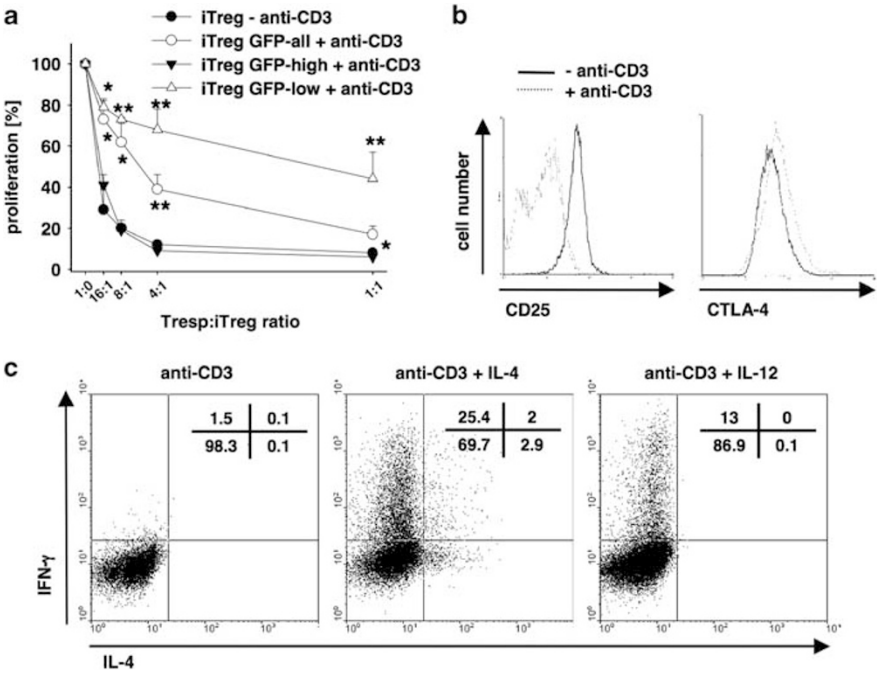


Figure 5 TCR-religation triggers conversion of iTreg cells. (**a**) T-cell suppression assay using iTreg cells directly after *in vitro* expansion or after restimulation with anti-CD3 for further 4 days in the presence of neutralizing anti-CD95L. Anti-CD3 restimulated iTreg cells were either used in bulk (iTreg GFP-all) or split into iTreg GFP-high and iTreg GFP-low cells. Naïve CD4^{+} responder T cells were cultured either alone or with different ratios of iTreg cells, irradiated APCs and soluble anti-CD3 for 72 h. Proliferation was assessed by ^{3}H-thymidine incorporation and normalized to proliferation of responder T cells cultured in the absence of iTreg cells. Symbols represent means of $n=4$ (separated iTreg) $n=8$ (bulk iTreg) independent experiments performed in triplicates $\pm$ S.E.M. (**b**) Expression of CD25 and CTLA-4 on iTreg cells directly after induction (-anti-CD3) or after additional 4 days of anti-CD3 restimulation in the presence of neutralizing anti-95L ($+$anti-CD3). One representative histogram plot out of three independent experiments yielding similar results is shown. (**c**) Sorted iTreg cells were restimulated with crosslinked anti-CD3 and neutralizing anti-CD95L, either alone (left), in the presence of IL-4 (Th2 conditions, center) or IL-12 (Th1 conditions, right) for 4 days. Expression of IL-4 and IFN-γ was assessed by flow cytometric analysis. Data are representative of three independent experiments with similar results. *$P\leq0.05$; **$P\leq0.01$

Figure S5A). Therefore, the total number of regulatory Ly5.2$^+$CD4$^+$Foxp3-GFP$^+$ T cells in mice that received iTreg or nTreg cells was still comparable at the time of analysis, reflecting also the comparable treatment success (Supplementary Figure S5B).

Surprisingly, however, although weight and histological score were equal in both groups treated with different types of Treg cells, the cytokine expression profile in Ly5.1$^+$CD4$^+$ Tcon cells differed significantly between the groups. In mice treated with iTreg cells more IFN-γ^+ Tcon cells were detected, compared with animals that had received nTreg cells. Conversely, in the latter group IL-17A$^+$ Tcon cells were more abundant (Figure 6d). Consistent with our *in vitro* analysis (Figure 5), iTreg cells also started to gain the capacity to produce IFN-γ or IL-17 after adoptive transfer. This ability was much less pronounced in nTreg cells (Figure 6e) confirming their stronger lineage commitment.

Silencing of Foxp3 expression by TCR-signaling. To gain more insight into the reprogramming process, we assessed the methylation status of critical regions within the Foxp3 gene locus.[11] Our analysis suggested that TCR-religation by anti-CD3 challenge did not significantly increase the degree of methylation at tested sites, suggesting that alternative mechanisms are engaged under these conditions to shut down Foxp3 (Supplementary Figures S6A and B).

Previous reports indicated that inhibition of PI3K/mTOR-signaling allows TGF-β-independent Foxp3 expression in Tcon cells,[22] whereas AKT or ERK activation limits Treg commitment.[23,24] Thus, we wondered whether blocking these signaling networks might prevent Foxp3 downregulation in iTreg cells after TCR-restimulation. Hence, iTreg cells were restimulated with anti-CD3mAbs in the presence of IL-2 and anti-CD95L mAb to preserve their survival. Foxp3 expression was followed over time in the absence or presence of the PI3K-inhibitor LY294002, the mTOR-inhibitor Rapamycin or the MEK1/2-inhibitor U0126. Indeed, inhibition of TCR relegation-triggered activation of the AKT/PI3K/mTOR-signaling pathway attenuated the downregulation of Foxp3 expression but inhibition or ERK-signaling proved more effective. In contrast, inhibition of calcineurin/NFAT signaling by cyclosporine A had no such effect (Figure 7a).

Although inhibition of AKT/PI3K/mTOR- or ERK-signaling impaired Foxp3 downregulation in iTreg cells after anti-CD3 restimulation, this phenomenon was only well observed in the presence of neutralizing anti-CD95L mAbs and IL-2, as all these inhibitors rapidly induced apoptosis (Figure 7b), precluding a possible validation of these findings in adoptive transfer settings.

Discussion

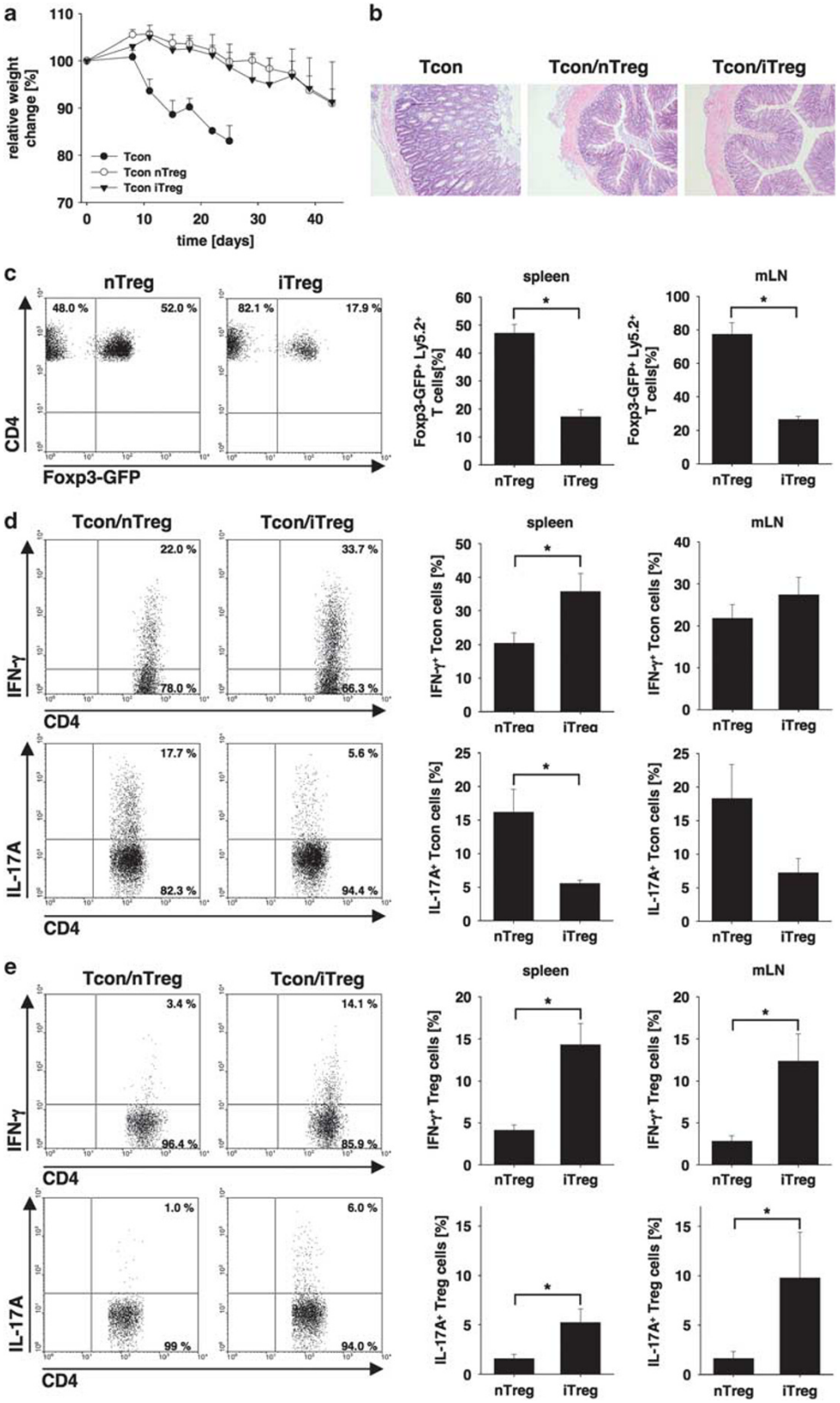
As iTreg cells carry high potential as cellular therapeutics for multiple human pathologies, precise knowledge about factors defining lineage commitment and lifespan is critical to facilitate clinical application. Our study revealed that mouse iTreg cells are highly susceptible to ACAD, but poorly susceptible to AICD, when compared with Tcon cells (Figures 1 and 2). Although the former observation is consistent with findings in nTreg cells that also show strong IL-2 dependence for survival,[25] the observed resistance of iTreg cells to CD95-killing contrasts observations made in human and mouse nTreg cells that are highly susceptible to this type of cell death.[26] This difference was associated with reduced expression levels of CD95 and CD95L in the former (Figure 1), but not *cFLIP* (data not shown), an inhibitor of caspase-8.

Reduced sensitivity to CD95 ligation led us to investigate AICD where TCR-religation drives autocrine production of CD95L, leading to auto- and paracrine T-cell death.[27] Surprisingly, TCR-restimulation caused only a gradual sensitization of iTreg cells to CD95-killing, whereas simultaneously rendering them less dependent on IL-2 for survival (Figure 1). During the down-phase of an immune response such a mechanism seems beneficial, as it might secure the delayed demise of iTreg cells once the antigen has been cleared and IL-2 becomes limiting. As in Tcon cells, apoptosis after cytokine deprivation depended on Bim[28] and possibly other BH3-only proteins such as PUMA,[29] as Bcl-2 overexpression provided superior protection from ACAD, as did loss of Bim (Figure 3). Consistently, loss of Bim partially rescued the loss of Treg cells in IL-2-deficient mice.[30]

Delayed clearance of iTreg cells after TCR religation may hence prevent unwanted residual activity of contracting effector T cells and may assist memory formation. However, upon TCR religation, experienced during chronic infection or repeated encounter of auto-antigens, this reduced sensitivity to CD95 killing harbours the potential problem that, depending on the cytokine milieu present, iTregs can be converted into effector T cells (Figure 5). iTreg cells appear particularly vulnerable to conversion as their lineage commitment does not appear to be as definite as that of nTreg cells (Floess *et al.*[11] and Supplementary Figure S6). This feature of iTreg cell biology appears particularly problematic when considering adoptive transfer of auto-antigen-specific cells into patients. Scrutinizing this hypothesis, we assessed iTreg function and stability in a T cell driven model of colitis.[31] Although both nTreg and iTregs efficiently prevented colitis, most iTreg cells lost Foxp3-GFP expression after adoptive transfer and gained the capacity to produce inflammatory cytokines (Figure 6), similar to what we observed after anti-CD3 restimulation of iTreg cells *in vitro* (Figure 5). This raises the question why mice treated with iTreg cells did not develop severe signs of colitis. First the total amount of Ly5.2$^+$CD4$^+$ iTreg cells detected after 7 weeks in the animals (Supplementary Figure S5A) was significantly higher than that of Ly5.2$^+$CD4$^+$ nTreg cells. This phenomenon may result from continued expansion of iTreg cells after transfer *in vivo* or their increased survival. The latter seems rather unlikely, given their high susceptibility to spontaneous death *in vitro* (Figure 3a). Regardless, due to this effect, the absolute numbers of functional iTreg and nTreg cells *in vivo* was comparable and may have compensated for the loss of iTreg cells lost due to reprogramming (Supplementary Figure S5B).

Interestingly, the cytokine production potential by Tcon cells derived from nTreg or iTreg-treated animals showed marked differences. In mice that received nTreg cells more IL-17A$^+$ Tcon cells were found, whereas in mice that were treated with iTreg cells Tcon cells produced preferentially IFN-γ (Figure 6d). Differences in iTreg survival and stability of lineage commitment *in vivo* may explain why in some disease

models iTregs are curative but fail in others.[32] Downregulation or loss of Foxp3 often correlates with reduced suppressive capacity that was also noted in our experiments (Figure 5a). Reprogramming of these cells to cytokine-producing effector T cells might contribute to augmented inflammation or the development of autoimmunity, limiting their therapeutic



potential. Interestingly, a recent report demonstrates that myelin-reactive iTreg cells can be converted into Th1-like effectors, but fortunately elicited only mild CNS-pathology upon adoptive transfer.[33] Nonetheless, general validity of such benign behavior of converted iTreg cells remains to be established and these observations, together with our

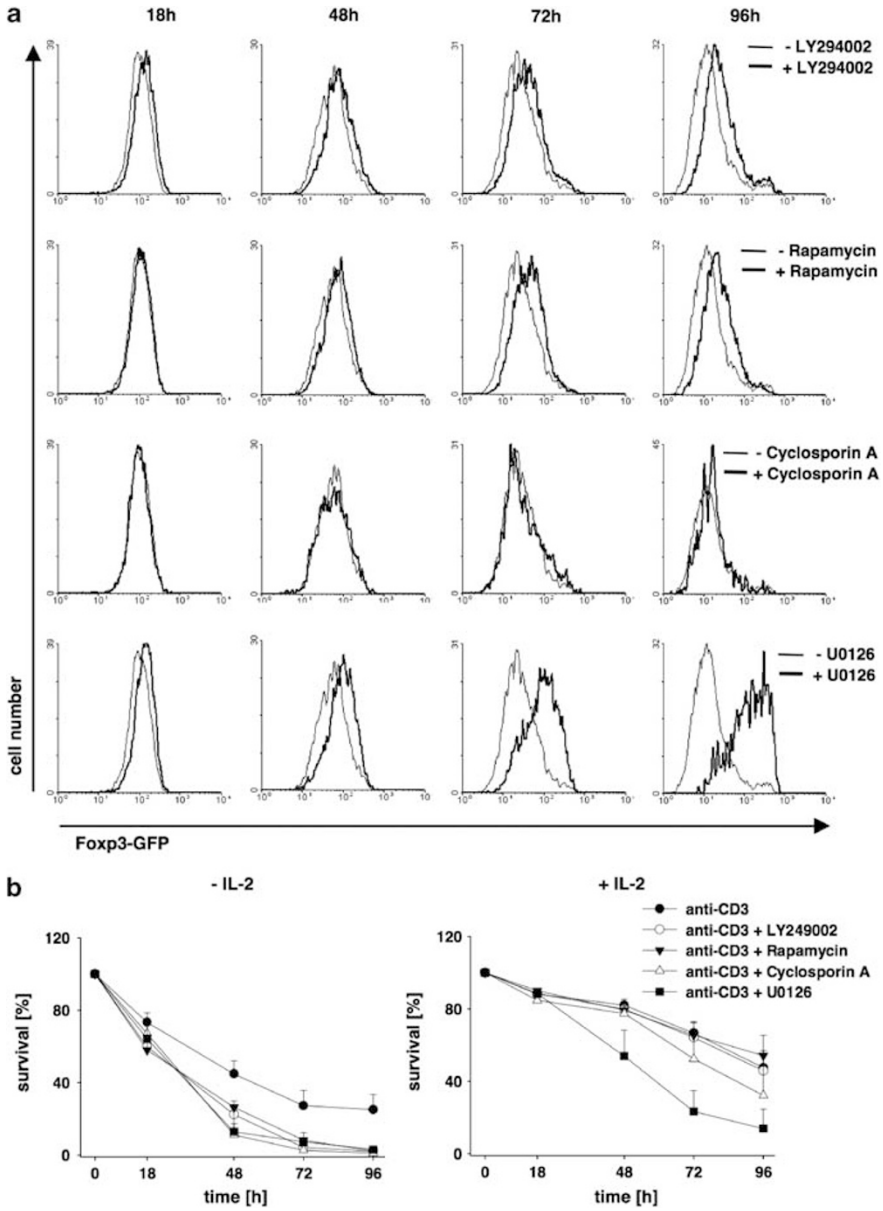


Figure 7 Inhibition of MEK, PI3K and mTOR signaling prevents loss of Foxp3 but induces T-cell apoptosis. (**a**) Sorted iTreg cells were restimulated with cross-linked anti-CD3 mAb, IL-2 and neutralizing anti-CD95L in the presence or absence of the indicated inhibitors. Foxp3-GFP expression was monitored at the indicated time points by flow cytometric analysis. Histogram overlays for representative examples of three independent experiments are shown. (**b**) Sorted iTreg cells were restimulated with cross-linked anti-CD3 in the presence of neutralizing anti-CD95L mAb and the indicated inhibitors in the absence (left column) or presence (right column) of IL-2. Cell survival was analyzed at the indicated time points by AnnexinV/7AAD staining. Only AnnexinV$^-$7AAD$^-$ cells were considered alive. Depicted are means ± S.E.M. ($n \geq 3$ per group)

Figure 6 iTregs prevent colitis induction but are reprogrammed *in vivo*. 3×10^5 Ly5.1$^+$CD45RB^{high}CD4$^+$CD25$^-$ Tcon cells were injected i.p. into Ly5.2 RAG1$^{-/-}$ mice, either alone (control group), or together with 1×10^5 Ly5.2$^+$CD45RB^{low}CD4$^+$Foxp3-GFP$^+$ nTreg or 1×10^5 Ly5.2$^+$CD4$^+$Foxp3-GFP$^+$ iTreg cells ($n = 3$–4 mice per group). Animals were killed when weight loss reached >20% (control) or 7 weeks after adoptive transfer (mice receiving either nTreg or iTreg cells). (**a**) Relative weight change in body weight normalized to the starting weight before transfer. (**b**) Representative H&E stained mid colon sections from untreated (Tcon only), iTreg or nTreg-treated animals (100 ×). (**c**) Foxp3-GFP expression in Ly5.2$^+$CD4$^+$ nTreg or iTreg cells 7 weeks after adoptive transfer. Depicted are representative dot plot (left column) from the spleens of animals that had received Tcon cells either together with nTreg or iTreg cells. Right panel displays percentages of Ly5.2$^+$CD4$^+$Foxp3-GFP$^+$ nTregs and iTreg cells among all Ly5.2$^+$CD4$^+$ T cells in the spleen and mLN ($n \geq 3$ per group). (**d**) Representative dot plots of IFN-γ (upper line) or IL-17A (lower line) expression in Ly5.1$^+$CD4$^+$ Tcon cells isolated from spleen (left column) and quantification of these cells in the spleen and mLN ($n \geq 3$ per group). (**e**) Ly5.1$^-$CD4$^+$ Treg cells analyzed as in (**d**) ($n \geq 3$ per group). Bars represent means ± S.E.M. *$P \leq 0.05$

own findings and previously published results on nTreg conversion,[12,13] clearly constitute a *caveat* for the therapeutic use of autoantigen-specific iTreg cells, if recapitulated in the human setting.

Of note, loss of Foxp3 expression was accompanied by a gradual decline in suppressive capacity but TCR-religation-mediated reprogramming did not associate with increased methylation of the *Foxp3* promoter (Supplementary Figure S5B), suggesting that alternative mechanisms can account for loss of Foxp3 expression. Regardless, these events are related to TCR religation-dependent activation of the ERK-signaling cascade and, to a lesser extent, the PI3K/mTOR-network, as inhibition of either pathway delayed conversion (Figure 5). This finding may actually also explain in part the immunosuppressive properties of Rapamycin, originally thought to primarily act negatively on effector T-cell proliferation.[34] In line, constitutive activation of AKT reportedly prevents commitment to the Treg cell lineage,[23] and loss of MEKK2/3 signaling causes an accumulation of Treg cells in the periphery.[24] These genetic studies clearly support our *in vitro* observations using pharmacological inhibitors of these pathways (Figure 7) and suggest that targeting these kinases may be exploited therapeutically.

To our surprise, we observed that TGF-β prevents conversion by triggering apoptosis in TCR-restimulated iTreg cells by counteracting IL-2 synthesis and promoting induction of mRNA encoding BH3-only proteins *Bim* and *Bmf* (Figure 2). The latter feature was previously noted only in primary gastric or malignant mammary epithelial cells.[35] Hence, iTreg-dependent cellular therapy may benefit from co-administration of TGF-β to limit conversion. However, as TGF-β also spares Tcon cells from CD95-mediated cell death (Figure 1), an overall beneficial effect of such a strategy remains questionable. Of note, our observations may provide a possible explanation how TGF-β can act also in an immune-promoting manner[36] by rescuing Tcon cells from AICD and/or triggering of Bim-dependent iTreg cell death when IL-2 levels are suboptimal. In line, prosurvival effects of TGF-β on T lymphocytes have been described to depend in part on induction of Bcl-xL after co-stimulation[37] or inhibition of CD95L-induction during the down-phase of an immune response.[21] We also noted that TGF-β inhibited CD95L induction in restimulated iTreg cells, as shown before in Tcon cells,[21] but in the former this coincided with the induction of *Bim* and *Bmf* mRNA, indicative for a rewiring of the apoptotic machinery in iTreg cells after TCR-restimulation. Consistently, Bim-deficient or Bcl-2-overexpressing iTreg cells were refractory to TGF-β-mediated death after TCR religation (Figure 3).

In summary, our findings demonstrate that TGF-β can exert opposing roles during iTreg generation *versus* maintenance, depending on the levels of IL-2 present and TCR activity. We propose that TGF-β-mediated apoptosis of iTreg cells limits their conversion into effector T cells after TCR religation that mainly depends on ERK and, to a lesser extent, PI3K/mTOR-signaling. Tcon cells can be rescued under such conditions from AICD and this phenomenon, together with iTreg depletion, may help to preserve immune function. However, when apoptosis is impaired, this also bears the risk of ongoing inflammation contributing to the development of immune-pathology.

Materials and Methods

Mice. *Foxp3^{gfp}* reporter mice[18] were purchased from Jackson Labs (Bar Harbor, ME, USA) and intercrossed with *Bim*$^{-/-}$,[28] *vav-Bcl-2*[38] or CD95-deficient *lpr* mice.[39] All mice were maintained on C57BL/6 Ly5.2 background. C57BL/6 Ly5.1 mice were used for intravenous adoptive transfer of 5×10^5 Ly5.2$^+$ iTreg cells. At the same time one group received 20 μg of anti-CD3 mAb (2C11) i.v. After 3 days, animals were killed and abundance of Ly5.2$^+$CD4$^+$TCRβ^+ T cells was analyzed by flow cytometry. *RAG1*$^{-/-}$ mice were a kind gift from A Moschen, Department of Internal Medicine II. All animal experiments were performed in accordance with the Austrian 'Tierversuchsgesetz' (BGBl. Nr. 501/1988 i.d.F. 162/2005) and have been granted by the Bundesministerium für Bildung, Wissenschaft und Kultur (bm:bwk).

Cell culture, cell sorting and cell death analysis. For induction of CD4$^+$Foxp3-GFP$^+$ iTreg cells, CD4$^+$Foxp3-GFP$^-$ naïve conventional T cells were purified from the spleen by cell sorting and cultured in the presence of 5 μg/ml plate-bound hamster anti-mouse CD3 mAb (2C11), 1 μg/ml hamster anti-mouse CD28 mAb (37.51, Biolegend, Fell, Germany), $\pm$5 ng/ml recombinant hTGF-β1 and 100 U/ml recombinant hIL-2 (both Peprotech, Vienna, Austria) for 72 h at 37 °C, 5% CO_2 in RPMI1640 containing 10% FCS, 100 U/ml Pen/Strep, 2 mM glutamin, 1 mM pyruvat, non-essential amino acids, 50 μM beta-mercaptoethanol. After 3 days of culture, living iTreg and Tcon cells were separated from dead or contaminating cells by cell sorting using a FACS$_{Vantage}$ cell sorter (Becton Dickinson, Heidelberg, Germany; BD Pharmingen, San Diego, CA, USA). Purity of isolated cell populations was usually $\geq$98%.

To induce AICD, wells were pre-coated with 20 μg/ml mouse anti-armenian and anti-syrian hamster IgG1-specific antibody (HIG-632, BD Pharmingen) followed by 1 μg/ml hamster anti-mouse CD3 mAb (2C11) (referred to as 'cross-linked anti-CD3'). For neutralization of CD95L, 10 μg/ml hamster anti-mouse CD95L mAb (MFL3, BD Pharmingen) and for IL-2 neutralisation 10 μg/ml rat anti-mouse IL-2 mAb (JES6-5H4, Biolegend) were used. To induce apoptosis by CD95, cells were cultured in the presence of 100 ng/ml hCD95L (Alexis, Vienna, Austria), cross-linked with 1 μg/ml anti-FLAG M2 mAb (Sigma, Vienna, Austria). For conversion, iTreg cells were restimulated in the presence of cross-linked anti-CD3 mAb and neutralizing anti-CD95L for 4 days. Cells were left either untreated (Th0) or were cultured in the presence of 2 ng/ml recombinant mouse IL-12 (R&D Systems, Vienna, Austria) (Th1), or with 25 ng/ml recombinant mouse IL-4 (Peprotech) (Th2).

Antibodies, cytokines and chemicals. Fluorochrome-labelled antibodies and reagents used are listed in the Supplementary Text S1. For intracellular staining of cytokines, cells were stimulated with 20 ng/ml PMA (Fluka Biochemika, Buchs, Switzerland) and 1 μg/ml ionomycin (Sigma) for 6 h. During the last 3 h of cell culture Monensin (Biolegend) was added. Cells were fixed and permeabilized according to the manufacturers instructions (Biolegend) and analyzed in a FACS$_{Calibur}$ (BD Pharmingen).

T-cell transfer model of colitis. Ly5.1$^+$CD4$^+$CD45RB^{high}CD25$^-$ conventional T cells were isolated from congenic C57/BL6 mice and injected i.p. into 6–10-week-old Ly5.2$^+$C57BL/6 *RAG1*$^{-/-}$ immunodeficient recipients (3×10^5 cells/mouse). 1×10^5 (per mouse) wild-type natural Ly5.2$^+$CD45RB^{low} CD4$^+$Foxp3-GFP$^+$ or induced Ly5.2$^+$CD4$^+$Foxp3-GFP$^+$ Treg cells were co-injected *i.p.* where indicated. Mice were monitored every 3–4 days for wasting disease. Mice were killed either losing $>$20% of its initial body weight or 7 weeks after cell transfer.

Histological assessment of intestinal inflammation. Samples of proximal colon, mid-colon and distal colon were fixed in buffered 4% formalin solution. Paraffin-embedded sections of 3 μm were cut and stained with hematoxylin and eosin.

T-cell suppression assay. iTreg cells were tested either after 3 days of *in vitro* induction or after anti-CD3 restimulation for additional 4 days in a co-culture suppression assay. Triplicates of different iTreg cell ratios were co-cultered with 1×10^4 splenic CD4$^+$Foxp3-GFP$^-$ responder T cells, 4×10^4 APCs (irradiated splenocytes, 30 Gy) and 0.5 μg/ml soluble hamster anti-mouse CD3 mAb in 96-well U-bottom plates for 72 h. 1 μCi ^{3}H-thymidine/well (Hartman Analytic) was added for the last 16 h. Cells were transferred onto glass fiber filters using a Combi Cell-harvester (Molecular Devices, Sunnyvale, CA, USA) and proliferation measured by scintillation counting in a β-counter (Beckman Coulter, Vienna, Austria).

RNA isolation and quantitative RT-PCR. Isolation of total RNA (Zymo Research Quick-RNA MicroPrep kit, Eichgraben, Austria) and subsequent cDNA synthesis (BioRad iScript cDNA Synthesis kit, Vienna, Austria) were performed according to the manufacturers instructions. Information on the primer sequences can be found in the Supplementary information available online. qRT-PCR was performed using a Mastercycler Gradient (Eppendorf, Vienna, Austria) and the DyNAmo Flash SYBR mastermix (Finnzymes, Vantaa, Finland) according to the manufacturer's instruction. The results were normalized to β-actin expression and evaluated using the $^{-\Delta\Delta}$CT quantification method.

DNA methylation analysis. Genomic DNA was isolated from purified T cells using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany). Bisulfite modification was performed using the EZ DNA Methylation-Gold Kit (ZymoResearch). MethyLight PCR analysis was done as described previously.[40] Three Foxp3 assays (two reactions for DNA methylation analysis and one for internal reference) were determined with the assistance of the computer program Primer Express version 2.0.0 (Applied Biosystems, Foster City, CA, USA). Information on primers and amplicons can be found in the Supplementary Text S1, available online.

Statistics. Estimation of statistical differences between groups was carried out using the Mann–Whitney *U* test or repeated measurement ANOVA analysis, where indicated. *P*-values of ≤ 0.05 defined statistically significant differences.

Conflict of Interest

The authors declare no conflict of interest.

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